Characterisation of anthelmintic resistance in a multiple drug resistant *Teladorsagia circumcincta* isolate

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Abstract

Anthelmintic resistance has become widespread in small ruminant farming systems across the world and reports of multiple resistance to all available broad spectrum anthelmintics are increasing. The small brown stomach worm, *Teladorsagia circumcincta,* is considered to be the major cause of parasitic gastroenteritis (PGE) in temperate regions and multidrug resistance (MDR) has been reported in this species in the UK and in other parts of the world. The purpose of this study was to undertake detailed molecular and phenotypic characterisation of a MDR isolate of *T. circumcincta* (*MTci5*) with particular focus upon the mechanisms underlying benzimidazole (BZ) resistance. *MTci5* was isolated from a farm in Central Scotland, which employed a suppressive anthelmintic dosing regime and was closed in 2002 when control of the parasite population became unsustainable. Underpinning all of the experiments in this study was an anthelmintic selection process whereby the *MTci5* isolate was pressurised individually with three broad-spectrum anthelmintics (benzimidazole, ivermectin and levamisole). All characterisation experiments involved analysis of the parent *MTci5* isolate and the three drug-selected F1 generations derived from it.

There are three main areas of investigation in this study, the first being an investigation of the population genetic structure of a MDR isolate. A central question was whether the MDR phenotype of *MTci5* is conferred by the inheritance of genes present in a single interbreeding population or whether there is genetic sub-structuring, whereby discrete sub-populations of the isolate each show resistance to different anthelmintics. Microsatellite analysis was employed to investigate the population genetic structure of the *MTci5* isolate. The results suggest that the *MTci5* isolate is a single, freely interbreeding population with triple resistance, showing no evidence of genetic sub-structuring. The second area of investigation was the role of the F200Y isotype I β-tubulin mutation in the determination of BZ resistance and the potential involvement of this mutation in resistance to ivermectin (IVM) and levamisole (LEV). There was no evidence of an effect of IVM or LEV selection upon the F200Y isotype I β-tubulin mutation. In contrast, there was strong evidence of selection by BZ upon this mutation suggesting that this is a major determinant of BZ resistance. This is consistent with the findings of previous studies. However, the BZ resistance phenotype of the *MTci5* isolate cannot be explained entirely by the F200Y isotype I β-tubulin mutation. This is because a large proportion of $P200^{Phe/Tyr}$ genotypes consistently survived *in vivo* and *in vitro* BZ

selection, suggesting that either the F200Y mutation is not recessive in the *MTci5* genetic background and/or that there are other loci involved in determining BZ resistance. Furthermore, a small proportion of surviving $P200^{Phe/Phe}$ genotypes was observed following *in vivo* and *in vitro* BZ selection, which might also suggest the presence of other contributory BZ resistance mechanisms in *MTci5*. Therefore, a study of all previously implicated BZ resistance mechanisms was undertaken in the *MTci5* isolate to determine if these could be contributing to the BZ resistance phenotype. The F167Y and E198A isotype I β-tubulin mutations reported previously in BZ resistant *T. circumcincta* and *Haemonchus contortus* populations were not found in *MTci5*. Furthermore, deletion of the isotype II β-tubulin locus which has been reported previously in BZ resistant *H. contortus* was also ruled out in *MTci5* (partial sequence of the isotype II β-tubulin gene is presented here for the first time in *T. circumcincta*). However, an investigation of the role of two non-specific xenobiotic removal mechanisms, P-glycoprotein (P-gp) and cytochrome P450 (CYP) oxidative enzymes, in the expression of BZ resistance in *MTci5* revealed interesting findings. Inhibition of these pathways *in vitro*, by the use of appropriate inhibitors (verapamil hydrochloride and piperonyl butoxide), reversed the resistance phenotype of *MTci5*. This implies that in the absence of P-gp and CYP activity, the F200Y isotype I β-tubulin mutation could not confer BZ resistance. This provides some evidence for the hypothesis that detoxification and drug efflux pathways could play a role in MDR in the *MTci5* isolate.

The third area of investigation was the origin and diversity of BZ resistance alleles in the *MTci5* isolate. Single strand conformation polymorphism (SSCP) analysis of a small region extending through exons 1 and 2 and intron 1 of the isotype I β-tubulin gene was used to assess the genetic diversity of this locus in the *MTci5* isolate and of five other UK *T. circumcincta* populations. Alleles from the *MTci5* isolate were then selected for sequencing based on the SSCP data. The genetic diversity of the isotype I β-tubulin locus in the *MTci5* isolate was found to be very high with 7 resistance alleles and 13 susceptible alleles identified. In comparison with previous studies of this kind, these results show a striking level of genetic diversity, particularly in the number of resistance alleles present. This is proposed to reflect the large amount of animal movement inherent in the UK sheep farming industry and the sequence and phylogenetic analysis suggest a hypothesis that resistant haplotypes have arisen in multiple locations and have been brought together by gene flow as a consequence of animal movement. Hence, overall, these results are consistent with the theory of multiple, independent, spontaneous mutations at the P200 locus of the isotype I β-tubulin gene.

Author's declaration

I declare that the work presented in this thesis is my own original work, except where otherwise stated, and it has not been submitted for any other degree or professional qualification.

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Table of contents

List of Figures

Chapter 1 Figures

Chapter 2 Figures

Chapter 3 Figures

Chapter 4 Figures

Chapter 5 Figures

Chapter 6 Figures

List of Tables

Chapter 2 Tables

Chapter 3 Tables

Chapter 4 Tables

Chapter 5 Tables

Abbreviations

°C	degrees Celsius
AChE	acetylcholinesterase
AMOVA/ ANOVA	Analysis of Molecular Variance/ Analysis of Variance
AFLP	Amplified Fragment Length Polymorphism
APS	ammonium persulphate
BSA	bovine serum albumin
BZ	benzimidazole
cDNA, mtDNA	complimentary, mitochondrial deoxyribonucleic acid
CET	controlled efficacy test
CYP	cytochromes P450
dNTP	deoxynucleotide
dH_2O	distilled water
DEPC-treated	diethylpyrocarbonate treated to remove RNases
DMSO	dimethylsulphoxide
$ED_{50,\ 96\ \&\ 99}$	egg development values (TBZ concentration at 50, 96 & 99% hatch failure)
EDTA	ethylenediaminetetraacetic acid
epg	eggs per gram (faeces)
EHA	egg Hatch Assay
EST	expressed sequence tag
FBZ	fenbendazole
FEC	Faecal Egg Count
FECRT	Faecal Egg Count Reduction Test
GABA-Cl	gamma aminobutyric acid-gated chloride channel
GDA	Genetic Data Analysis
GluCl	glutamate-gated chloride channel
Hd	haplotype diversity
Ho, He	observed & expected heterozygosities
IgA	immunoglobulin A
IPTG	isopropyl-β-D-thiogalactopyranoside
ITS	internal transcribed spacer
IVM	ivermectin
KCl	potassium chloride

l	litre
L_1, L_3, L_4	first, third and fourth stage larvae
LB	L broth
LEV	levamisole
LFIA	larval feeding inhibition assay
LMIA	larval migration inhibition assay
M, μM	molar, micromolar
ML	macrocyclic lactone
$MgCl_2$	magnesium chloride
MOX	moxidectin
mm, μl, μg	millimetre, microlitre, microgram
MTci1, 2, 3, 4 & 5	Moredun *Teladorsagia circumcincta* isolate 1, 2, 3, 4 & 5
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
PAGE	polyacrylamide gel electrophoresis
PB	piperonyl butoxide
PBS	phosphate buffered saline
PCA	principle coordinates analysis
PCR	polymerase chain reaction
PGE	parasitic gastroenteritis
PPR	periparturient rise
PPRI	periparturient relaxation in immunity
P-gp	P-glycoprotein
RFLP	Restriction Fragment Length Polymorphism
Rpm	revolutions per minute
ScKiTc	St Kilda, Soay sheep *Teladorsagia circumcincta* isolate
SNP	single nucleotide polymorphism
SSCP	Single Strand Conformational Polymorphism
TAE/TBE/TE	tris-acetate-EDTA buffer/ tris-borate-EDTA buffer/tris-EDTA buffer
TBZ	Thiabendazole
TEMED	N,N,N',N'- tetramethylethylenediamine
TRIS	tris(hydroxymethyl)amino methane
VPL	verapamil hydrochloride
WAAVP	World Association for the Advancement of Veterinary Parasitology
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1.0 Chapter 1 General Introduction

1.1 Teladorsagia circumcincta: a parasite of veterinary importance

1.1.1 Classification and life history traits

In temperate areas of the world, the small brown stomach worm, *Teladorsagia circumcincta,* is the most economically important nematode parasite affecting sheep and goats. Indeed, it is the most common cause of parasitic gastroenteritis of sheep in the UK. *T. circumcincta* belongs to the Phylum Nematoda, Order Strongylida, Superfamily Trichostrongyloidea and Family Trichostrongylidae. This genus was only recently reclassified to distinguish it from the Genus Ostertagia based upon some key morphological differences: bursal ray patter, absence of the proconus (swelling of the genital cone) and ventral ridges (Lichtenfels *et al.,* 1988). Occasionally, *T. circumcincta* is still referred to as *Ostertagia circumcincta.*

T. circumcincta is a diploid, obligate sexual species, which is known to mate with several individuals and has a direct life cycle. This nematode inhabits the abomasum of goats and sheep, however, this species has a wide reservoir host range (Anderson, 1992). It has a heterogonic life cycle, whereby the infective third stage larvae (L_3) are ingested from contaminated pasture (see Figure 1.1). *T. circumcincta* L_3 are known to persist on pasture for up to two years and still remain viable. When the L_3 are ingested and reach the acidic environment of the abomasa, they exsheath, then seek out and enter the gastric glands. Here, they feed and undergo a moult to become fourth stage larvae (L_4). After about 10 days they emerge into the lumen and mature to adulthood on the mucosal surface after a minimum of 18 days post-infection. The L_4 stage is able to undergo hypobiosis (larval arrest) in the abomasal gland for long periods of time and this is thought to assist the survival of this short-lived parasite when the environmental conditions are less favourable for transmission (Anderson, 1992). Hypobiosis may also afford parasites a survival advantage whereby anthelmintic treatment bypasses them (either via limited contact with the drug in the tissues or limited uptake of the drug into the worm). Incidentally, this may exert a selection pressure for resistance or introduce already resistant nematodes into a population via new stock. The stimulation of arrested larvae to resume their development often occurs during pregnancy or

lactation of the ewe. This is known as the peri-parturient rise and is accompanied by a relaxation in host immunity as resources are invested in reproduction. Anderson (1992) postulated that hypobiosis until the point of the peri-parturient relaxation in immunity (PPRI) is an evolutionary strategy which maximises the output of parasites onto the pasture when the newborn, non-immune lambs are most susceptible to infection.

1.1.2 Veterinary and economic importance of *Teladorsagia circumcincta*

The disease caused by *T. circumcincta* is classified into two syndromes, Type I and Type II teladorsagiosis. Type I teladorsagiosis is more common, typically occurring in young animals (termed 'ill thrift' in lambs) in the early summer season, which have ingested a large quantity of larvae from the pasture over a short period. Clinical symptoms include watery diarrhoea, fatigue and weight loss. Type I disease is easily treatable if identified in the early stages (two or three days after onset of clinical symptoms). Type II teladorsagiosis is rarer in sheep, but its cattle counterpart ostertagiosis is commonly found in animals of all ages. Type II disease is caused by the simultaneous emergence of larvae from the hypobiotic state; this typically occurs in the late winter and early spring months often in housed animals. Clinical symptoms include loss of appetite and associated weight loss, as well as diarrhoea and thirst. Animals are at risk of losing protein through damage to the gut and in severe cases, a submandibular oedema can result (Scott *et al.,* 1998), although, this is a more commonly recognised symptom of heavy infections of the blood feeding nematode, *Haemonchus contortus*. Type II ostertagiosis is a far more serious condition and the risk of mortality is high, even if treatment is applied. Hence, cattle farmers tend to employ a suppressive regime of helminth control to avoid this situation. Economic considerations of these diseases include mortality, reduced carcass weights and poor wool quality. The cost of parasitic gastroenteritis (PGE) to UK sheep farmers was estimated at £65million per annum based on assumed sub-clinical production losses, labour and anthelmintic costs (R.L. Coop & F. Jackson, personal communication). The fraction of this total which is spent on anthelmintics is estimated at £10million per annum (F. Jackson, personal communication).

1.2 Anthelmintic Resistance

1.2.1 Anthelmintic drugs

Effective control of *T. circumcincta* and other trichostrongylid nematode species has been achieved primarily through the use of broad spectrum anthelmintic drugs that are effective against a range of species and different developmental stages since these drugs were first introduced in the 1960s. However, the emergence of anthelmintic resistance in several gastrointestinal parasite species is now a major issue worldwide (see Figure 1.2). There are three main broad-spectrum anthelmintics available to the UK market, these are the benzimidazoles/ pro-benzimidazoles (BZ), the macrocyclic lactones (includes the avermectins and the milbemycins e.g. ivermectin and moxidectin, respectively), and the imidazothiazoles/ tetrahydropyrimidines (e.g. levamisole and pyrantel, respectively). These groups are also referred to as the white, clear and yellow drenches, respectively. One other narrow spectrum family: the salicylanilides contain an anthelmintic called closantel, which is effective against blood feeding helminths such as *Fasciola* and *Haemonchus spp.* The development of novel anthelmintics is a slow process as profitability of these compounds is declining. For instance, it is estimated that development and registration of a novel anthelmintic in the United States through the Food and Drug Administration Agency (FDA) will take 15 years, with an investment of around $400 million and only one in 5,000 anthelmintics will reach the market (Johnstone, 1998). Therefore, the preservation of efficacy of existing anthelmintics is a primary aim of current research in anthelmintic resistance.

1.2.2 Emergence of anthelmintic resistance

The first case of BZ resistance in the UK was reported on a Cheshire sheep farm (Britt, 1982). A subsequent survey detected resistance to thiabendazole (TBZ, the first BZ drench introduced) on a number of farms, but crucially also side-resistance to fenbendazole (FBZ), albendazole (ABZ) and oxfendazole (OFZ) amongst *T. circumcincta* populations (Cawthorne & Whitehead, 1983). According to a more recent survey, 80% of lowland farms and 45% of hill farms in Scotland are now resistant to BZ (Bartley *et al.,* 2001). Ivermectin resistance was first reported in South Africa in 1985, just four years after its introduction to the market (Carmichael *et al.,* 1987). The first report of IVM resistance in the UK was in 1992 on an

experimental goat farm in Scotland (Jackson *et al.*, 1992a,b,c) and following a recent small scale survey, it is now thought to be prevalent on 30% of UK sheep farms (Bartley *et al.*, 2006). The first case of LEV resistance in sheep was found in SW England (Hong *et al.*, 1996) and since then, several more cases have been highlighted in UK sheep farms (Coles & Simkins, 1996; Coles *et al.*, 1998). Cases of BZ and LEV resistant worm populations have been reported from two goat farms (Jackson *et al.*, 1992 a,b,c; Coles *et al.*, 1996). Moreover, triple resistance has recently been reported and confirmed on two Scottish sheep farms (Bartley *et al.*, 2004), an Angora goat herd from the Scottish borders (Jackson *et al.*, 1992a, b, c), one English sheep farm (Yue *et al.*, 2003) and one English goat farm (Coles *et al.*, 1996). Surveys of anthelmintic resistance are rare and it is likely that the prevalence of MDR is more widespread.

1.2.3 Factors leading to the development & spread of anthelmintic resistance

Anthelmintic resistance is known to occur in response to selection and there are a number of factors which have been implicated as agents of selection. First of all, we should consider the three ways in which resistance alleles could arise in a population:

1. via selection upon naturally occurring (perhaps rare) pre-existing alleles, which happen to have a selective advantage for resistance against one or more anthelmintics.
2. via gene flow: the introduction of resistance alleles via animal movement.
3. via spontaneous mutation events, which happen to have a selective advantage for resistance against one or more anthelmintics.

Adding to the first and last principles, there is also the possibility that a suite of mutations or alleles in one or more loci are responsible for the development of a resistance phenotype. Moreover, these mutations could accumulate over time without a selective advantage, but without a fitness cost either. Furthermore, the rate of occurrence of these resistance alleles and the rate at which they spread within a population are dependent upon the following factors (Wood and Bishop, 1981):

- Number of genes or mutations involved, which is dependent on relative mutation rate
- Dominance or recessiveness of allele(s)

- Extent of selection pressure(s) e.g. frequency and duration of drug exposure or *in vivo* pharmacokinetic effects
- Reproductive strategy e.g. changing population size, frequency of generations, ability to self-fertilise.
- Rate of gene flow (in introducing or diluting resistance alleles)

Having established the possible genetic mechanisms by which resistance alleles can arise and spread, it is important to consider the common farming practices which are responsible for the selection and spread of anthelmintic resistance.

1.2.3.1 The 'Dose and Move' strategy

In the 1970s, the advice given to farmers to reduce parasitism in their flocks was to dose their animals and move them onto a 'clean pasture', that is, pasture which had preferably not been grazed by sheep for at least a year or had been grazed by cattle (Stubbins *et al.,* 2003). Unfortunately, this system applied a severe selective pressure, whereby only the resistant survivors of treatment were transferred onto new pastures. Essentially this practice was responsible for propagating populations comprising mostly of resistant worms. Currently, advice is given to drench animals well in advance of moving them onto 'clean pasture' to allow small numbers of both resistant and susceptible parasites to be transferred. As an alternative, animals could be drenched on so-called 'safe pastures' which contain low numbers of larvae (which are predominantly susceptible) to impose a dilution effect upon the resistant worms being released. The idea of having a susceptible population *in refugia* is now the favoured option, as the scientific and farming communities have accepted that eradication is not possible, and strategic control measures should be used instead. It is also advised that ewes should never be dosed-and-moved onto either 'clean' or 'safe' pastures as the selection pressures imposed are too great. Instead, it is advised that only immature or underweight animals should receive treatment, since older non-lactating ewes should be immune and should display resilience towards parasitism. Advice is also given to monitor the faecal egg output of lambs, and if burdens are high, then there are two possible courses of action. The first is that lambs should be dosed several days prior to being moved, again, with the aim of moving a mixed resistant/susceptible worm population to the new pasture (since resistant worms will inevitably arise, hence diluting them with susceptible worms is the best strategy). Secondly, it is recommended that selective drenching of lambs be carried out, where only a

proportion of the animals are drenched- these would constitute the lightest and least healthy-looking members of the flock. This would permit the survival of susceptible worms from the heaviest and healthiest lambs to maintain the population *in refugia* (Stubbins *et al.,* 2003). This constitutes the approach of the 'targeted selective treatment' advocated by EU PARASOL (European Union Parasite Solutions, 2006).

1.2.3.2 Frequency of treatment

It is generally accepted that use of a suppressive regime of control to manage flocks with a high frequency of treatments will only serve to propagate a highly resistant suprapopulation (Martin *et al.,* 1982; Barton, 1983). Furthermore, modelling studies have suggested that the principle cause of selection for resistance is high treatment frequency (Barnes *et al.,* 1995, Dobson *et al.,* 1996), therefore, targeted chemotherapy for individuals who are showing clinical symptoms is a better strategy for worm control. A multiple drug resistant (MDR) isolate, *MTci5* (the characterisation of which is central to this thesis), was derived from a farm with a suppressive treatment regime and this served to propagate resistance to all three broad-spectrum anthelmintics in just five years (Sargison *et al.*, 2007). This is discussed further in Section 3.1.1.

1.2.3.3 Quarantine and treatment of new stock

As point 3 suggests, the adequate quarantine and treatment of new stock on a farm is imperative to prevent the introduction of resistant worms (via gene flow). Current UK advice is that, since BZ resistance is widespread, treatment should include more than one broad spectrum anthelmintic (ML + LEV) to give the best chance of removing resistant worms. Combination drenches are now commercially available in Australia and New Zealand for this purpose, e.g. Triton which contains the recommended dose rate of BZ, IVM and LEV anthelmintics. However, these are not available in the UK, therefore, sequential (and not simultaneous) doses of different anthelmintics are recommended. This advice is intended to stop farmers from mixing different drugs whose carrier formulations may not be miscible. Following the use of the two anthelmintics, UK farmers are advised to keep animals off pasture for a minimum of 24 hours following treatment. Moxidectin (MOX) is the preferred ML in this case due to its high efficacy amongst UK parasite populations and the unknown level of IVM resistance. Furthermore, the anthelmintic used for quarantine drenching should

not be the same anthelmintic used for the prophylactic treatment of the whole flock (Stubbins *et al.,* 2003).

1.2.3.4 Under-dosing and co-grazing

Under-dosing is thought to be one of the greatest contributors to the development of anthelmintic resistance. By under-dosing, the potential of survival of partially resistant worms is much higher. For instance, a heterozygous worm may carry a resistance allele which is partially dominant and the survival of these heterozygotes will give rise to a greater number of (fully) resistant homozygotes. There is an example of this in the literature whereby heterozygous worms which are usually susceptible to BZ at the recommended dose rate have been shown to survive treatment at one quarter of the recommended dose rate (Silvestre *et al.,* 2001). Under-dosing also applies a selection pressure for spontaneous mutations as point 3 suggests. There are two common farming practices that may enhance the rate of selection of resistance. Firstly, many farmers simply estimate maximum bodyweight - a practice that has been shown to result in under-dosing, since the estimates are inevitably underestimates. Secondly, farmers often administer doses to the whole flock on the basis of the average weight of the animals which again results in under-dosing. The current best practice advice is to calculate the dose for the heaviest member of the flock and use that dose for the entire flock to prevent under-dosing.

It is also thought that sub-optimal treatments for goats have contributed to the rise of anthelmintic resistance in the UK. Goats are known to metabolise anthelmintics at a much faster rate than sheep and rumen by-pass also occurs more frequently in goats. These two factors reduce the bioavailability of anthelmintics in goats resulting in less effective treatments, thus promoting heterozygote survival. Goats require 1.5 to 2 times more anthelmintic than sheep (Hennessy, 1994). The under-dosing of goats which are co-grazed with sheep has also been a likely cause of the spread of anthelmintic resistance alleles across the UK. Thus, co-grazing sheep and goats is currently not advised (SCOPS, 1995).

1.2.3.5 Rotation of anthelmintic treatments

Advice has been given in the past to rotate the anthelmintics used on an annual basis to prevent the worm population having sufficient time to acquire resistance to each drug. This is only effective if the anthelmintics being used in rotation belong to different drug classes.

However, it is thought that poor knowledge transfer to farmers regarding which anthelmintics belong to each class has led to over-dependence upon single classes, thus contributing to resistance (Stubbins *et al.,* 2003).

1.2.4 Benzimidazole resistance

There are four generic mechanisms by which resistance can develop towards a drug (Wolstenholme *et al.,* 2004), these are:

- Modification of target receptor to reduce binding affinity of anthelmintic
- Increase in number of receptor sites: sequestration of the drug
- Up-regulation of pre-existing xenobiotic removal mechanisms via increased expression of a protein or a gene duplication event
- Enzymatic modification of drug

The mechanisms of resistance towards each of the broad-spectrum anthelmintics will be due to one or more of the above changes (Wolstenholme *et al.,* 2004).

1.2.4.1 Tubulin: target site of the benzimidazoles

The target site of the BZ group is the protein tubulin. Tubulin molecules aggregate to form microtubules (see Figure 1.3), which are structures critical to the function of all cells including those found in nematodes e.g. cellular shape, motility, transport and secretion, mitosis, co-ordination and nutrient absorption (Dustin, 1984). BZ causes the depolymerisation of microtubules and thus, interferes with vital cell function (Lacey, 1988). Lethality is greatest in cells that are actively dividing or growing. Microtubule polymerisation begins with two soluble subunits, α- and β-tubulin, which bond to form heterodimers. These stack upon each other by means of opposite charge to form a protofilament structure. This first stage of polymerization is now thought to involve head to tail arrangements of the subunits in a sheet, which subsequently closes to form the hollow, cylindrical microtubule (Lacey, 1988, Desai & Mitchison, 1997). Subunits are then added at either end of the microtubule in what is known as the 'elongation phase'. In the way that the dimers are positioned, the overall structure is polarised. Microtubule assembly will only begin when the cellular concentrations of the dimers are above a certain threshold and if the optimal conditions (e.g. temperature and pH)

are in place (Desai & Mitchison, 1997). Molecules of guanidine triphosphate (GTP) are bound to the α- and β- subunits. When polymerization is occurring, the GTP bound to β-tubulin molecules become hydrolysed to guanidine diphosphate (GDP), whereas the GTP-α-tubulin complexes remain unchanged. The GTP molecules bound to the subunits, which are exposed at each end of the microtubule, act as a stabilizing force for the elongation process and when this is removed, the underlying GDP-dimer complexes are exposed and depolymerisation ensues (Lacey, 1988, Desai & Mitchison, 1997, Oxberry *et al.,* 2001). Microtubule assembly and disassembly is a process of dynamic equilibrium and several chemical regulators are known to be involved in the control of microtubule activity, however, these will not be discussed here.

Tubulin molecules have three primary binding sites, the colchicine, taxol and vinblastine sites. It was proposed that BZ binds to the colchicine site forming a tubulin-BZ complex, the structure of which depends on the isotype involved, preventing the dimers from aggregating (Lacey, 1988). It has been further suggested that BZ stimulates the production of a GTPase enzyme in the heterodimers, thus precluding microtubule assembly (Oxberry *et al.*, 2001) as was previously demonstrated with colchicine (Lacey, 1988). The actual mechanism remains contentious, however, there is much evidence to suggest that a reduced affinity of BZ to β-tubulin from resistant populations of *H. contortus* occurs (Lubega & Prichard, 1991a, b, c).

The effects of BZs have been investigated at the ultrastructural level as well as the molecular level. Jasmer *et al.*, (2000) reported multiple lethal effects of FBZ in *H. contortus*; namely disintegration of the anterior intestine, fragmentation of nuclear DNA in this locality and the presence of undigested host erythrocytes. It was suggested that the secretory vesicle transport system in these worms is governed by microtubules and the disruption of these by FBZ led to the inappropriate release of digestive enzymes causing damage to parasite tissues instead of digestion of erythrocytes. However, not all parasites which show susceptibility to the action of BZ are blood feeders, and thus, there may be varying degrees of susceptibility to gut damage.

Both α- and β- tubulin have many isoforms, between species and even between tissues of the same organism (Lacey, 1988). It is thought there are two isoforms of β-tubulin in parasitic

nematodes: isotype II represents the β8-9 gene and isotype I represents the β12-16 gene of *H. contortus* (Geary *et al.,* 1992; Lubega *et al.,* 1993). Subsequent studies in *H. contortus* revealed a third gene: β12-164, which differs from β12-16 by only 23 nucleotides, however, these are regarded as members of the same isotype class (Geary *et al.,* 1992). The β8-9 gene differs dramatically from the other genes in the carboxy terminus (Geary *et al.,* 1992). Three β-tubulin genes have also been reported from *Caenorhabditis elegans*; these are *tub-1* (Gremke, PhD thesis, 1986, cited in Driscoll *et al.,* 1989), *ben-1* (Driscoll *et al.,* 1989) and *mec-7* (Savage *et al.,* 1989). Since BZ can be used safely when mammalian tubulin is also at risk of depolymerisation, there must be some difference in binding properties (Lacey, 1988) between nematode and mammalian β-tubulins. Whilst mammalian tubulins are sensitive to other microtubule inhibitors e.g. colchicine, nematode tubulins are far less so, thus, there are obviously important differences in binding receptors. Interestingly, *mec-7* shares the highest homology with mammalian tubulins and this is compounded by its sensitivity to similar inhibitors (Chalfie *et al.*, 1986, Savage *et al.,* 1989). For instance, despite its resistance to BZ, *mec-7* microtubules are sensitive to colchicine and podophyllotoxin in contrast to *ben-1* or *tub-1* products.

1.2.4.2 Early reports of BZ resistance

Resistance emerged soon after the introduction of thiabendazole (TBZ) to the market in 1961 (Brown *et al.,* 1961). The first case of resistance was reported in *H. contortus* in 1964 in Australia (Drudge *et al.,* 1964). This was not an isolated case as more resistance reports emerged in 1972 (McFarland, 1972) and BZ resistance is now considered widespread throughout the world in trichostrongylid nematodes of sheep (McKellar & Jackson, 2004). It is thought that TBZ resistance developed so quickly due to the short-acting nature of the drug and the way it was administered (Prichard *et al.,* 1978). Hence, Le Jambre *et al.*, (1981) suggested adding BZ to a sustained release device. However, resistance also developed after the use of 100-day slow release BZ formulations during the 1990s (Dobson *et al.*, 1996). Reports of side-resistance emerged in the 1970s (i.e. following selection with one BZ drug, resistance is apparent soon after the introduction of other drugs within the BZ class), and this indicated that the binding mechanism seemed to involve the core part of BZ molecule, and not the whole structure (Hotson *et al*, 1970, Colglazier *et al*, 1975).

1.2.4.3 Mechanism of BZ resistance

Early studies with the free living nematode *C. elegans* were pivotal in the elucidation of the mechanism of BZ resistance. Susceptible animals appeared to have slow growth and uncoordinated motility in the presence of benomyl, a benzimidazole derivative (Driscoll *et al.,* 1989). Using mutagenesis techniques, this study demonstrated 28 non-lethal, recessive mutations in the *ben-1* gene (a β-tubulin gene), which conferred resistance upon *C. elegans* as determined by locomotive activity on benomyl-treated agar. Moreover, a deletion event of *ben-1* also conferred resistance and as the BZ mutants were still fully viable, this would suggest that *ben-1* is functionally redundant and that it actually reduces fitness when active in the presence of BZ (Driscoll *et al.,* 1989). Most of the 28 mutations were dominant or semi-dominant, but this was temperature-dependent. For instance, at 15°C, resistance in heterozygotes was absent, but at higher temperatures the benomyl drug had reduced efficacy. Furthermore, at concentrations greater than 12.5μM benomyl, heterozygotes appeared to be susceptible. However, it is likely that any resistance mechanism has a saturation point. Dominance in *C. elegans* appears to be related to the stability of the BZ-tubulin complex, which is in turn temperature-dependent. A similar phenomenon was also observed in baker's yeast, *Saccharomyces cerevisiae* (Thomas *et al.,* 1985). Therefore, some alleles appear to have partial sensitivity and there may be a number of mutations that reduce the effects of benomyl binding. This may be important in the development of resistance, affording the chance of survival to partially resistant phenotypes, which could then give rise to fully resistant progeny.

However, Schatz *et al.* (1986) argued that β-tubulins were not involved in BZ resistance in yeast, but instead, an increased gene dosage of α-tubulin was involved and, concomitantly, a lower dosage increased susceptibility. The authors suggested that by increasing the number of binding sites, the drug was essentially being sequestered. Schatz (1986) postulated that the intracellular ratio of α- and β-tubulins might determine the degree of sensitivity to BZ. This theory would not apply to parasitic worms, however, as the development of BZ resistance appears to occur quickly and not gradually (since one would expect gene duplication events to take time). For example, one selection event with BZ in a laboratory maintained susceptible isolate of *H. contortus* increased the BZ resistant phenotype (Roos *et al.,* 1990). Furthermore, the involvement of α-tubulin and actin was investigated in *H. contortus* during the process of

resistance development (Roos *et al.,* 1990) and no changes were observed in the restriction patterns of either locus.

By the mid 1990s, the discovery of a point mutation at codon 200 of the isotype I β-tubulin gene appeared to provide a reliable marker for BZ resistance. The same point mutation has since been observed in resistant isolates of *T. colubriformis* (Kwa *et al.,* 1994); *T. circumcincta* (Elard *et al.,* 1996) and in several species of cyathostome (Reviewed by Von Samson-Himmelstjerna & Blackhall, 2005). This point mutation leads to an amino acid substitution of phenylalanine to tyrosine (TTC to TAC). Kwa *et al.,* (1994) designed the first allele-specific PCR for *H. contortus* to determine the amino acid present at codon 200. A multiplex PCR (see Section 2.4.3) was designed to improve upon this assay including an internal control to allow confidence in the efficiency of the PCR (Elard *et al.*, 1999). Not only was this mutation a convenient marker, but it was actually responsible for the development of resistance (Kwa *et al.,* 1994). This was unequivocally demonstrated by means of transgenic experiments in the free-living nematode, *C. elegans* (Kwa *et al.,* 1995). Transfection of benomyl-resistant *C. elegans* with a susceptible isotype I β-tubulin transcript from *H. contortus* conferred susceptibility upon individual animals. Furthermore, subsequent *in vitro* mutagenesis of these animals resulted in the Phe to Tyr mutation at codon 200, which restored the BZ resistant phenotype. This demonstrated that the β-tubulin gene was functionally active and it also suggests that the F200Y isotype I β-tubulin mutation was not genetically linked to another resistance gene, since this association would have been broken down in the process of gene transfer to another organism. This is at odds with the findings of Oxberry *et al.,* (2001), which suggested that β-tubulin mutations are convenient markers linked to actual resistance-causing mutations in α-tubulin, which affect the binding affinity of the drug. This remains an unresolved and contentious issue.

1.2.4.4 Theories regarding role of F200Y isotype I β-tubulin mutation in BZ resistance

There have been numerous studies concerning the binding of BZ to nematode tubulin. Instability of the BZ-tubulin complex; a reduction in the number of high affinity receptors and a depolymerisation of microtubules as a consequence of BZ 'mopping up' free dimeric tubulin subunits have all been implicated as potential mechanisms. The mechanism, by which the F200Y isotype I β-tubulin mutation might effect a change in the binding potential of BZ, continues to perplex molecular biologists as modelling predicts that these residues are buried

(inaccessible) within the $\alpha\beta$ complex (Robinson *et al.,* 2004). The F200Y isotype I β-tubulin mutation is located near nucleotide binding domain II (amino acids 203-206) and it has been suggested that this may lead to a small conformational change that affects the binding of BZ. Minotti *et al.,* (1991) observed that an increase in polymerised tubulin correlates with resistance to anti-mitotic drugs, whilst Schibler and Huang (1991) found that microtubule stability is enhanced overall upon exposure to various antimitotic drugs. For instance, colchicine-resistant *Chlamydomonas reinhardtii* mutants were also cross-resistant to vinblastine and dinitroanilines. However, BZ-resistant worms do not appear to be resistant to other anti-mitotic drugs (Lacey, 1988).

Robinson *et al.* (2004) proposed a mechanism for a change in conformation resulting from the F200Y isotype I β-tubulin mutation, whereby a hydrogen bond could form in the tyrosine side chain (replacing a p-oxygen atom in phenylalanine) and bond with the serine at codon 165. The resultant conformational change would block the hydrophobic region at the base of the BZ binding cleft thus preventing the stable formation of a BZ-tubulin complex. The instability of the BZ-tubulin complex was previously suggested by Lacey (1988), but the foundation of this claim was not explained. This is in contrast to findings by Nare *et al.* (1996), which showed that binding of a photoactive BZ analogue occurred solely in the region of amino acids 63 to 103. This region would presumably be unaffected by the conformational change suggested downstream involving codon 200. The function of the F200Y isotype I β-tubulin mutation in conferring resistance is still contentious, however, as Ghisi *et al.* (2007) indicated, the mammalian isotype I β-tubulin gene encodes tyrosine at codon 200. Hence, the mutation in the parasite equates to that of the host, rendering the drug ineffective. In recent years, there have been suggestions that the F200Y isotype I β-tubulin mutation is not the sole determinant of BZ resistance, and this will be dealt with next.

1.2.4.5 Other changes in the isotype I and II β-tubulin genes associated with BZ resistance

Many studies have been employed to measure the variation in β-tubulin isotype composition in BZ-resistant versus -susceptible populations, particularly using *H. contortus* as a model (Roos *et al.,* 1990; Kwa *et al.,* 1993, 1994, 1995; Lacey & Gill, 1994; Otsen *et al.,* 2001). Roos *et al.* (1990) demonstrated (using restriction fragment length polymorphism, RFLP) that a loss of variation was associated with the developing resistance phenotype and later studies

have similarly shown that reduced variation of the isotype I and II β-tubulin loci occurs. Furthermore, with continued selection, it was apparent that a complete loss of the isotype II β-tubulin locus occurred as resistance progressed (Kwa *et al.,* 1993, 1994; Roos *et al.,* 1995). This is consistent with the findings in *C. elegans* whereby deletion of the *ben-1* (BZ susceptible) gene was a by-product of selection (Driscoll *et al*, 1989). Perhaps *ben-1* is a homologue of β-tubulin isotype II found in parasitic nematodes? However, Beech *et al.,* (1994) failed to observe such a deletion event with extreme BZ selection of a different *H. contortus* population. Furthermore, in the fungus, *Physarum polycephalum,* BZ resistant β-tubulin was shown to be incorporated into functioning microtubules irrespective of the BZ concentration applied throughout this process, which suggests that inactivation of a more sensitive isotype is not important here, rather, modification of an existing tubulin is occurring (Foster *et al*, 1987). However, it is possible that there are several mechanisms of BZ resistance which come into play at different levels of BZ selection and, as Beech *et al.,* (1994) conceded, the loss of the isotype II locus was most likely prohibited by the overall loss of variation in the population. This loss of variation perhaps did not occur to the same extent in the populations examined by Kwa *et al.* (1993, 1994).

In contrast to a loss of the isotype II β-tubulin locus, Beech *et al.,* (1994) found the F200Y allele at a frequency of 12% in the isotype II β-tubulin locus in one BZ resistant population. It does appear that there are numerous potential mechanisms for BZ resistance and that some mutations are more likely than others. Perhaps there are fitness costs associated with some of the more rare mutations and these are only observed in artificially selected lines. The question of fitness is an important one. For instance, in the absence of selection, assuming BZ resistance did have a fitness cost, then one would expect the level of resistance to decrease over time. However, there appeared to be no reversion to susceptibility from a BZ resistant population monitored for 10-15 years at MRI in the absence of selection (F. Jackson, personal communication). However, this was measured phenotypically using controlled efficacy tests, faecal egg count reduction tests and egg hatch assays; thus, it would be worthwhile monitoring the F200Y isotype I β-tubulin genotype frequencies over some years of a closed population without selection to determine if the genotypes revert back to Hardy-Weinberg Equilibrium.

Not only are there multiple genes involved in the development of high-level BZ resistance, but other SNPs of the isotype I β-tubulin gene have been highlighted in association with BZ

resistance. For instance, Silvestre & Cabaret (2002) observed the F167Y isotype I β-tubulin mutation amongst two resistant populations of *H. contortus* and one of *T. circumcincta* from farms in SW France. There were two interesting observations regarding this novel SNP, the first being that it appeared to confer resistance in the absence of the F200Y isotype I β-tubulin mutation and secondly, that resistance did not appear to be recessive as it appears in the case of F200Y, that is, a $P167^{Tyr/Tyr}$ and a $P\ 167^{Phe/Tyr}$ genotype conferred resistance. Moreover, the $P167^{Phe/Tyr}$ worms survived equally as well as the $P200^{Tyr/Tyr}$ worms under BZ selection, showing that the $P167^{Tyr}$ allele is dominant. Prichard (2001) has also reported resistant *H. contortus* worms with $P167^{Phe/Tyr}$ or $P167^{Tyr/Tyr}$ genotypes, occurring in the absence of the F200Y isotype I β-tubulin mutation. Furthermore, recombinant β-tubulin incorporating the F167Y isotype I β-tubulin mutation was shown to suppress the binding of BZ, again, in the absence of the F200Y isotype I β-tubulin (Prichard *et al.,* AAVP abstract, 2000). This mutation has been found in other organisms, for instance, benomyl resistant *Neurospora crassa* also show the F167Y isotype I β-tubulin mutation (Orbach *et al.*, 1986).

Recently a third isotype I β-tubulin mutation (E198A) has been found in association with BZ resistance in *H. contortus,* occurring in the absence of either F167Y or F200Y mutations (Ghisi *et al.*, 2007). These populations were isolated from S. Africa and Australia, suggesting that the E198A isotype I β-tubulin mutation had arisen independently in these areas. The E198A isotype I β-tubulin mutation has also been reported from BZ resistant strains of fungi, including *Monilinia fructicola* (Ma *et al.,* 2003).

1.2.4.6 Heritability of BZ resistance

The heritability of the F200Y isotype I β-tubulin mutation is of great importance in determining the rate of its spread throughout a population. Early studies by Le Jambre *et al.* (1979) on the inheritance of TBZ resistance rejected the possibility of resistance being a sex-linked and single locus trait. For instance, since males of *H. contortus* are heterogametic, then a simple cross would show whether resistance was sex-linked. If so, then the male progeny of resistant males crossed with susceptible females would only be susceptible. This was not the case. Furthermore, the authors found evidence of a matroclinous influence upon resistance. This means that progeny with a resistant female parent crossed with a susceptible male were more resistant than those resulting from a cross of a susceptible female and a resistant male. The authors suggested that this influence could manifest in the egg shell and/or cytoplasm as

this is provided by the female and it was pointed out that these structures contain DNA. However, there is a possibility that this form of resistance is not inherited genetically, but due in part to some physical change in the eggs induced by the female worm under drug selection. This could make the eggs more resistant in phenotype, e.g. with a less permeable egg shell. If this is an environmental influence and does not have a genetic basis, then TBZ resistance may still be regarded as a single locus trait. Furthermore, Martin *et al.* (1988) demonstrated through backcrossing of resistant and susceptible *T. colubriformis* adults, that resistance was inherited co-dominantly and was multigenic. Herlich *et al.* (1981) also suggested that resistance in *H. contortus* was incompletely dominant and that expression of BZ resistance at its fullest involved more than one locus. Hence, there are two issues here regarding BZ resistance, which remain contentious in the literature. These are the involvement of multiple loci and the proposed recessiveness of the resistance allele. The transgenic experiments by Kwa *et al.* (1995) showed that a single mutation could confer resistance, which is at odds with the work of Le Jambre *et al.* (1979), Martin *et al.* (1988) and Herlich *et al.* (1981). Secondly, Elard *et al.* (1998; Elard & Humbert, 1999) showed that the survival of only $P200^{Tyr/Tyr}$ genotypes was possible following BZ selection at the recommended dose rate, suggesting that the BZ resistance allele must be recessive. Again this is contrary to the findings described above. It is crucial to investigate this further, as a monogenic trait is much easier to pass on to subsequent generations than a polygenic trait and a dominant or partially dominant trait will also spread faster within a population. These factors will determine the rate at which resistance develops.

1.2.4.7 Evidence for other non-tubulin resistance mechanisms to BZ

In recent years, yet more mechanisms of resistance to BZ have been implicated in parasitic nematodes, and these involve the inherent mechanisms of dealing with xenobiotics from the environment. For instance, P-glycoproteins (P-gp) are ubiquitous, highly conserved transmembrane molecules, which mediate the ATP-dependent transport of lipophilic peptides across the cell membrane (Gottesman *et al.,* 1995; Higgins *et al.,* 1997). P-gp were first described in 1976 (Juliano & Ling) and belong to the ATP-binding (ABC) cassette protein family. Each P-gp gene comprises two halves, each of which encodes a protein consisting of a nucleotide binding domain (NBD) in which the ATP binding site is situated. There is also a hydrophobic region containing six transmembrane domains. An extracytoplasmic loop with an N-linked glycosylation site between transmembrane domains 1 and 2 in the N-terminal half

is also a highly conserved feature of these genes (Sangster, 1994). P-gp was first implicated in the process of anti-cancer drug efflux from mammalian tumour cells (van der Bliek & Borst, 1989). This was supported by studies whereby transfection of P-gp into susceptible mammalian cells produced a resistant phenotype to anti-cancer drugs (Hammond *et al.,* 1989). P-gp was also shown to be the first line of defence against ingested toxins in *C. elegans,* (Broeks *et al.,* 1995) since they are conveniently expressed in the gut (Lincke *et al.,* 1993). To date, 14 P-gp genes have been described in *C. elegans* (Geerts & Gryseels, 2000) compared with only two in humans (Kerboeuf *et al.,* 2003), suggesting that they have a vital role in these nematodes. There are at least seven known P-gp genes in *H. contortus* (Kerboeuf *et al.,* 2003) and expression is localised along the intestinal tract, primarily in the pharyngeal region (Smith & Prichard, 2002). In parasitic nematodes, P-gp may have a role in homeostasis (Valverde *et al.,* 1992). Also, control of osmolality, especially in blood feeders, presents a significant task as helminth cells are hypo-osmotic (Sangster, 1994). However, P-gp may also have a role in drug resistance, since it has been shown that these transporters can utilise BZ drugs as substrates for efflux.

By inhibiting P-gp with verapamil (VPL), it is possible to show the comparative influence of these transporters upon BZ resistance of parasitic nematodes *in vitro*. For example, a study of three BZ resistant and two susceptible *H. contortus* populations employing the egg hatch assay with VPL showed that the presence of this inhibitor increased the potency of albendazole (ABZ) and thiabendazole (TBZ) anthelmintics in all five populations. Complete reversal of resistance, however, was not achieved in the resistant populations, suggesting that this is a secondary resistance mechanism (Beugnet *et al.,* 1997). In order to confirm the action of the inhibitor, Kerboeuf *et al.* (1999) utilised two P-gp substrate transport probes, Rhodamine 123 and Verapamil-Bodipy, in flow cytometry analysis of uptake by resistant vs. susceptible *H. contortus* eggs. The results showed that the resistant eggs had a far higher affinity for these compounds, showing similar activity to MDR cancer cells. Furthermore, similar findings were achieved with resistant *T. circumcincta* eggs suggesting that higher constitutive expression of P-gp may be a common resistance mechanism (unpublished data, Jackson & Kerboeuf, personal communication). Thus, it appears that there are a number of mechanisms which contribute towards the BZ resistance phenotype of nematodes at all levels of resistance, however, it is not known how the up-regulation of P-gp occurs or at what stage of resistance it

is most likely to develop. Furthermore, there are probably other mechanisms which contribute to the removal of all xenobiotics that have not yet been discovered.

A second potential mechanism has also been suggested recently, and that is the cytochromes P450 (CYP) metabolic enzymes. CYP are involved in the oxidation of endogenous compounds and xenobiotics (Guengerich, 1991) and it is known that most BZ anthelmintics are suitable for oxidative metabolism by CYP. For instance, TBZ, which is employed in the egg hatch assay, is known to induce CYP1A1 class enzymes (Galtier *et al.,* 1997). Although the metabolism of BZ by CYP has not been demonstrated in parasites, there seems to be no obvious reason why this would not occur in parasitic nematodes (Kotze *et al.,* 2006). Thus, we might expect CYP activity to contribute towards the metabolism of BZ, however, we do not know the extent to which this mechanism may contribute towards resistance and whether this mechanism is up-regulated in resistant worms. An example of how these enzymes may be up-regulated in response to chemical selection pressures was observed in *Drosophila melanogaster*. It was shown that DDT resistance in these insects was up-regulated by 10 to 100 times, as a consequence of the increased transcription of a CYP enzyme, *Cyp6g1*. This was caused by the insertion of a single Accord transposon in the promoter region (Daborn *et al.,* 2002). This resistance allele was found amongst a number of geographically disparate populations, suggesting that this mutation had occurred on more than one occasion, and may have been a pre-adaptation for resistance.

1.2.4.8 The origin of BZ resistance

The number of mutations and mechanisms potentially associated with BZ resistance seems ever-increasing, however, it appears that the expression of BZ resistance can at least be explained by a single mutation: either F200Y or F167Y or E198A of the isotype I β-tubulin gene (Kwa *et al.,* 1995; Prichard, 2001; Ghisi *et al.,* 2007). High level BZ resistance seems to involve a number of loci and these can vary amongst populations. It is crucial that the mechanisms underlying BZ resistance are better characterised as this will allow us to address another important issue: the origin of BZ resistance. As mentioned previously, there are three ways in which resistance can arise in a population, via: pre-adaptation; spontaneous mutation or gene flow. An understanding of the mechanism(s) by which resistance arises, is a pre-requisite for the development of effective sustainable strategies for control. For instance, if it was apparent that resistance in the UK was primarily caused by gene flow, via inadequately

quarantined stock, then the farming industry could take steps to prevent this happening for the other drug classes. If we discover that resistance is a pre-adaptation and not a product of spontaneous mutation, this is a more worrying situation and the combination drench approach should be considered as a primary course of action. Intuitively, the concept of pre-adaptation is more likely to be associated with a monogenic trait. A polygenic resistance trait should be more difficult to select, however, resistance to BZ in particular, arose quickly with reports within three years of the introduction of BZ to the market (Drudge *et al.,* 1964). This may indicate that BZ resistance is primarily caused by a single mechanism, i.e. F200Y, F167Y or E198A of the isotype I β-tubulin gene. Given that the F200Y isotype I β-tubulin mutation has been found amongst a vast range of species and nematode isolates across the world, this seems to suggest that this is the most important BZ resistance mutation. However, does this also suggest that the F200Y isotype I β-tubulin mutation is an ancient polymorphism, a pre-adaptation for resistance? Whilst this seems highly possible, we must consider the alternative that the F200Y isotype I β-tubulin locus represents a mutational hotspot. Furthermore, perhaps several mutations arise frequently within the isotype I β-tubulin gene, but most of these have a fitness cost and thus, do not persist.

1.2.5 Ivermectin resistance

The mechanism(s) responsible for the development of resistance to ivermectin (IVM) are poorly understood. A number of candidate genes have shown an association with IVM selection, although none has been proven unequivocally to cause resistance in parasitic nematodes (reviewed by Gilleard, 2006). IVM is a ubiquitous anthelmintic with applications for the control of ecto- and endo- parasites of humans and animals. Unsurprisingly, due to its extensive usage, resistance developed soon after its introduction to the market and was first reported in South Africa in 1988 (Van Wyk & Malan, 1988) and soon after in Brazil (Echevarria & Trindade, 1989). Moreover, the emergence of moderate levels of IVM resistance on UK sheep farms appeared to be more rapid than the time taken for similar levels of BZ resistance to emerge. The first cases of BZ resistance in sheep were reported in Scotland in 1983 (Scott *et al.*, 1990) and in the first survey conducted some 8 years later, about 24% of the surveyed lowland farms had BZ resistance (Mitchell *et al.*, 1991). By way of contrast, the first case of ivermectin resistance in sheep in Scotland was reported in 2001

(Sargison *et al.*, 2001), but in a small scale survey conducted in Scotland in 2004 over 30% of the surveyed properties showed evidence of ivermectin resistance (Bartley *et al.*, 2006). Perhaps this suggests that MDR mechanisms evolved during the development of resistance to BZ (which are non-specific in terms of substrate), or perhaps there is genetic linkage between the BZ and IVM resistance mechanisms, giving parasites a survival advantage prior to IVM exposure. Unlike BZ resistance, it has been suggested that IVM resistance is a dominant trait (Dobson *et al.*, 1996) and this may also explain the rapid development of IVM resistance in trichostrongylid nematode populations. The candidate resistance mechanisms which have been investigated previously will now be discussed.

1.2.5.1 Potential IVM resistance mechanisms caused by modification at or near the target sites

Modification of the binding site is one possible mechanism for IVM resistance. Two chloride channels have been implicated in association with IVM resistance in *H. contortus*: specifically the glutamate-gated chloride channel (GluCl), and the γ-aminobutyric acid (GABA)-gated chloride channel (Blackhall *et al.*, 1998, 2003). Glutamate and GABA are neurotransmitters that regulate muscle contraction through binding of their respective receptors. IVM binds irreversibly to the α-type subunit of these chloride channels in pharyngeal and somatic muscle cells and in doing so, prevents the generation of action potentials (Cully *et al.*, 1994). This causes hyperpolarisation and leads to paralysis of the somatic musculature as well as inhibition of the pharyngeal pump, which prevents the worm from feeding. However, [H^3]IVM binding studies utilising membrane preparations from IVM-selected and non-selected lines of *H. contortus* indicated that there was no alteration of the target region in resistant worms (Rohrer *et al.*, 1994). Another subunit, *HG1*, of a putative GABA-Cl was shown to display variation in allele frequencies between resistant and susceptible *H. contortus* (Prichard, 2001), however, none of these studies have shown causal associations with IVM resistance. In contrast, a comparative study of the GluClα subunit between resistant and susceptible *Cooperia oncophora* populations did provide more substantive evidence of a role for these genes in IVM resistance (Njue *et al.*, 2004). The GluClα sequences of resistant populations differed from the susceptible sequences by three non-synonymous mutations. Expression of these cDNA sequences in *Xenopus* oocytes showed that the cDNA from resistant populations was less sensitive to IVM. It was subsequently shown that one of these mutations, L256F, was responsible for this loss of IVM sensitivity, demonstrating a clear

functional role for this locus in resistance. However, this mutation is not considered to be the only mechanism of IVM resistance and it has yet to be found in other nematode species e.g. *H. contortus* and *T. circumcincta* (P. Skuce, personal communication). This may suggest that there are a number of possible mutations within this gene or amongst other genes which could influence IVM resistance status.

Earlier studies in *C. elegans* have shown multiple locus contributions to IVM resistance. For instance, simultaneous mutations occurring in three genes in response to *in vitro* mutagenesis (*avr-14, avr-15* and *glc-1*) encoding GluClα-type subunits (i.e. homologues of *GluClα3*, *GluClα2* and *GluClα1*, respectively) were the only survivors of IVM exposure (Dent *et al.*, 2000). Interestingly, severe loss of function mutations in the two latter genes alone did not confer IVM resistance, however, a mutation in *unc-7* (a gene encoding a gap junction or innexin), in combination with the three mutations described above, conferred an even higher level of IVM resistance. Dent *et al.* (2000) also observed that the hyperpolarisation effect of IVM upon extrapharyngeal neurons was transmitted to the pharyngeal neurons via gap junctions, which inhibits pumping of the pharynx. It was hypothesised that a mutation in *unc-7* could prevent the spread of hyperpolarisation between neighbouring cells, thus, limiting the effect of IVM to the superficial cells.

It appears from these studies that IVM has targets that involve a number of different genes. This also suggests that under continual drug-selection, an accumulation of mutations in these different genes could proportionally increase resistance. Therefore, there may be several alleles circulating within and between field populations of parasites, each exerting a mild de-sensitising effect of IVM upon receptors. Thus, populations could accumulate resistance to IVM proportionally with the spread of such alleles. This view should be treated with some caution, since these experiments were performed using *in vitro* mutagenesis of a model organism (*C. elegans*) and thus, they are not representative of the situation with parasitic nematodes which are under a different set of selection pressures in the field. Furthermore, one might surmise that the mechanism of IVM resistance in the field may be less complex, since multiple mutations developing simultaneously would intuitively be rare occurrences, unless they were genetically linked (and this does not appear to be the case in *C. elegans*). Hence, the rate of evolving resistance would have to be slower than that of the BZ story for example. Given that this does not seem to be the case, perhaps there is some commonality between BZ

and IVM drug targets, or, there is one major mechanism of IVM resistance in the field, which is yet to be discovered. Indeed, other mechanisms of resistance which do not involve modification of the target sites of IVM have been implicated in recent years and these are the same mechanisms which are thought to contribute to BZ resistance.

1.2.5.2 Involvement of non-target site mechanisms

P-gp were mentioned previously for their potential role in BZ resistance, however, they may also be important in IVM resistance. IVM is a confirmed substrate for P-gp transport (Didier & Loor, 1996). A comparison of one such gene, *Pgp-A*, between ML-resistant and susceptible isolates of *H. contortus* revealed a reduction in the number of alleles associated with IVM resistance (Xu *et al.,* 1998). Three more studies similarly observed selection of particular P-gp alleles in association with ML resistance in a number of different *H. contortus* isolates (Blackhall *et al.,* 1998; Sangster *et al.,* 1999; Le Jambre *et al.,* 1999), however, the alleles under selection were not always consistent between populations (Blackhall *et al.,* 1998). Hybridisation/backcrossing studies have shown that in at least one case, there was no causal association between changes in P-gp allele frequency and ML resistance (Le Jambre *et al.,* 1999).

One potential mechanism linking P-gp and IVM resistance in parasitic nematodes is over-expression, leading to enhanced rates of efflux (Xu *et al.,* 1998; Blackhall *et al.,* 1998). In *Plasmodium falciparum*, the *pfmdr1* gene is over-expressed and thought to cause anti-malarial resistance by sequestration of the drug before it reaches the binding site (Pussard & Verdier, 1994). This seems a feasible hypothesis given that the transfer of extra P-gp genes into susceptible cancer cells conferred resistance to anti-cancer drugs (Hammond *et al.,* 1989). However, if over-expression was the cause of resistance, one would expect to find mutations in the promoter region, however, mutations have mainly been described in the transmembrane regions. Although, increased P-gp mRNA concentrations were observed in IVM-resistant populations compared to non-resistant populations of *H. contortus;* RFLP analysis of these mRNAs generated different banding patterns (Xu *et al,* 1998). However, Smith & Prichard (2002) failed to show any difference between the P-gp mRNA expression levels in the pharyngeal region of ML resistant vs. susceptible *H. contortus*. The proviso of the latter study was that the authors used a probe specific to the ATP binding region of *Pgp-A,* and whilst it shares some homology (between 50 and 70%) with other known P-gp genes, it is possible that

they may have been looking at a less important P-gp, or a less important location of P-gp expression in these worms. Stage-specific expression of P-gp has already been demonstrated in some parasite species (Huang & Prichard, 1999), thus, there may be a relatively simple mechanism to enhance P-gp expression in response to drug selection. For example, *Onchocerca volvulus* shows a higher level of expression of two P-gp genes (*ovpgp-1* and *ovplp-1*) in adulthood compared with the larval stage (Huang & Prichard, 1999). This is thought to account for the higher tolerance of IVM treatment that adults show compared with larvae (de Silva *et al.*, 1997), however, this was not proven.

Another study examined the effects of IVM exposure upon a P-gp gene of *O. volvulus* and found a change in allele frequencies correlated with IVM exposure (Eng & Prichard, 2005). Again, it is not clear whether these results were a consequence of genetic hitchhiking or epistasis, however, this study and subsequent experiments showed a strong association between IVM resistance and the isotype I β-tubulin gene in both *O. volvulus* and *H. contortus* (Eng *et al.*, 2006). Three amino acid substitutions (M117L, V120I and V124A) located in the H3 helix of the isotype I β-tubulin gene of *Onchocerca volvulus,* have been found consistently in IVM-selected worms. Furthermore, an effect of IVM selection across the region encoding amino acids 195 to 235 of the isotype I β-tubulin gene of *H. contortus* was reported. Moreover, an increase in the frequency of $P200^{Phe/Tyr}$ (but not $P200^{Tyr/Tyr}$) genotypes was apparent following IVM selection in *H. contortus* (Eng *et al.*, 2006). As yet, these finding have not been confirmed in other species or isolates.

There is a need to identify the common molecular mechanisms underlying resistance to provide markers for unambiguous diagnosis at the individual worm level. However, if IVM resistance in the field is caused by a multitude of mechanisms, which may vary dramatically between isolates, then this will undoubtedly impede the search for a reliable marker for IVM resistance.

1.2.6 Levamisole resistance

Resistance to the levamisole (LEV) group is the mechanism we know least about. Studies have focused on assumed modification of the target region. Levamisole (LEV) is chemically

related to, and acts by mimicking, acetylcholine, the neurotransmitter that causes muscles to contract at the neuromuscular junction (Sangster *et al.,* 1991). Hence, these drugs are agonists of the nicotinic acetylcholine receptors (nAChR) and cause an over-contraction of somatic musculature resulting in a 'spastic paralysis' of the worm (Aubry *et al.,* 1970; Coles *et al.,* 1975). nAChR receptors are composed of multiple subunit cation channels and these are well characterised in vertebrates, but less so in nematodes. Numerous nAChR genes have been isolated from *C. elegans*, two of which have been expressed in *Xenopus* oocytes (*unc-38* and *acr-2*) to demonstrate LEV-gated cation channel activity (Lewis *et al.,* 1987). Moreover, *unc-38* mutants were shown to be highly LEV-resistant along with six other genes involved in the production of these receptors (Lewis *et al.,* 1987). It was thought that a change in the number of receptors or a change in the binding affinity of these receptors was involved in resistance of parasitic nematodes (Lewis *et al.,* 1980; Sangster *et al.,* 1988). However, the genes encoding the various components of the nAChR receptor have been studied extensively and as yet no resistant alleles have been found in species of veterinary importance, again suggesting that *C. elegans* is a poor model for resistance in parasitic nematodes. For instance, Hoekstra *et al.* (1997a) examined *Hca1*, a homologue of the *unc-38* subunit between resistant and susceptible *H. contortus* populations using RFLP and found no allelic association with LEV resistance. This method has been criticised when mixed population of worms are used and exposed to an anthelmintic. For instance, if the resistance allele was present, it would have to be common before it was identified due to the relative insensitivity of RFLP (Sangster, 1996).

The question of dominance of the LEV resistance trait also appears inconsistent amongst different species, suggesting that there may be more than one mechanism involved. For instance, it was shown that LEV resistance was a sex-linked (X-chromosome) incompletely recessive trait in *T. colubriformis* controlled by one gene or a closely linked group of genes, however, it was described as an autosomal recessive trait in *H. contortus* (Martin & McKenzie, 1990; Dobson *et al.,* 1996). Hence, a recessive (LEV) resistance trait that is not sex-linked will be slower to develop (i.e. in *H. contortus*) due to the male worms only expressing one copy of the gene phenotypically (Le Jambre, 1985). These findings were supported by field observations that LEV resistance was more widespread in *T. colubriformis* populations than co-existing *H. contortus* populations of Australia (Waller *et al.,* 1995). Furthermore, the pattern of development of LEV resistance in a laboratory population of *H. contortus* was consistent with an autosomal recessive trait (Hoekstra *et al.,* 1997b).

Interestingly, this study also revealed a fitness cost of high LEV resistance, in terms of reduced fecundity per female as well as reduced female survival, which suggested that reversion to susceptibility may occur when selection ceases (Hoekstra *et al.,* 1997b). Furthermore, the results appeared to suggest that a very small proportion of the starting susceptible population propagated the subsequent resistant generations (which was consistent with the findings of Waller *et al.,* 1985), meaning that the high-level resistance alleles were rare in the susceptible isolate. These observations may account for the comparatively low incidence of LEV resistance in the field. Little is known about LEV resistance in *T. circumcincta*.

Finally, suggestions have been made which link the resistance mechanisms of LEV with BZ. For instance, the egg hatch assay was used to show that a BZ resistant *H. contortus* population was twice as resistant to LEV, compared to a susceptible isolate (Hubert & Kerboeuf, 1992). This suggests that the effect of BZ treatment was to select for a sub-population, which also showed an inherently increased level of resistance to LEV prior to exposure with this anthelmintic. This may indicate some linkage between the BZ and LEV resistance loci, perhaps due to location on the same chromosome. However, this is inconsistent with the findings of Donald *et al.* (1980) who showed that a LEV-selected isolate of *T. circumcincta* had a decreased level of resistance to BZ in the egg hatch assay when compared with the same isolate that had been selected with BZ (Donald *et al.,* 1980). If there is linkage between BZ and LEV resistance loci, the implications of such an association would be expected to vary between species due to the apparent difference in trait dominance and probable involvement of multiple mechanisms.

1.3 Phenotypic resistance detection techniques

Phenotypic measures of anthelmintic resistance remain an important part of current laboratory practice, especially considering that the elucidation of mechanisms of resistance is far from complete. They are generally considered to be less accurate than genetic assays (Martin *et al.,* 1989), however, they are generally more cost-effective and easier to run. The benefits of commonly used phenotypic assays for resistance will be addressed in turn.

1.3.1 Faecal egg count reduction test (FECRT)

The FECRT was the first laboratory test for anthelmintic resistance (Borgsteede, 1982) and is still widely used due to its simplicity, cost-effectiveness and applicability to all anthelmintic groups. Two samples are required per farm, one prior to the administration of the anthelmintic and one following treatment with different post-treatment sampling times depending on the drug class (Coles *et al.,* 2006). The reduction in the number of eggs per gram of faeces between these periods gives an indication of the efficacy of the anthelmintic, thus providing a crude measure of drug resistance. Most FECRTs are conducted by veterinarians or specialised laboratories, however, there is now a commercially available faecal egg counting kit (manufactured by FECPAK International Ltd), which farmers can use to self-monitor faecal egg counts and anthelmintic efficacies.

1.3.2 Egg hatch assay (EHA)

The egg hatch assay was conceived by Le Jambre (1976) to monitor the effect of *in vitro* BZ exposure upon the development of nematode eggs. Adaptations of this assay have also been used for assessment of LEV resistance (Dobson *et al.,* 1986). The proportion of eggs which fail to hatch to the L_1 stage at varying drug concentrations is used to plot a dose-response curve, from which egg development values (ED) values can be estimated. For instance, the $ED_{50,\ 96\ \&\ 99}$ values are universal estimates of the concentration of drug required to prevent 50, 96 & 99% of eggs from hatching. Thus, the egg hatch assay was the first method which could ascertain the degree of BZ resistance exhibited by a parasite population. This has provided a useful adjunct to molecular assays to determine whether the gene(s) examined are correlated with the phenotypic expression of resistance.

1.3.3 Larval migration assay (LMA)

The larval migration assay was developed to monitor the *in vitro* response of larvae to anthelmintics which induce paralysis (Wagland *et al.,* 1992). It has been primarily adopted for studies of LEV resistance, however, it has also been used to study the effects of IVM and other ML anthelmintics. This assay involves incubation of larvae in different drug concentrations for a certain period, e.g. two hours, to allow the drug to take effect. The larvae

are then placed above a filter submerged in water, through which uninhibited larvae can normally migrate to reach the bottom of the well. Larvae are left for another period of time, e.g. 16 hours, to allow migration of the resistant worms to occur. The proportion of migrating larvae at different drug concentrations can be used to construct a dose-response relationship. This can be used to estimate $LM_{50,\ 96\ \&\ 99}$ values, which are useful in comparing the resistance phenotype between populations, or in studying the effects of drug inhibitors or synergists.

1.3.4 Larval feeding inhibition assay (LFIA)

A more recent innovation in the field of resistance diagnosis is the larval feeding inhibition assay, which was developed at Moredun Research Institute to examine the phenotypic expression of resistance in *T. circumcincta* against macrocyclic lactones, such as IVM (F. Jackson, personal communication). Since one of the major consequences of IVM exposure is inhibition of the pharyngeal pump, this assay was designed to measure the ability of parasites to feed following IVM exposure. The L_1 stage is used in this assay for two reasons: firstly, they are easy to obtain and maintain under laboratory conditions and secondly, when hatched *in vitro*, they have not had the prior opportunity to feed. Since the free living stages of these parasites (L_1 and L_2) feed on bacteria, then by incubating them with fluorescently labelled *Escherichia coli*, feeding can be determined by examining larvae using a fluorescence microscope. As in all other anthelmintic resistance bioassays, data generated in this assay can be used to calculate LFI values which can in turn be used to compare the phenotypic resistance exhibited by different isolates. Populations can also be fractionated using this technique so that it is possible, for example, to select drug concentrations that allow the most resistant or susceptible fractions of the population to feed. Genetic comparisons can then be made between these two subpopulations to search for genes associated with resistance or susceptibility.

1.3.5 Tubulin binding assay

Assays were developed to determine the magnitude of tubulin depolymerisation by BZ (Lacey, 1988; Lacey & Snowdon, 1988; Lacey & Gill, 1994). At a specific pH and in the presence of GTP, magnesium and calcium ions, tubulin will polymerise to form microtubules at 37°C. Spectrophotometric assays were commonly used to monitor the depolymerisation

process following the addition of various radio-labelled antimitotic drugs. The extent of binding to a known amount of nematode tubulin could be estimated and sensitivity to the drug would relate to the extent of depolymerisation. This technically demanding assay is regarded as being no more sensitive than the egg hatch assay (Martin *et al.,* 1989).

1.4 Molecular resistance detection techniques

Genetic assays are extremely powerful in estimating the extent of BZ resistance within worm populations. Non-genetic assays such as the Faecal Egg Count Reduction Test, *in vitro* egg hatch assay and the tubulin binding assay only have the power to detect resistance when ~ 25% of the population are phenotypically resistant (Martin *et al.,* 1989). This implies that at least 25% of the population are homozygous resistant (due to the apparently recessive nature of BZ resistance at F200Y isotype I β-tubulin mutation). Also, for this to occur, there must be numerous heterozygous individuals each with the opportunity to give rise to more $P200^{Tyr/Tyr}$ progeny. However, the allele-specific PCR can detect resistance alleles when they are at a very low level in the population (i.e. down to the individual worm level). The ability to detect resistance during the earliest stages in its development provides time to employ alternative control strategies. Unfortunately, BZ resistance is now widespread in *T. circumcincta* and amongst other parasites of veterinary and medical importance and so its use in the field will be restricted to those species which have yet to develop resistance.

1.4.1 Diagnostic PCR of F200Y isotype I β-tubulin mutation in BZ Resistance

The BZ-resistant allele appears to be recessive. Elard & Humbert (1999) demonstrated that treatment of all possible genotypes in a resistant population of *T. circumcincta* ($P200^{Phe/Phe}$, $P200^{Phe/Tyr}$ & $P200^{Tyr/Tyr}$) with BZ permitted the survival of only the resistant homozygotes. A diagnostic PCR was subsequently developed to detect F200Y in the β-tubulin isotype I gene (Elard *et al.,* 1999). This assay is based upon the principle that, under stringent conditions, the extreme 3' base of a primer determines its specificity to the template. Thus, if there is not a 3' match between the allele-specific primer and the template, the corresponding fragment will

not be amplified. This multiplex reaction incorporates two non allele-specific primers and two allele-specific primers and the pattern of amplified fragments allows genotyping to be carried out (see Section 2.4.3 for detailed method).

1.4.2 Pyrosequencing of F200Y isotype I β-tubulin mutation in BZ Resistance

Pyrosequencing is a versatile new sequencing technology that can be applied to several molecular approaches including single nucleotide polymorphism (SNP) analysis, DNA methylation, species identification sequencing and inter-population polymorphism analysis. SNP analysis was used in this study to analyse the frequency of F200Y isotype I β-tubulin mutation (see Figure 1.4). The assay requires the initial generation of a biotinylated PCR product (using one modified i.e. 5' biotin-labelled and one unmodified primer). The length of the PCR product does not need to be very large (typically 200bp), but the sequence does need to be accurately known and the position of the SNP predetermined. All elements of primer and assay design are automatically available through the associated Pyrosequencing software (Biotage). The biotinylated PCR products are then bound to streptavidin-coated sepharose beads and denatured. A nested sequencing primer is hybridised to the single stranded DNA template to complete the sample preparation step. The Biotage program is informed of the expected sequence including all possible mutations or indels. When the SNP run begins, an enzyme and substrate mix is dispensed into each reaction. This contains the following: DNA polymerase, ATP sulfurylase, ATP luciferase, ATP apyrase and substrates: adenosine 5' phosphosulphate (APS) and luciferin. Each nucleotide is dispensed into the well sequentially, and if it is not complimentary to the template sequence and incorporated into the new strand, it is degraded by the apyrase enzyme before the next base is added. The DNA polymerase catalyses the incorporation of each nucleotide into the complimentary strand by the same process as in regular PCR. This reaction releases pyrophosphate (PPi), which is converted to ATP by ATP sulfurylase via the substrate APS. The ATP then drives the reaction of luciferin to oxyluciferin by luciferase, releasing light energy that is captured by a CCD (charge coupled device) camera in the machine. The amount of light generated is proportional to the amount to PPi and ATP released; therefore, the height of the peaks (in the Pyrogram output) corresponds to the number of nucleotides of the same type incorporated in turn. The function of the apyrase enzyme is to degrade excess nucleotides and ATP continually, thus, there is no

interference when the next nucleotide is incorporated. If there are mutations to detect, the expectation of all possible complimentary bases is entered by the user. For example, in the case of the F200Y isotype I β-tubulin mutation, the third base expected is either a T or an A, therefore, either an A or T nucleotide is predicted to be incorporated, but all four bases will still be added, one by one, to the reaction. If a G or C nucleotide was incorporated, or the A/T signal was too weak, the sample would fail through the machine's in-built quality control. At the beginning of each assay, the Pyrosequencer automatically releases a non-complimentary nucleotide into the reaction first to ensure that there is no false positive signal. Other controls are included by the user, for instance: sequence primer only; water only; PCR negative controls and a heterozygote positive control.

1.5 Population genetics analysis of parasitic nematodes

Population genetics has been defined as 'the study of structure, function and inheritance of genes and genomes in natural populations' (J. Conway, Chapter 5 in Thompson, 2000). Genes and genomes are influenced by evolutionary forces, including genetic drift, selection, mutation, migration and non-random mating (Hartl, 2000). Commonly, population genetics is used to study the effects of artificial and natural selection upon species and also in the epidemiology of disease. There have been relatively few population genetics studies of parasitic nematodes, with much of the research being applied to bacteria, fungi and viruses of medical importance. Population genetics analysis of parasitic nematodes can aid our understanding of how anthelmintic resistance develops and spreads within and between populations, which is fundamental to our strategies for control.

1.5.1 Population dynamics of nematodes

The population dynamics of nematodes is very complex, and this is predominantly due to their scattered distribution, which maximises the likelihood of transmission to other hosts. Population dynamics also depends upon the number of hosts required to complete the life cycle, the spatial distribution of hosts, the nature of the external environment and upon host factors. In the case of *T. circumcincta*, there is one host required (direct life cycle) and the distribution of hosts depends on the intensity of the farming system. For example, hill farms

would tend to have a more scattered distribution of parasites than lowland farms. This is supported by findings that hill farms in Scotland show lower levels of BZ resistance than lowland farms (Bartley *et al.,* 2001). In terms of the environment, climatic conditions are important for *T. circumcincta*, the free-living stages are more suited to temperate conditions (reviewed by O'Connor *et al.,* 2006). Hence, prolonged heat waves and drought may have a detrimental impact upon the population and possibly the hosts. Host factors also affect the distribution of parasites; for instance, age, sex, reproductive cycle, immunity and feeding behaviour. These factors tend to result in many hosts carrying small parasite burdens whilst a few hosts will have large burdens. This is known as over-dispersal (Anderson & Gordon, 1982). Moreover, at particular times of the year, e.g. in the breeding season, the number of hosts carrying larger burdens may increase as a consequence of the peri-parturient relaxation in immunity.

Categorisation of the different life stages of parasites is necessary for the study of population dynamics. The entire population of nematodes existing within one ecosystem (e.g. on one farm) is regarded as the 'suprapopulation' (Zander & Reimer, 2002), whilst the population living within hosts (on that farm) is termed the 'infrapopulation' (Huyse *et al.,* 2005). The populations distributed across a much wider geographical area, between which gene flow can occur (e.g. on a number of Scottish farms), are known collectively as the 'metapopulation' (Huyse *et al.,* 2005).

1.5.2 Genetic diversity of nematode populations

High levels of genetic diversity are commonly associated with trichostrongylid nematode populations (reviewed by Anderson *et al.,* 1998). Furthermore, it is well documented that trichostrongylid nematodes show considerably greater within-population genetic diversity than that among-populations (Blouin *et al.,* 1992, 1995). The features which prevent them from becoming genetically differentiated are thought to include: their high infrapopulation size; the persistence of free-living infective larval stages; the ability to undergo hypobiosis; the high levels of fecundity; the obligately sexual and promiscuous nature of the adult population; the ability of parasites to colonise different environments; the presence of wild animal reservoirs and the direct life cycle (since paratenic hosts add another tier of selection pressures) (Anderson *et al.,* 1998; Nadler, 1995). All of the above features have the effect of maintaining

a large *Ne* which, under the model of drift-mutation equilibrium is a limiting function of genetic diversity (π). That is, $\pi = 4Ne\mu$, where μ is the mutation rate (Nadler, 1995). Hence, an increase in the effective population size or in the mutation rate will serve to increase genetic diversity. Consider a scenario where there are 500 hosts on a farm, with each host having a modest parasite burden of 1000 female worms, with each female producing 500 eggs per day. Denver *et al.,* (2004) predicted that two mutations occur per genome, per generation in *C. elegans* and assuming that the *T. circumcincta* genome is 100Mb, as in *C. elegans*, then every one worm in a population of 1×10^8 will have two mutations. Hence, the above scenario would constitute a daily output of 2.5×10^8 worms per day and this shows that the resistance allele could potentially arise on a daily basis under selection.

One might argue that the dispersal of *T. circumcincta* offspring occurs in concentrated sites, thus, the individuals within each site would presumably be more closely related (Boag *et al.,* 1989). This might be expected to propagate the rare genotypes that are in existence as aggregations of larvae may well end up (in)breeding in the new host (Saul, 1995). Thus, why is it common to find so little genetic differentiation between hosts? For example, Braisher *et al.* (2004) reported that most of the variance between the three *Teladorsagia spp.* populations studied in the UK occurred within hosts (97.65%), as opposed to within populations (0.75%) or even among populations (1.6%) implying that the worms constituting the infrapopulation were no more closely related to each other than they were to external populations (i.e. in other hosts). However, the wide-ranging nature of host grazing and the accompanied larval intake would prevent inbreeding of the *T. circumcincta* infrapopulation. Furthermore, as mentioned previously, these parasites show great promiscuity and a high level of fecundity, thus increasing the genetic diversity of their offspring. Hence, even though relatively few individuals, in terms of the suprapopulation, are successful in breeding, the effective population size (*Ne*) of such a parasite species is large, and this is likely to maintain the high level of genetic diversity. The size of a trichostrongylid infrapopulation often numbers in the tens of thousands (Grenfell *et al.,* 1987), and thus, an *Ne* of millions is not an unfathomable possibility.

1.5.3 Genetic structuring of nematode populations

Elucidating the genetic structure of parasite populations is important for a number of reasons. For instance, it can indicate whether a species is undergoing divergence which can aid phylogenetic classification. It can also be used to analyse the proportion of organisms in a population with a particular phenotypic characteristic, such as high pathogenicity or drug resistance. This is particularly useful when the genetic loci responsible for these changes are unknown, thus, random or neutral markers which may show an association with these genes can indicate divergence.

An example of population sub-structuring has been reported in a *T. circumcincta* isolate. Gasnier & Cabaret (1996) used an allozyme survey to reveal significant population subdivision within a *T. circumcincta* population from a goat farm in Southern France. The authors proposed that a cryptic species had formed via genetic drift, as this isolate was derived from a flock which had been closed for many years (i.e. no new stock had been introduced since the farm was established). Later experiments using multiple markers (β-tubulin isotype I, ITS-2 of rDNA, MDH-2 locus and the ND4 locus of mtDNA) showed this isolate to comprise two populations: a *T. circumcincta* population capable of infecting both goats and sheep, and a second goat-specific *Teladorsagia spp.* population which could not persist beyond one generation in sheep (Leignel *et al.,* 2002).

Geographical isolation is not the only influence which could lead to population sub-structuring. Given that the free-living stages of trichostrongylid populations are susceptible to hot and cold temperatures and to desiccation which must occur on some occasions (reviewed by O'Connor *et al.,* 2006) and that frequent bottleneck events of the infrapopulation occur due to anthelmintic treatment, it is surprising that very little genetic structuring is observed between isolates distributed though geographical space and time. However, there have been relatively few populations sampled for population genetics analysis, although, those which have been studied include populations separated by continents. For instance, mtDNA studies showed relatively little genetic differentiation (hence, lack of population sub-structuring) between populations of *T. circumcincta* across the United States (Blouin *et al.*, 1992, 1995), and even between the USA and Australia (Constantine, cited in Anderson *et al.*, 1998). This is thought to be a consequence of large *Ne* and also of gene flow, resulting from the extensive movement of stock across continents occurred in recent history (Blouin *et al.*, 1992, 1995).

Interestingly, the global population structure of *H. contortus* does not share the same pattern. Genetic differentiation between populations is generally high, and this is accompanied by genetic sub-structuring, even within the same geographical area (Troell *et al.,* 2006). Whilst being highly speculative, it is thought that in temperate regions, there is a very small *H. contortus* population *in refugia* due to the poor ability of this species to survive winter. Hence, this species follows a more "epidemic" pattern amongst farms in temperate regions, i.e. rapid expansions of the population followed by severe bottlenecks. Hence, despite the high fecundity exhibited by this species, the lack of gene flow and frequent population crashes lead to genetic drift, whereas, *T. circumcincta* generally has a more stable population.

Nadler's (1995) review describes the factors which can increase genetic structure and those which are relevant to *T. circumcincta* include: sedentary definitive hosts (this is analogous to high stocking density) and small *Ne* (which may be affected by anthelmintic selection combined with removal of the free-living population for example by the 'dose and move' practice or by environmental desiccation). Conversely, those factors hypothesised in this review to reduce genetic structure include: resilient life stages with high longevity (either developmental arrest or persistence of the free-living population); low host specificity and opportunities for reservoir hosts and frequent extinction of the free-living population followed by rapid recolonisation events (following extreme environmental conditions for example). The last factor seems counter-intuitive, since the effective population size would be reduced by such an event, but Nadler argues that the comparatively high infrapopulation (which may include arrested larvae) of trichostrongylid parasites which can be endured by the host maintains the level of genetic diversity.

1.5.4 The importance of measuring gene flow amongst nematode populations

Analysing gene flow between populations is fundamental to our understanding of the spread of resistance mutations (Nadler, 1995). *Ne* is affected by gene flow, and thus, *Ne* can be used to estimate the spread of a resistance allele within a population. Furthermore, one can even calculate the probability (P) that the resistance allele will go to fixation using *Ne* in Kimura's (1962) equation: $P = 1 - e^{-2Nsp} / 1 - e^{-2Ns}$, whereby s is the selection coefficient, p is the frequency of the advantageous allele, and N is *Ne*. If this resistance allele has only one copy

in the population, then the probability of it becoming fixed is actually lower than that of a selectively neutral allele. This is because most new alleles arising in a large population will be lost via genetic drift, but if the population is small, and hence, less genetic variation is present, then a new allele may become more frequent (through stochastic evolution) and with continued selective advantage, it will eventually become fixed*. Furthermore, the time that it takes for an advantageous mutation to become fixed is less when *Ne* is lower. Inevitably, when an effective anthelmintic is used it removes 90-100% of the parasite infrapopulation (and if the 'dose and move' practice is employed, the free-living population is also removed); this leads to a severe bottleneck in the parasite suprapopulation. Subsequently, this provides resistance alleles with an opportunity to spread. Some have suggested that flooding resistant parasite populations with unselected worms will restore the overall susceptibility (hence, genetic variation) of the population, allowing us to retain efficacy of the anthelmintics for targeted therapeutic use in the future (Van Wyk & Schalkwyk, 1990).

Returning to the findings of Blouin *et al.,* (1992, 1995), whereby genetic differentiation between populations in different continents was lower than expected, we must assess the evidence that this observation was due to gene flow. Blouin *et al.* (1999) also compared the level of genetic diversity amongst wild animal parasite populations versus domestic animal parasites. The wild animal populations showed far higher levels of differentiation consistent with geographical distance, suggesting that gene flow was responsible for the lack of differentiation between trichostrongylid populations on different continents. In essence, gene flow reduces the rate of genetic differentiation.

1.5.5 Population genetics techniques: Microsatellite analysis

One of the techniques commonly used in population genetics studies is microsatellite analysis. Microsatellites are short, repetitive sequences of DNA usually less than 150bp in length. They can comprise repeats of between one and six base pairs and constitute the most variable regions of DNA in the genome. The vast polymorphic potential of microsatellites between individuals of the same species and of the same population make them suitable markers for studies of relatedness, hence their role in DNA fingerprinting. The number of microsatellites

* Similarly a deleterious mutation also has some probability of becoming fixed in a small population; this explains why some inbred communities experience genetic disorders at a higher frequency than outbred populations (Nadler, 1995).

in an organism is correlated with the overall size of the genome (Hancock, 1996). The high rate of mutation (between 10^{-3} and 10^{-5} per locus and per generation) is a feature of repeated sequences confusing the DNA replication process, causing further error. Intuitively, the larger the repeat sequence is, the higher the mutation rate, hence, the polymorphic potential of the marker (Chakraborty *et al.*, 1997). Microsatellites can be found within both coding and non-coding regions of DNA and play a considerable role in the evolution of the genome by generating quantitative genetic variation. If present in the promoter region of a gene, microsatellites can affect transcription (Kashi *et al.,* 1997), however, the majority of repeats are found in non-coding regions (Hancock, 1995). Some of the uses of microsatellites in population genetics analysis of nematodes will now be discussed.

1.5.5.1 Use of microsatellites in assessing gene flow

There is little doubt as to the usefulness of microsatellites in population genetics analysis of parasites. One particular study in *Echinococcus multilocularis* involved the use of only two markers but the results revealed that this hermaphroditic species does cross-fertilise *in vivo* (Nakao *et al.,* 2003); a matter of long standing contention in the field. These questions are very important in any parasitological field as inbreeding also applies to sexually reproducing species which, as mentioned previously, may undergo population bottlenecks in response to anthelmintic selection and accompanying environmental hazards.

1.5.5.2 Use of microsatellites in analysing population genetic structure

Not only are microsatellites useful in determining the extent of genetic variation in a population as described above, but also in characterizing population structure. For instance, Grillo *et al.* (2007) used microsatellite markers to analyse a French *T. circumcincta* isolate which had previously been shown to contain a cryptic species (Leignel *et al.,* 2002). The results showed great genetic differentiation of this isolate when compared with 12 other *T. circumcincta* populations from the UK and France, showing that microsatellite analysis is a useful technique. Analysis of sub-structuring could be beneficial in studies of MDR populations. If the development of resistance to anthelmintics is not deleterious to the overall fitness of a population then a population will continue accumulating resistance mechanisms to every anthelmintic it is exposed to. However, it is not clear whether MDR of a population is exhibited at the individual worm level or whether there are distinct sub-populations which are

each resistant to one or perhaps two drugs. The latter would imply population sub-structuring, which can be examined using microsatellites.

1.5.5.3 Use of microsatellites as markers for genes under selection

Since microsatellites are ubiquitous and randomly dispersed throughout the genome, they may be linked with genes affected by various selection pressures e.g. "resistance genes" and can thus be regarded as potential markers. If a resistance gene is genetically linked with a 'neutral' marker, then a study of the microsatellite loci between unselected and drug-selected populations of the same parent isolate would demonstrate changes in allele frequencies at that locus only in the drug-selected population.

1.5.6 Population genetics techniques: Single Strand Conformation Polymorphism (SSCP) analysis

SSCP involves the electrophoretic separation of single-stranded nucleic acids on a non-denaturing polyacrylamide gel. Single-stranded DNA is unstable and it takes on a specific conformation which varies depending on the precise nucleotide sequence. Hence, differences in the primary sequence, including subtle point mutations, deletions etc. can affect the secondary structure and conformation of the single-stranded DNA molecule (see Figure 1.5). The conformation and length of the single-stranded DNA fragment used determines the extent of the electrophoretic migration and appearance of each allele on a polyacrylamide gel (Orita *et al.,* 1989). This method can resolve alleles that differ by as little as a single point mutation. Under optimised conditions the migration of these alleles and subsequent banding pattern is consistent, and thus, each allele can be assigned an arbitrary identity. This allows the frequency and combination of alleles to be compared between populations. The benefit of this technique is that it removes the requirement for sequencing a large number of amplicons. Instead, a representative of each allele identified by its SSCP banding pattern can be sequenced. The requirements of a candidate gene for SSCP are that it should be reasonably polymorphic and readily amplifiable by PCR under stringent conditions. The region should ideally be small, between 100 and 300bp, although some studies have shown that regions of over 600bp can be used if there is only one SNP present (Kukita *et al.,* 1997).

1.5.6.1 Advantages of SSCP over other techniques

This technique is rapid, straightforward and high throughput and has many applications in molecular biology. SSCP has been used for species identification, population genetics analysis, molecular evolution (Reviewed by Gasser & Chilton, 2001), diagnosis of medical conditions (Sozen *et al.,* 2004; Chinchang *et al.,* 2005; Tagaki *et al.,* 2005) and analysis of candidate drug resistance genes (Blackhall *et al.,* 1998, 2003; Eng & Prichard, 2005; Eng *et al.,* 2006). It has many advantages over traditional methods such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and direct sequencing, which are known to be either less sensitive, more complicated or time consuming. For instance, RFLP analysis does not always highlight sequence variation when it is present depending on the size of the fragment and the position of the restriction sites relative to the mutations. AFLP is a DNA fingerprinting technique (Vos *et al.,* 1995) which is more sensitive than RFLP and does not require prior knowledge of the sequence composition of the gene of interest. However, AFLP involves a complicated assay design and the use of radioisotopes which raises safety concerns. Moreover, both RFLP and AFLP rely on pooled samples of genomic DNA, thus, rare alleles shown by individual worms may be overlooked by these methods (Beech *et al.,* 1994; Gasser & Chilton, 2001). Direct sequencing, whilst informative, can lead to loss of sequence types through cloning bias and generation of PCR artefacts, which is both time consuming and expensive. SSCP does not involve a complex assay design or the use of radioisotopes, it is very sensitive to mutations and it highlights variation at the individual worm level. Whilst sequencing is required at first to identify the sequences and to subsequently confirm the identity of alleles constituting each haplotype, thereafter SSCP can be used as a simple, rapid diagnostic assay for the presence or absence of particular alleles.

1.5.6.3 Common uses of SSCP

SSCP has already been used in a number of studies to analyse the effects of drug selection upon candidate resistance genes. For instance, a study of the gene encoding the α-subunit of a glutamate gated chloride channel between two unselected, one MOX-selected and two IVM-selected isolates of *H. contortus* was carried out (Blackhall *et al.,* 1998). This technique was also used to analyse the involvement of six candidate resistance genes in IVM-selected versus unselected adults of *Onchocerca volvulus* (Eng & Prichard, 2005). Furthermore, SSCP has been employed in studies of genetic diversity, for example, Ruiz *et al.,* (2004) analysed the

genetic diversity of two geographically disparate *H. contortus* populations (from North America and Spain) in terms of five cysteine protease genes. Four out of five loci were polymorphic and there were highly significant differences between the allele frequencies of each isolate.

This technique has also been used in species identification studies, for example, of the *Schistosoma haemotobium* complex (Kane *et al.,* 2002); of *Trichinella spp.* inter- and intra-specific differences (Gasser *et al.,* 1998) and of the Elaphostrongylinae from cervids of N. America (Huby-Chilton *et al.,* 2006). Moreover, SSCP has been shown to be a reliable method for medical diagnosis of several diseases, for instance, in mutation-detection diagnosis of Thalassemia, (Chinchang *et al.,* 2005) and Hypercholesterolaemia (Sozen *et al.,* 2004).

1.6 Aims of study

This study is concerned with the molecular characterisation of anthelmintic resistance in a MDR *T. circumcincta* isolate *(MTci5).* It is important to understand the mechanisms by which resistance develops and the selection pressures involved. As discussed previously, current non-molecular laboratory tests have insufficient power to detect resistance at lower levels (Martin *et al.,* 1989); this is especially pronounced when heterozygous worms that are phenotypically susceptible cannot be detected. Therefore, sensitive detection of resistance is imperative and provides scope to introduce management techniques which can prevent and/or limit further development and transmission of resistance. Demonstrating proof of concept, BZ resistance can now be diagnosed at the individual worm level possibly providing time to employ appropriate control strategies. Unfortunately, BZ resistance is now so widespread amongst trichostrongylid nematodes of sheep and goats, that this capability is "too little, too late". Nonetheless, the technology and information surrounding the occurrence and spread of BZ resistance is still applicable to other species for which the mechanisms of resistance are likely to be similar and are not yet widespread e.g. *Ostertagia ostertagi* in cattle. Furthermore, this diagnostic test can be exploited to give information on the population genetics and the inheritance of resistance alleles amongst BZ-resistant isolates, which may aid our understanding of how IVM and LEV resistance develops and spreads within a population and whether there is any evidence of cross-resistance at this locus. This research represents part of

a wider study seeking to characterise one of the first ovine triple resistant isolates in the UK. A genetics approach is now required to compliment previous studies on the phenotypic expression of anthelmintic resistance in the *MTci5* isolate.

The aims of each results Chapter are as follows:

- Chapter 3 involves characterisation of the population genetic structure of the *MTci5* isolate using neutral markers and examines the effects of drug selection upon the F200Y isotype I β-tubulin genotype and BZ resistance phenotype of this population.
- Chapter 4 examines the importance of the F200Y isotype I β-tubulin mutation in the expression of BZ resistance of the *MTci5* isolate and explores the relative contribution of other previously implicated resistance mechanisms to BZ resistance.
- Chapter 5 describes a study of the origins and diversity of BZ resistance alleles within the *MTci5* isolate and includes a survey of the genetic diversity of the isotype I β-tubulin gene amongst five other UK *T. circumcincta* isolates.

Chapter 1 Tables & Figures

Figure 1.1: Life cycle of *Teladorsagia circumcincta*. Photo credit: F. Jackson.

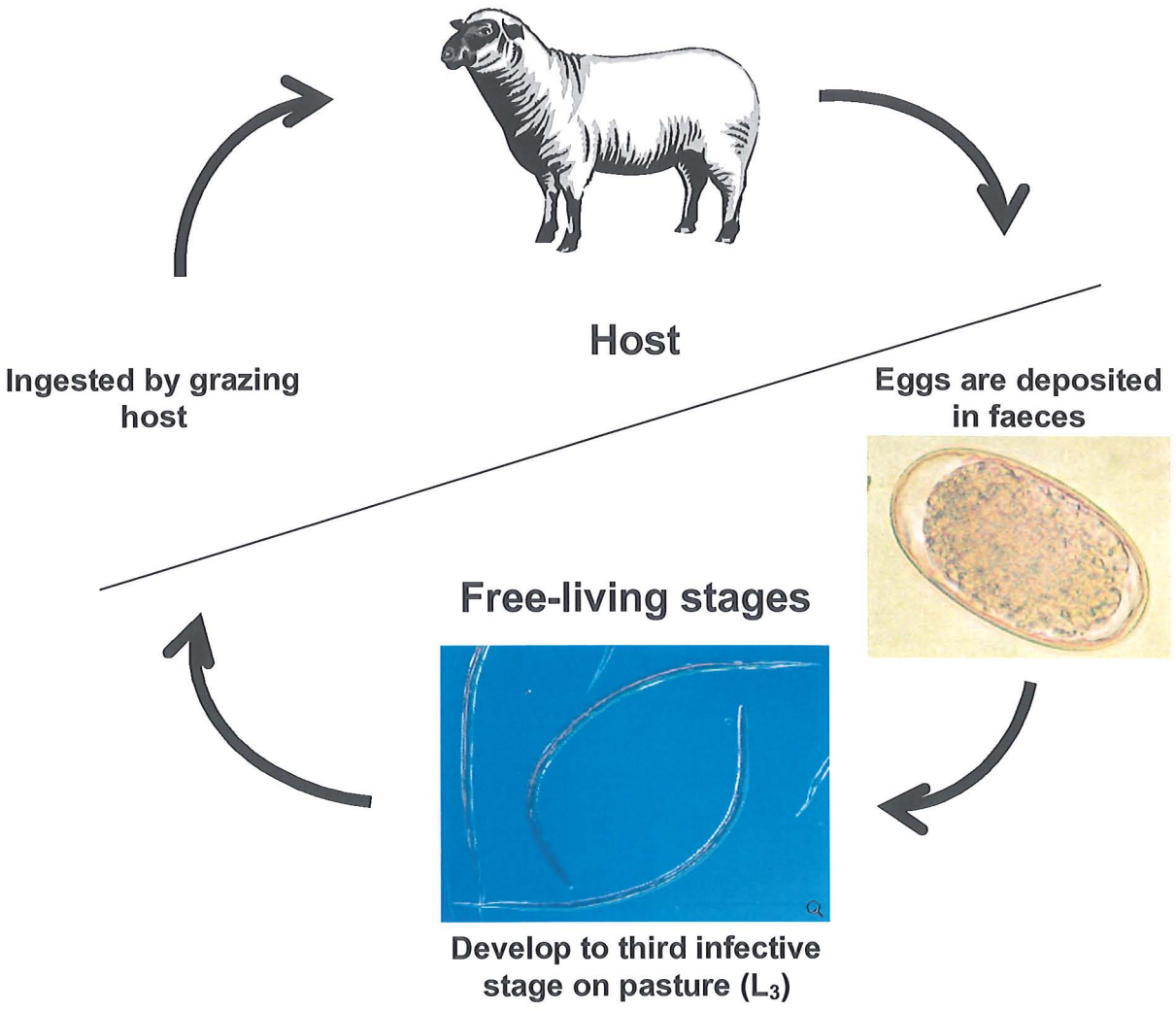


Figure 1.2: Prevalence of anthelmintic resistance across the world in terms of percentage of farms surveyed. BZ, IVM and LEV refer to benzimidazole, ivermectin and levamisole broad-spectrum anthelmintics, respectively. The anthelmintic resistance data was obtained from McKellar & Jackson (2004), Bartley *et al.*, 2004 and Sargison *et al.*, 2001. Map courtesy of www.theodora.com/maps, used with permission.

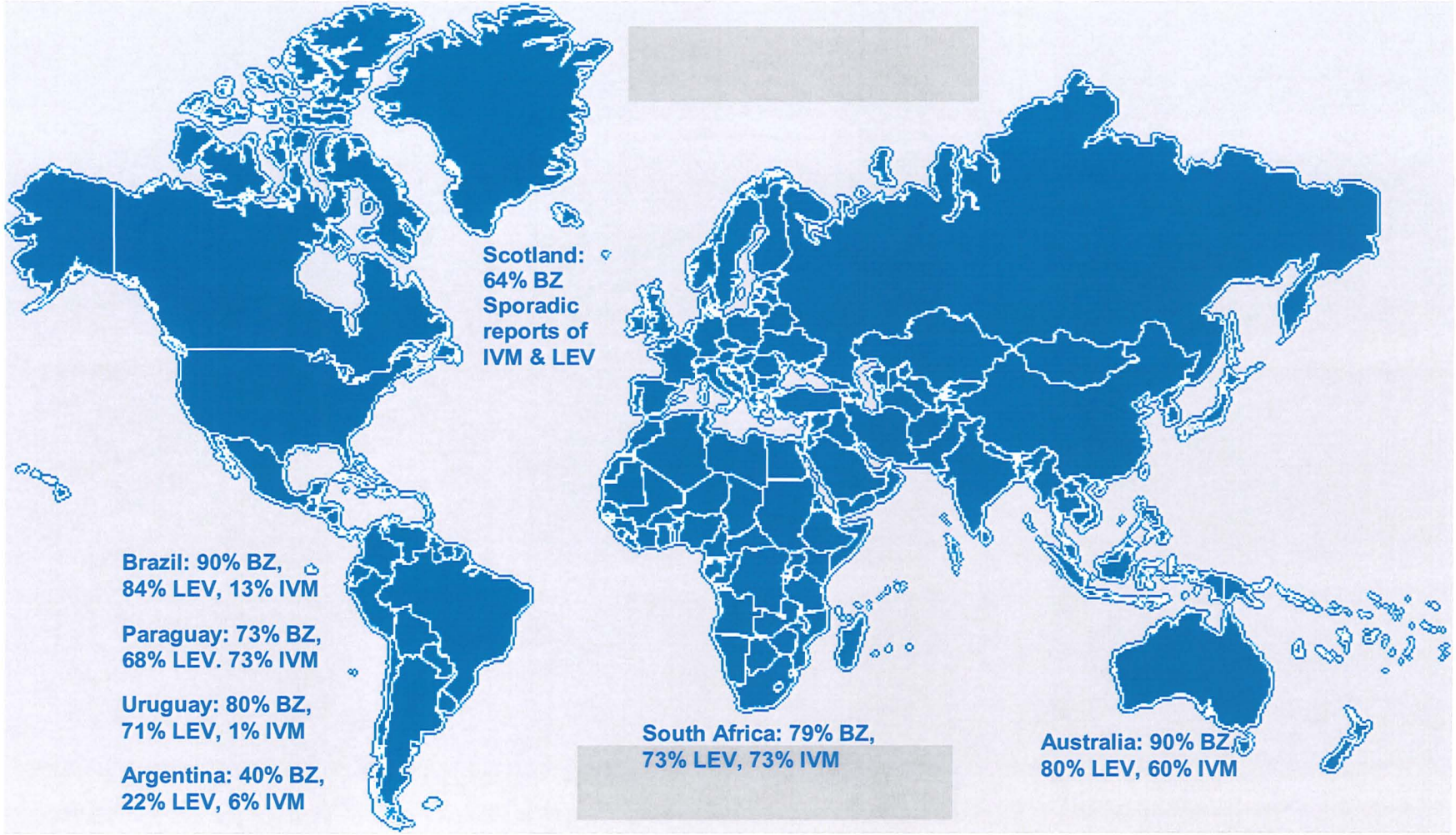


Figure 1.3: Microtubule assembly. There are many isoforms of α- and β- tubulin, which vary between tissues and species (Lacey, 1988); thus they are represented here as a simplified cartoon. The process of dynamic equilibrium shown here was hypothesised by Desai & Mitchison (1997).

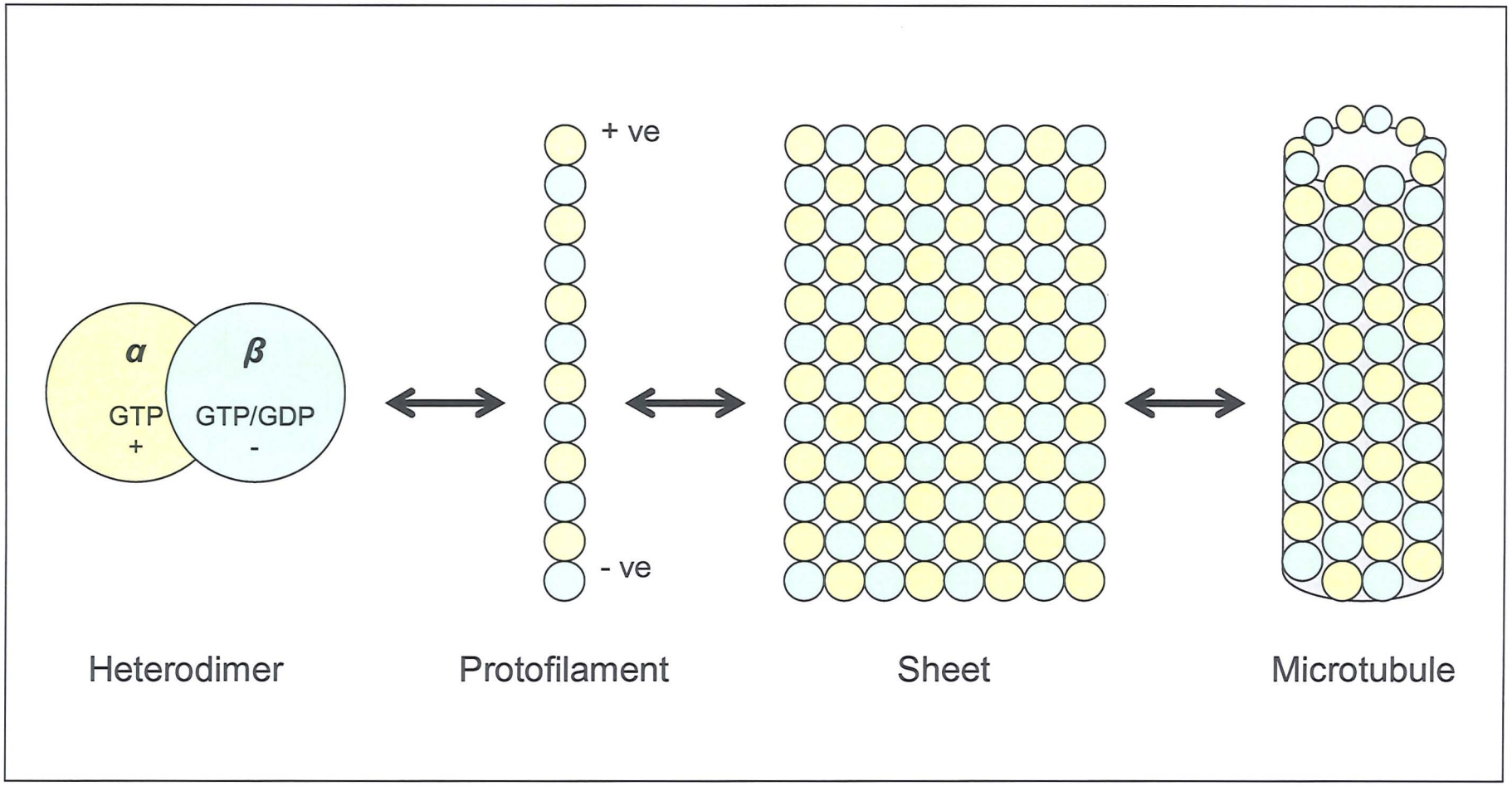


Figure 1.4: Pyrosequencing chemistry. SNP denotes single nucleotide polymorphism. An unexpected (control) nucleotide is always dispensed first, which is not shown here. This is the actual F200Y isotype I *β*-tubulin assay; it is in reverse and SNP 1 is the F200Y polymorphism. SNP 2 is another common mutation found in the *MTci5* isolate. Note that the reaction driven by luciferin (L) produces oxyluciferin (OL) as well as light energy, which is detected by the CCD camera and relayed to the computer which generates a Pyrogram. The Pyrogram shows a single height peak when one nucleotide is incorporated and a double height peak when two of the same type are incorporated sequentially. This diagram is adapted from that shown on the Biotage website (www.biotage.com).

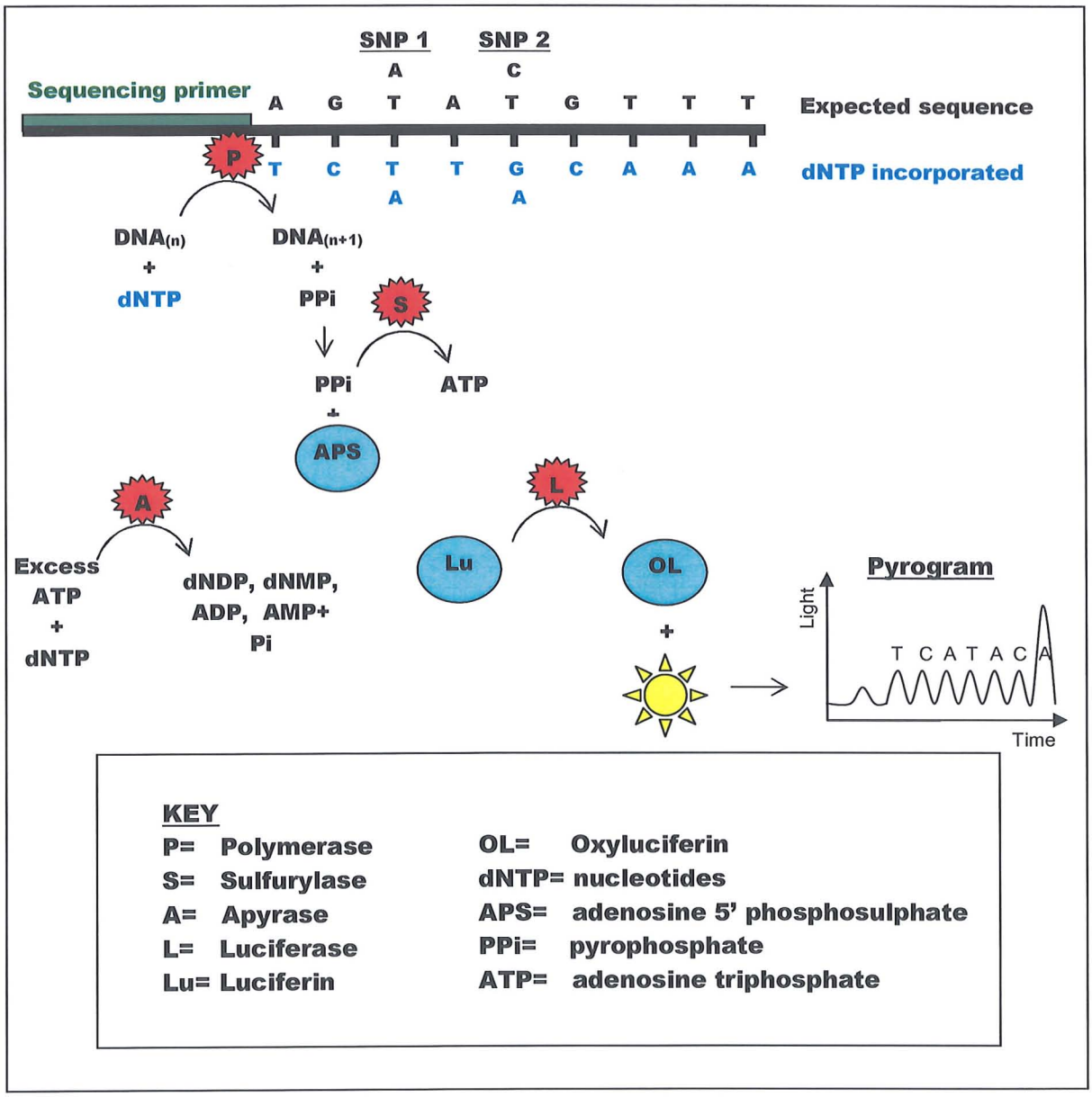
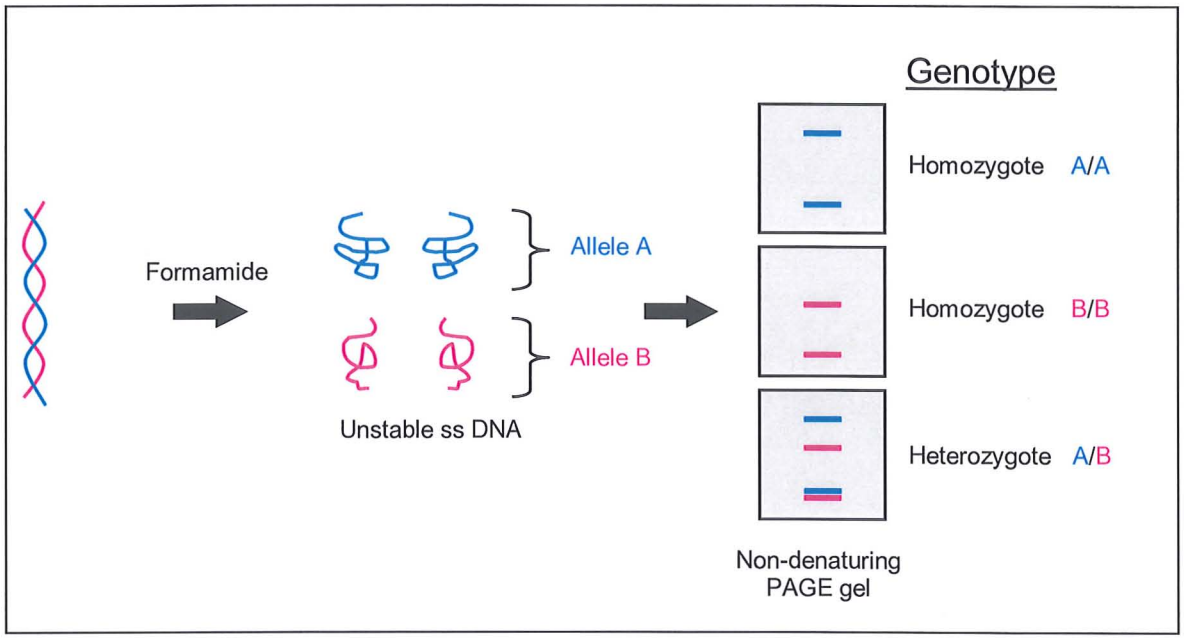


Figure 1.5: Single strand conformation polymorphism (SSCP). This diagram shows the process of double stranded DNA becoming denatured by the addition of formamide and subsequently how this affects the secondary conformation of the single stranded DNA molecules on a polyacrylamide gel. The resulting banding patterns are used to genotype each individual thereafter.



2.0 Chapter 2 Materials & Methods

2.1 General Parasitological techniques

2.1.1 Culture, storage and passage of parasites

2.1.1.1 Infection procedure & passage

To obtain the correct dosage of larvae, the cultures were mixed thoroughly and a sub-sample was collected and centrifuged to pellet the larvae at the base of the tube. The supernatant was removed and the pellet was reconstituted with a known volume of water, i.e. 10ml. After mixing the solution to ensure thorough dispersal of the larvae, three 10μl aliquots were collected and streaked out onto a glass slide. L_3 were counted under the x40 objective and the average number determined for a volume of 10μl. The number of L_3 in the stock solution could thus be estimated. The stock solution was then concentrated/ diluted as necessary to obtain the desired number of L_3 in a small volume and re-counted by the above method. A volume of between 5 and 10ml was best for delivery of parasites to the host. Doses were administered using a syringe (without the needle) over the back of tongue, ensuring that the animal's head was held back to encourage the swallow reflex. Only those holding a Home Office license were permitted to infect sheep, this was carried out by F. Jackson, D. Bartley or A. Donnan. The frequency and number of donors per passage at MRI depends on the isolate (see Section 2.1.6), however the dose of larvae per donor is usually between 10,000 to 15,000 larvae.

2.1.1.2 Coproculture and storage of L_3

Donor sheep were harnessed to allow the continual collection of faeces, from which the parasite eggs were cultured. The faeces were turned out into a shallow tray and covered with a polythene bag, which was punctured to allow air flow. If the faeces were not in pellet form, they were separated into balls and vermiculite was mixed with faeces to absorb excess water in particularly loose samples. The greater the surface area, the more efficient the larval migration was out of the faeces. After a period of ten days at 22 °C, the faeces were flooded with warm tap water (~25 °C) and allowed to soak for 2 -3 hours to allow the larvae to emerge

from the pellets. Then the water was collected and filtered through Baermann apparatus with high wet-strength filter paper (Kleenaroll) to capture the larvae. The filters were then placed into a container with fresh warm tap water ensuring the filter paper was submerged. Larvae were allowed to migrate through the filter for up to 4 hours and would settle at room temperature. The water was aspirated using a vacuum line and the larvae were resuspended in fresh, cold tap water. Larvae were stored at 4°C in plastic flasks with loosened lids. The water was changed every two weeks.

2.1.1.3 Cyropreservation techniques

Pools of L_3 were cryopreserved from each of the treatment groups for the purpose of continuing the selection process in the future. The Van Wyk *et al.* (1990) method for cryopreservation was adopted, whereby larvae were suspended in phosphate buffered saline and snap frozen in liquid nitrogen before transferral to the nitrogen storage facility. Previous tests had shown that 50% of larvae survived the cryopreservation process (D. Bartley, personal communication, unpublished data).

2.1.2 Species identification

Eggs can be identified to family level (Trichostrongylidae) by size and general shape, however, identification of L_3 to genus level is only possible by examination of the head shape and the gap between the tail point and the end of the sheath. Species identification of adult male worms was based upon the spicule characteristics described in the Manual of Veterinary Parasitology Techniques (MAFF, 1986). The issue of cryptic species in the *Teladorsagia* genus is still contentious, the variation in spicule morphology does not necessarily imply a genetic difference, but could be environmental. Just as height varies in humans, spicules could be a plastic indicator of phenotypic variation.

2.1.3 Faecal egg counts

The faecal egg count (flotation) method was based on that described by Christie & Jackson (1982). Faecal samples were collected in polythene bags and weighed. Water was added at 10ml per gram and the pellets were broken up using a stomacher (Seward Medical Ltd) to

allow release of the eggs into solution. A 10 ml sub-sample of the agitated solution was collected and sieved (aperture 1mm) to remove the bulky faecal debris. The filtrate was centrifuged at 1,000 rpm for 2 minutes (Beckman) to pellet the eggs and debris and allow the sample to be cleaned by removal of the supernatant. The pellet was thoroughly resuspended with saturated salt solution and centrifuged again. The supernatant, which contained the eggs, was collected by clipping the flexible centrifuge tube with tweezers just beneath the meniscus and pouring into a cuvette. The cuvette was topped up with more saturated salt solution, sealed with a lid and placed on the bench horizontally to allow the eggs to float up to the surface. The number of eggs (which represents the number of eggs per gram of faeces or epg) was counted using a compound microscope under the x40 objective.

2.1.4 Mass egg extraction

Fresh faeces were collected from the rectum (to ensure egg development had not begun) and were washed through a series of sieves (aperture: 1mm, 500μm, 212μm, 100μm & 38μm). Eggs trapped in the 38μm sieve were separated from the faecal debris by two successive salt flotations (see faecal egg count method, Section 2.1.3) and resuspended in distilled water. The number of eggs recovered was determined by transferring a 100μl sub-sample of the well mixed suspension to a cuvette, which was topped up with saturated salt solution and counted in the same manner as the faecal egg count method. Eggs were refrigerated to prevent development for a maximum of three hours before use in the egg hatch assay. Eggs were allowed to embryonate and hatch at room temperature for use in the larval feeding inhibition assay.

2.1.5 Recovery of adult worms from host

All animals were slaughtered according to the Home Office guidelines by individuals licensed to do so (F. Jackson or D. Bartley). An incision was made along the ventral midline and the entire abomasum was removed by cutting at the junctions of the omasum and ileum. The abomasum was transferred to a clean, 10 litre bucket, opened lengthwise and the folds were washed thoroughly with warm saline (0.85%) to dislodge the adult worms.

2.1.5.1 Manual collection vs. agar technique

There were two methods used to isolate the worms from the digesta. The manual method involved screening a small amount of abomasal contents at a time in a Petri dish under a compound microscope at the x10 objective. Worms were picked out using a hypodermic needle bent into an L shape and transferring to a tube containing fresh 0.85% saline solution. The agar technique is far less labour intensive and time-consuming and involved mixing the digesta with warm, molten agar. The molten agar was made by adding 9g agar powder (Sigma-Aldrich) to 500ml of warm saline and heating to melt the agar. The agar was cooled to 45°C, then mixed with the digesta (up to 1 litre in volume: 1 part sediment to 2 parts saline supernatant). The mixture was then poured onto a mesh on top of a shallow plastic tray (the lid from a 10 litre bucket was used) and allowed to set. The agar-digesta slab was then transferred to a large filter funnel containing warm physiological (0.85%) saline (39°C) ensuring that the fluid was in contact with the agar slab. An incubation of four hours at 39°C allowed the worms to migrate out of the agar into the saline. A tap at the base of the funnel was opened and the saline containing the clean worms was collected. Adult worms sedimented and could be concentrated in a small volume and stored in liquid nitrogen. The latter method yielded worms with much less effort, however, it is thought that the recovery is less than with the manual method.

2.1.5.2 Cyropreservation and defrosting of adult worms

Adults were always suspended in physiological (0.85%) saline and were archived in clumps of around 100 worms. Cryovials with screw cap lids (Nunc, Denmark) were used. Tubes were dropped into a flask of liquid nitrogen to freeze instantly and transferred to the nitrogen storage facility where they were maintained at a temperature of ~ -196°C until required. When adults were removed from storage for DNA extraction, cryovials were allowed to defrost on ice for 30 minutes and then at room temperature for a further 30 minutes to prevent the adults bursting through extreme temperature change.

2.1.6 *Teladorsagia circumcincta* isolates

A summary of the information regarding the *T. circumcincta* isolates used in this study is given in Table 2.1.

2.1.6.1 MTci5 isolate

The *MTci5* isolate originated from Glencorse Farm (a Scottish lowland sheep farm) and initial characterisation showed that *T. circumcincta* was the predominant species. Resistance against all three broad spectrum anthelmintics was reported from this farm and the isolate was brought into MRI for culture in 2002. This isolate is passaged two to three times per year at MRI. The *MTci5* isolate has been the primary focus of the characterisation work in this study (see Section 3.1.1 for more information on the history of this isolate).

2.1.6.2 Other resistant isolates (MTci3 & MTci4)

MTci3 is a BZ resistant isolate which was obtained from lambs at Firth Mains farm (near Penicuik) in 1983. *MTci4* is resistant to both BZ and IVM and was isolated from goats on Sourhope farm (near Kelso) in 1991. Both isolates are passaged at least once a year at MRI (see Section 5.1.5.1 for more information on the history of these isolates).

2.1.6.3 Susceptible isolates (MTci1, MTci2 & ScKiTc)

MTci1 is a drug susceptible isolate, which was collected in 1979 from lambs on a Midlothian farm (Central Scotland). It has been maintained in the laboratory and passaged at least twice a year at MRI. *MTci2* was obtained from the Central Veterinary Laboratories (Weybridge) in 2000 and is passaged three to four times per year at MRI. It is known to be susceptible to IVM and LEV, however, mild BZ resistance is suspected. Adult worm lysates comprising a mixture of *Teladorsagia* spp, (supplied by V. Grillo, J. Gilleard, B. Craig and J. Pemberton, Universities of Glasgow and Edinburgh) represent the *ScKiTc* isolate. These worms were isolated from the wild *ScKiTc* sheep on the island of St Kilda, and whilst this is classed as a susceptible isolate, it is thought that some sheep may have been dosed with anthelmintic in the past. Therefore, it is uncertain how much selection these parasites have encountered (see Section 5.1.5.2 for more information on the history of these isolates).

2.2 In vitro bioassays and associated data analysis

2.2.1 Egg hatch assay

Following the mass egg extraction procedure (Section 2.1.4), 24 well plates were set up with increasing concentrations of thiabendazole (TBZ, Sigma-Aldrich) dissolved in neat dimethyl sulphoxide (DMSO, Sigma) with approximately 100 eggs per well. A stock solution of TBZ at 1000µg/ml was used to prepare the dilutions in neat DMSO. Stock solutions were disposed of after three months and dilutions were disposed of after one day's use. Each concentration was carried out in duplicate. DMSO and water controls were also included. Plates were incubated for 48 hours at room temperature to allow hatching and samples were fixed with 20µl of helminthological iodine (see Appendix) to preserve samples for enumeration of eggs and first stage larvae (L_1). Subsequently, universal ED_{50}, ED_{96} & ED_{99} values (concentration of TBZ at which 50, 96 & 99% of eggs fail to hatch, respectively) were calculated based on the WAAVP method (cited in Coles *et al.,* 1992). This assay was performed upon unselected and drug-selected populations. The results were analysed to show the extent of increased phenotypic expression of BZ resistance in each of the groups. A resistance factor (RF) was calculated (see below) within the isolate using the control group for comparison and a second RF was determined using a known BZ susceptible isolate (*MTci1*).

Resistance factor (RF) = mean ED_{50} value of test group/ mean ED_{50} value control

Latterly, it was discovered that the TBZ disassociated in part (~40%) with the DMSO after a period of 24-48 hours (J. Small, personal communication, von Samson-Himmelstjerna *et al.,* WAAVP abstract, 2005). Thus the concentrations of TBZ that the eggs were exposed to were likely to be at least 40% less than expected. However, since many of the egg hatch assays were performed in the same manner, the error is likely to be consistent within and between these assays, and thus, these data are still informative. The data were not corrected for the reduction in TBZ concentration, as this could not be accurately determined. Latter experiments (which reveal more accurate ED values) have not been compared with earlier assay data. In the more recent assays (Section 4.6), the TBZ solution was made up in DMSO and kept at room temperature for 48 hours before high performance liquid chromatography

was used to accurately determine the concentration. Oxibendazole was used as an internal standard and these experiments were performed by J. Small and D. Jones at MRI.

2.2.1.1 DNA extraction from eggs and L_1 from the EHA

On occasion, eggs and L_1 were required from the egg hatch assay for F200Y isotype I β-tubulin genotyping to provide a comparison with the observed phenotype of the population as determined by the assay. In this case, the samples were not fixed with helminthological iodine (as this would preclude efficient amplification of DNA), but were instead counted in a Petri dish and transferred individually into 25μl lysis buffer containing proteinase K as described in Section 2.3.1.

2.2.1.2 Use of inhibitors in the EHA

A variation of the egg hatch assay was performed to determine the effects of addition of inhibitors upon the *in vitro* expression of BZ resistance. Piperonyl butoxide (PB), an inhibitor of the oxidative metabolic enzymes cytochromes P450, and verapamil hydrochloride (VPL), an inhibitor of p-glycoprotein, were used (Sigma-Aldrich). Further information regarding the use of these inhibitors is given in Section 4.6.

2.2.2 Faecal Egg Count Analysis and Reduction Tests

Faecal egg counts (FEC) were conducted (see Section 2.13) at regular intervals for approximately ten days prior to, and following, drug treatment. The standard faecal egg count reduction test (after Coles *et al.,* 1992) was performed, which is a phenotypic estimation of the efficacy of the drug upon reducing total egg output. In this analysis, the mean egg counts were calculated for each animal before and after treatment. The WAAVP method was employed, whereby the treated animals' mean egg counts were corrected relative to that of the control. Then the percentage reduction for each group was calculated across those means. According to the WAAVP guidelines, if an anthelmintic showed less than 95% efficacy in reducing FEC, this was evidence of resistance.

Statistical analysis was also performed on the FEC data using Minitab 13. These analyses were based on a minimum of five pre- and post- treatment data points. These data were first tested for equal variances in Minitab v14 (2006) and then analysed via paired t-tests to

indicate whether there were any significant reductions in faecal egg count following each drug treatment. This test was carried out for each donor animal (thus each *MTci5* population) and for each trial.

2.3 General molecular techniques

2.3.1 DNA extraction and storage

2.3.1.1 Extraction from immature stages

Eggs, L_1 and L_3 were lysed for DNA extraction by the same method, although the L_3 required an initial step to exsheath them. This was achieved by adding 200μl Milton's sterilising fluid (active ingredient: sodium hypochlorite) to 5ml water containing the larvae and incubating at room temperature until most of the larvae had exsheathed. The progress of exsheathing was monitored under the microscope to ensure that the larvae were not exposed to sodium hypochlorite for a prolonged period which can be damaging. Subsequently, they were centrifugally washed in water to remove the sodium hypochlorite. Eggs, L_1 and exsheathed L_3 were individually transferred to 96 well plates containing aliquots of 25μl lysis buffer (50mM KCl; 10mM Tris (pH 8.3); 2.5mM $MgCl_2$; 0.45% Nonidet P-40; 0.45% Tween- 20; 0.01% gelatine and 5% proteinase K (Bioline). Plates were sealed and incubated at 60°C overnight to allow complete lysis to occur. The lysates were then heated for 15 minutes at 95°C on an ABI 2700 thermocycler to prevent further Proteinase K activity and stored at -20°C.

2.3.1.2 Extraction from single adult worms

Crude lysis of adult worms, by the method described above, did not allow efficient PCR amplification of DNA. Therefore, purified DNA extraction from single adult worms was achieved using minipreps (QIAquick DNA mini kit, Qiagen) and eluted in 100µl volumes. All worm lysates were given an identity and could thus be used in multiple applications to provide information on the same individuals. Only male adult worms were used in the molecular analyses to prevent contamination of genotypes that can occur from eggs when isolating DNA from females. Furthermore, morphological identification of males is easier than that of females, and thus, we have more confidence in male worms being *T. circumcincta* rather than *T. trifurcata*. DNA samples were stored at -20°C.

2.3.1.3 Extraction from pooled adult worms or L_3

Genomic DNA was extracted from pools of mixed sex adult worms for use in testing new primers/targets by PCR. Frozen pellets of worms were ground to a powder using a mortar and pestle. Liquid nitrogen was continually poured onto the worms to prevent them from defrosting. The Stratagene DNA extraction kit was used thereafter, which included Pronase and RNase enzymes. The DNA was precipitated to remove the protein using absolute ethanol and a number of centrifugation steps before being dissolved in TE buffer (pH 8, see Appendix) and stored at -20ºC.

2.3.2 Polymerase chain reaction

The same reagents and suppliers were used for all PCR assays. Platinum *Taq* DNA polymerase (Invitrogen) was selected for its high fidelity and high yield benefits and worked well with Roche dNTP mix. Primers were always supplied by MWG Biotech and molecular-grade water was obtained from Fisher Scientific. An ABI 2700 thermocycler (Applied Biosystems) was used for PCR cycling. Further information regarding individual PCR assays is given later.

2.3.2.1 Prevention of contamination

Contamination with commonly amplified PCR products is always a risk. Certain precautions were taken to minimise the number of contamination events, although they inevitably happened. A designated UV workstation (Thistle Scientific) was latterly introduced, which replaced a laminar flow UV hood. Contamination was more difficult to remove from the laminar flow cabinet; it is thought that somehow the DNA contaminants could persists in the air filtration system. The UV workstation had a dedicated set of equipment (pipettes, racks, consumables etc.) that did not leave the hood and no new equipment was introduced from contaminated areas. Contaminated areas included labs where post-PCR materials were used e.g. for gel electrophoresis, culture of transformed competent cells etc. The workstation was treated with UV for at least ten minutes prior to and following use. Additionally, the product DNA Zap (Ambion) was used to spray all surfaces and equipment and then rinsed with water and dried with disposable towels after using the workstation. Reagents were divided into aliquots in order that in the event of contamination, a small volume of each reagent (i.e. the aliquots currently in use) could be discarded without contaminating and wasting entire stocks.

Molecular grade water (Fisher Scientific) was used (stored in aliquots) to remove any risk of introduction of a contaminant from the in-house water purification system. Designated lab coats were provided solely for use at the UV workstation and any samples containing amplified or large amounts of DNA were stored in a different lab. Accordingly, the template for positive controls was added in another lab. During the set-up of a batch of reactions, negative controls (PCR mix without template; included 1 control per 10 reactions) were open to the air to ensure that any contamination would be detected if it were present.

2.3.3 Gel Electrophoresis and visualisation of products

2.3.3.1 Agarose gels

For examination of PCR fragments, 1.5 to 3% (depending on the resolution requirements) agarose gels were used. An agarose gel stock was made by dissolving powdered agarose (Sigma-Aldrich) in 1x TAE solution (see Appendix). Until recently, ethidium bromide (Sigma-Aldrich) was used to bind/stain DNA at a concentration of 1μg/ml, however, Gel Red stain (Biotium) was a safer alterative and showed greater resolution of bands under UV exposure (AlphaImager 2200, Alpha Innotech). Gel Red was diluted 1:3 (v/v) and used to make up the 1x TAE solutions to which the agarose powder was added. Electrophoresis was carried out in horizontal gel tanks (Thistle Scientific) and a 1kb DNA ladder (Roche) was included (per row of wells) to allow estimation of product size. Depending on the size of the gel and the number of combs used, electrophoresis was carried out for 30 minutes to an hour at between 50 and 100V, to ensure sufficient separation of fragments and markers. Agarose gels were viewed under UV exposure using an AlphaImager 2200 (AlphaInnotech).

2.3.3.2 Polyacrylamide gels

Polyacrylamide gels were required for the single strand conformational polymorphism (SSCP) technique. For optimal resolution, 20% (37.5:1) polyacrylamide: bisacrylamide (Severn Biotech) gels were prepared (see Appendix). Ethanol-cleaned (17 x 15cm) glass plates with 20 well combs were poured at an angle to ensure no air bubbles were present. Gels were allowed to solidify at room temperature before use. All gels were run for 24 hours at a constant voltage of 120V and 100mA in 1 x TBE buffer in vertical electrophoresis tanks (Thistle Scientific). Subsequently, gels were carefully removed from the glass plates and soaked in 1μg/ml ethidium bromide stain or latterly Gel Red (1:3 v/v, Biotium), for a

minimum period of 30 minutes and scanned using an AlphaImager 2200 (AlphaInnotech) using UV light at a low density setting. Images could be enlarged for analysis and background noise could be removed to sharpen the resolution using Quantity One software (v 4.5, Bio Rad laboratories).

2.3.4 Cloning procedure

2.3.4.1 Isolation & purification of DNA fragment

PCR products were always run on 1.5% agarose gels before cloning. If there were multiple bands, those of interest were excised from the gel and the DNA was extracted using the Qiagen Gel Extraction Kit and DNA was eluted in 30μl of molecular grade water (Qiagen). If a single band was generated, PCR products were cleaned directly using the Qiaquick PCR Purification Kit (Qiagen) and DNA was eluted in 30μl of molecular grade water (Qiagen).

2.3.4.2 Ligation

The PGEM-T Easy vector (Promega) kit, which contains a lac Z gene and a sequence encoding ampicillin resistance, was used to ligate all PCR products. Each 10μl ligation reaction (containing 5μl 2x Ligase buffer, 1μl PGEM-T vector, 1μl Ligase enzyme and 3μl of PCR product) was carried out overnight at + 4°C. Ligations were stored at -20°C.

2.3.4.3 Transformation & culture of competent cells

JM109 competent cells (Stratagene or Promega) were transformed with the ligated plasmid vectors. Competent cells were stored at -80°C and thawed on ice (re-frozen cells were never used for transformation). Sterile eppendorfs were chilled on ice before adding 30μl of competent cells (without vortexing) and 2μl of a ligation reaction. They were incubated on ice for 30 minutes before the heat shock procedure was carried out (exposed to 42°C in a water bath for 45 seconds and returned to ice) to allow the transformation to occur. To revive the cells, 200μl of SOC medium (see Appendix) was added to each eppendorf under aseptic conditions and shaken gently in an incubator for an hour at 37°C. A further 200μl of SOC medium was added before spreading out 200μl of the cell culture onto an agar plate. LB agar plates containing 1x Ampicillin (Stratagene) were used, the surfaces of which were treated with 40μl (40mg/ml) X-Gal (Promega) and 40μl 1M IPTG (Bioline) and allowed to dry slightly in a laminar flow dryer to prevent condensation forming on the lid. IPTG is the activating compound for the Lac-Z gene, whilst X-Gal is the substrate. These compounds

ensure that the bacterial colonies will appear white if the cell from which the colony derives was transformed successfully with a plasmid containing the Ampicillin resistance feature. Hence, when the colonies were selected for culture, only white colonies (as opposed to blue) were picked and incubated in LB broth (containing 0.1x Ampicillin).

2.3.4.4 Purification of DNA for sequencing

Between 1 and 5 ml of the broth culture was centrifuged at 13,000 rpm for 10 minutes to pellet the cells. The double stranded plasmid DNA was extracted from this pool of bacteria using the Wizard Plus SV Minipreps DNA purification kit (Promega) and eluted in 60μl of distilled water.

2.3.5 Preparation of cDNA

2.3.5.1 Total RNA extraction using Trizol reagent

A pool of cryopreserved adults (mixed sex) was used for extraction of total RNA, which was subsequently used to make cDNA. The pellet was first weighed and then ground into a fine white powder using a mortar and pestle; liquid nitrogen was frequently added to prevent the sample from thawing and degrading. When the liquid nitrogen had evaporated, 2ml of Trizol reagent (Invitrogen) was added per 100mg of material. The Trizol solidified and the sample was ground in the mortar until the liquid had become clear again. The samples were incubated at room temperature (between 15 and 30°C) for five minutes to allow complete dissociation of nucleo-protein complexes. The solution was split into 1ml aliquots and centrifuged at 12,000 x g for 15 minutes at 2-8°C. This separated the solution into three phases: a lower red phenol-chloroform phase; an interphase and a colourless upper aqueous phase. The upper aqueous phase constituted about 60% of the total volume and since the RNA was found exclusively here, this phase was removed to clean eppendorfs. Isopropanol was added at 500μl per 1ml Trizol originally used and the tubes were incubated at 15 to 30°C for 10 minutes. Samples were then centrifuged at 12,000 x g for 10 minutes at 2-8°C. The RNA formed a gel-like pellet at the base of the tubes and the supernatant was gently aspirated and discarded. The pellet was washed with 1ml 75% ethanol per eppendorf and mixed by vortexing. The pellet was re-formed by centrifuging at 7,500 x g for 5 minutes at 2 to 8°C. The pellet was air-dried in a sterile incubator to remove traces of ethanol and dissolved in 100μl DEPC-treated water

(Fisher Scientific). The same water sample was transferred to each tube carrying over the dissolved RNA so that it could be concentrated in a relatively small volume. The RNA sample was finally incubated at 55°C for 10 minutes before the concentration was estimated on a GeneQuant (Amersham Biosciences) spectrophotometer. The quality of the RNA was checked by running 2μl on a 1.5% agarose gel. An RNase inhibitor was added (1μl of RNasein added, Promega) to prevent the RNA being degraded by enzymes and was stored at -80°C.

2.3.5.2 cDNA synthesis using BD Smart kit

The BD Smart PCR cDNA synthesis kit (Clontech) was used to make cDNA from 1μg of pure RNA. The steps involved first strand cDNA synthesis by reverse transcription, followed by long distance PCR. Three reactions were carried out and later pooled to maximise the yield of cDNA without using too many PCR cycles. One cycle of 95°C for 1 minute was followed by 19 to 21 cycles (depending on concentration of RNA) of: 15 seconds at 95°C (denaturing phase); 30 seconds at 65°C (annealing phase) and 6 minutes at 68°C (extension phase). The double-stranded cDNA was visualised on a 1.2% agarose gel with ethidium bromide staining (Promega) to ensure quality. The Qiaquick PCR purification kit (Qiagen) was used to purify the cDNA before the concentration was estimated on a GeneQuant spectrophotometer. The cDNA was used for testing new primers and as a positive control and proved particularly useful when attempting to isolate isotype II β-tubulin (see Section 4.5).

2.3.6 Sequencing

The concentration of DNA was estimated using a Genequant spectrophotometer (Amersham Biosciences) and approximately 1ng of double-stranded plasmid DNA was sent for commercial sequencing using SP6 and T7 universal primers (MRI sequencing service/ Imperial College/ MWG Biotech). Direct sequencing of PCR products was occasionally employed in order to obtain the entire sequence length of the product.

2.4 Molecular assays and associated data analysis

2.4.1 Analysis of population genetic structure using microsatellites

2.4.1.1 PCR amplification of microsatellites

Five microsatellite markers derived from studies in *Haemonchus contortus* (provided by V. Grillo, University of Glasgow, Scotland) were used for population genetics analysis of adult male worms from the *MTci5* isolate (see Section 3.3). PCR amplification of microsatellites was carried out using the following recipe and primers. A 20µl reaction contained 2.5µl lysate (1:20 dilution of an adult male worm lysate), 11.1X reaction buffer (see Appendix), 2% Tween-20 (Sigma Aldrich), 0.5µM of each primer (MWG Biotech) and 0.03 units of Platinum *Taq* DNA polymerase (Invitrogen). PCR cycling was carried out on an ABI 2700 thermocycler (Applied Biosystems) with one cycle of 94°C for 3 minutes; 40 cycles of 94°C for 15 seconds, T_{ann}°C for 30 seconds and 72°C for 1 minute; followed by a final extension step of 15 minutes at 72°C. The annealing temperatures and primer sequences are given in Table 2.2 for each locus. The forward primers were labelled with either HEX or FAM fluorescent dyes (MWG Biotech). A subset of the PCR products was visualised on a 1.5% agarose gel to ensure the reactions had worked efficiently and to check for contamination in the negative controls.

2.4.1.2 Genotyping of microsatellites by capillary electrophoresis

PCR products were diluted 1:200 with distilled water and 1µl of each sample was mixed with 20µl HiDi Formamide (Applied Biosystems) and 0.25µl of Genescan ROX 400 internal size standard marker (Applied Biosystems) in a 96 well microtitre plate (Applied Biosystems). Samples were denatured on an ABI 2700 thermocycler (Applied Biosystems) for 5 minutes at 94°C and then stored at 4°C until ready for analysis. The genotyping was performed using a capillary sequencer (ABI 3100 Genetic Analyser, Applied Biosystems) in conjunction with Genescan software (Applied Biosystems), which gave an accurate estimation of PCR product size (accurate to 2 bases). The output was in the form of a chromatogram for each sample and these data were analysed using the ABI Prism Genotyper 3.7 NT software (Applied Biosystems). The product sizes were noted for each sample and in cases where the signal (peak height) was weak against the background or where contamination was evident, samples would be repeated to ensure the correct genotypes were obtained.

2.4.2 Microsatellite data analysis

2.4.2.1 Allele binning and data handling

Product sizes were assembled into their corresponding binning ranges, essentially their respective integers (set by V. Grillo, University of Glasgow, Scotland) for analysis (see Tables 2.3 to 2.7 for allele binning ranges per locus). These product sizes represented alleles, thus the data were assembled to determine the genotypes of each worm at each of the five microsatellite loci. The Microsoft Excel add-in program, Microsatellite Toolkit (Park, 2001), was used to convert the data into formats which could be entered into other statistical programs and to perform some basic descriptive statistics. The format most commonly used was Genepop 2D for use in the Genepop program (version 3.4, Raymond & Rousset, 1995). The one/two column format was also very helpful for transferring the data into GenAlEx, a more comprehensive microsatellite analysis tool (add-in software for Microsoft Excel, version 6, Peakall & Smouse, 2006). A third program, Convert (Glaubitz, 2004), was very useful for converting text files containing data into formats, which could be read by Arlequin (Schneider *et al.,* 2000) and GDA (Lewis & Zaykin, 1996). When using Convert, the option to compute as 'standard' data was selected as 'microsatellite' data had to adhere to a stepwise mutation model, which is not representative of the microsatellites used here.

2.4.2.2 Null alleles

Null alleles represent those samples that could not be amplified by PCR after numerous attempts. Locus MTG15 was known to display null alleles regularly from previous studies (Grillo *et al.,* 2006, 2007), therefore, all population genetics analysis in Chapter 3 presents the data both inclusive and exclusive of the MTG15 locus.

2.4.2.3 Linkage Disequilibrium analysis

Linkage disequilibrium refers to the non-random association of alleles at two or more loci, not necessarily on the same chromosome (e.g. linkage between microsatellite loci). This test determines the probability of certain allelic combinations occurring together at a frequency greater than expected by chance, i.e. greater than that expected from a population of randomly generated haplotypes. This was perhaps the most crucial of the analyses and was thus performed first. If there was non-random association of markers, these markers would not be suitable for population genetics analysis as each locus would not give a true and independent

representation of the variation between individuals or populations. This test was performed using Genepop option 2 (version 3.4, Raymond & Rousset, 1995) with Fisher's Exact test. Significant p values (<0.05) provide evidence of linkage between microsatellite loci.

2.4.2.4 Allele frequencies and patterns across MTci5 populations

Allele frequencies were calculated using Microsatellite Toolkit (Park, 2001). An allelic pattern summary was generated in GenAlEx, which described the following statistics for each population: the number of observed alleles; the number of private (unique) alleles; the number of less common alleles ($\leq$50%); the number of alleles with a frequency of $\geq$5% and the expected heterozygosity. Expected heterozygosity is also known as gene diversity and is a measure of inbreeding/outbreeding when compared to observed heterozygosity. The number of effective alleles was also estimated, which is the number of equally frequent alleles required to achieve a given level of gene diversity. This is used to compare populations with different numbers and distributions of alleles.

2.4.2.5 Hardy-Weinberg Equilibrium multiple locus analysis

Hardy-Weinberg law states that the genotype frequencies at a given locus will become fixed at a particular value after one generation of random mating. It predicts whether a population is evolving via selection at a particular locus. This model is based on several assumptions:

- The population size is infinite (or large enough for effects of genetic drift to be negligible);
- Subjects are diploid and sexually reproducing; allele frequencies are consistent between sexes (i.e. not sex-linked and containing different copy numbers between sexes);
- Mating occurs randomly within a single population (i.e. assortive mating is not occurring);
- There are no mutations and/or migrations which may affect the allele frequencies within the population and
- All genotypes have equal fitness, i.e. selection is not occurring at any particular locus

This dataset does not violate any of the above assumptions, thus probability tests were performed to determine whether there was any deviation from Hardy-Weinberg Equilibrium using Fishers Exact Test (10,000 runs). Tests were carried out using both Genepop (version 3.4, Raymond & Rousset, 1995) and GDA version 1.1 (Lewis & Zaykin, 2001) programs,

which were always in agreement. Bonferroni corrections were used to test the significance of probability values of less than 0.05, but greater than 0.01. This calculation is based on the assumption that at a significance level of 0.05, one in twenty samples will show significance by chance alone. Hence, by dividing this significance level (0.05) by all samples showing that level of significance, a new significance threshold is created against which the other probability values can be compared.

2.4.2.6 Genetic diversity of MTci5 populations

The population diversity statistics were generated using the Microsoft Excel add-in program Microsatellite Toolkit (Park, 2001) and included expected (He) and observed (Ho) heterozygosities, Wright's F_{IS} statistic (Weir & Cockerham, 1984 method) and associated probability (p) values. F_{IS} (also known as the inbreeding coefficient) is an inter-individual estimate of the deviation in genotype frequencies which is described in terms of heterozygote deficiency or excess.

2.4.2.6 Genetic differentiation between MTci5 populations: Principle Coordinates Analysis

PCA is a multi-locus genotype analysis tool calculated in the GenAlEx (version 6, Peakall & Smouse, 2006) add-in software for Microsoft Excel. This program produced a scatter plot with each point representing an individual worm. The analysis could also be performed at a population level. The pattern of the scattered points indicated whether the worms were closely related (evenly scattered) or showed evidence of sub-structuring (clumping of points in one coordinate). The output showed the information from the first two coordinates. When the cumulative variation in the data is largely explained by those first two coordinates one can have confidence in the analysis. The program used an algorithm originally described by Orloci (1978). The option to preserve individual genotypes (instead of breaking up the genotypes and analysing them at the population level) was used.

2.4.2.7 Genetic differentiation between MTci5 populations: Pairwise (Wright's) F_{ST}

Pairwise FST values were calculated in GDA version 1.1 (Lewis & Zaykin, 2001). Wright's F_{ST} (also known as the fixation index or coancestry coefficient) is an estimate of the loss of heterozygosity in a population that results from gene flow. Its main use is in showing the overall genetic divergence amongst sub-populations. FST values range between 0 and 1,

where 0 defines a panmictic population (i.e. no admixture) and 1 defines complete genetic isolation (non-interbreeding sub-populations).

FST values were interpreted using the following standard guidelines:

0 -0.05	little differentiation
0.05 - 0.15	moderate differentiation
0.15 – 0.25	great differentiation
>0.25	very great differentiation

2.4.2.8 Genetic differentiation between MTci5 populations: Analysis of Molecular Variance

A locus by locus analysis of molecular variance (AMOVA) with 10,000 permutations was carried out using Arlequin version 2.0 (Schneider *et al.,* 2001). AMOVA provides a way of examining the amount of genetic population structure based upon analysis of variance between gene frequencies. This analysis apportions the total variance observed into its covariance components (which are calculated in terms of Wright's F_{ST}) in relation to inter-population differences. It essentially creates a distance matrix between samples. It is a permutational analysis and makes few assumptions about the dataset.

2.4.3 Allele-specific PCR for F200Y isotype I β-tubulin genotyping

The PCR protocol was modified from a published method by (Elard & Humbert, 1999; Silvestre & Humbert, 2000; Humbert *et al.,* 2001; A. Silvestre (INRA, France) personal communication). This multiplex reaction incorporates two non allele-specific primers and two allele-specific primers and the pattern of amplified fragments allows genotyping to be carried out. As Figure 2.1 shows, there are three possible products generated by this combination of primers. This assay is based upon the principle that, under stringent conditions, the extreme 3' base of a primer determines its specificity to the template, and thus, if there is not a 3' match between the allele-specific primer and the template the corresponding fragment will not be generated. Non allele-specific primer 1 (P1) creates the largest fragment of approximately 800bp with non allele-specific primer 4 (P4). P1 also generates a fragment (~600bp) with an allele-specific primer for the susceptible allele (P2) where Phe is present (i.e. $P200^{Phe/Phe}$ and $P200^{Phe/Tyr}$ genotypes). Similarly, P4 generates a small fragment (~300bp) with the resistant

allele-specific primer (P3) where Tyr is encoded (i.e. $P200^{Tyr/Tyr}$ and $P200^{Phe/Tyr}$ genotypes). The P1↔P4 product also acts as an internal standard for the PCR as it is the largest fragment and will not be amplified preferentially over smaller fragments. All three fragments are produced if the individual is a heterozygote and only two fragments are produced for $P200^{Tyr/Tyr}$ and $P200^{Phe/Phe}$ genotypes.

However, the multiplex (four primer) reaction described above was not reliable in this case, therefore, an approach utilising two separate three primer reactions providing either the susceptible or resistant fragment together with the larger internal standard band was adopted. The reaction mixture contained 1/10th reaction volume of lysate (PCR template), 1x Platinum Buffer (10X, Invitrogen), 1.5mM Platinum $MgCl_2$ (50mM, Invitrogen), 0.32mM dNTP mix (10mM, Roche), 1.25μM of each primer: P1, P4 and one of P2S or P3R (MWG Biotech) plus 0.04U Platinum Taq DNA polymerase (5U/μl, Invitrogen) made up in molecular grade water (Fisher Scientific). The non allele-specific primers: P1 5'-GGA ACA ATC GAC TCT GTT CG-3' and P4 5'- GAT CAG CAT TCA GCT GTC CA-3' provided the internal standard band. Probes: P2S 5'-GTA CAG AGC TTC ATT ATC GAT (G/A) CA GA-3' and P3R: 5'-TTG GTA GAA AAC ACC GAT GAA AC (A/G) TA-3' formed the susceptible (P1 and P2S) and resistant fragments (P3R and P4) respectively. The probes are degenerate following close examination of *MTci5* β-tubulin isotype I cDNA sequences (pGEM-T vector, Promega). PCR cycling was carried out on a heat block (ABI 2700 thermocycler) with one cycle of 94°C for 2 minutes; 55°C for 55 seconds; 72°C for 55 seconds, followed by 30 cycles of 92°C for 55 seconds, 55°C for 55 seconds, 72°C for 55 seconds with a final extension step of 10 minutes at 72°C. Each reaction was run on a 1.5% agarose gel and the combination fragments present were used to genotype each worm.

2.4.4 Pyrosequencing

The Biotage package includes a computer with software to design and run assays and to store data from these assays. The PSQ Assay Design software (Biotage) required accurate pre-determined sequence information for the area surrounding the SNP(s) of interest. Approximately 100 bases of sequence on either side of the codon 200 SNP was entered into the program and the SNP was highlighted. The software then generated multiple sets of primers and scored them in terms of their specificity, similarity of melting temperature, and

likelihood of hairpin or dimer formation. These primers were all tested by PCR before the modified (Biotinylated) primers were ordered and the assay was run. Assays were run using Pyromark ID version 1.0 software. Refer to Section 1.4.2 for an explanation of the Pyrosequencing chemistry.

2.4.4.1 Generation of biotinylated PCR products

Biotinylated PCR products were generated using a Biotin-labelled primer and an unmodified primer. An excess of the unmodified primer ensured that all of the biotin primer was used up - an excess of biotin-primer can reduce the efficiency of the Pyrosequencing reaction. Each reaction contained 1/10th reaction volume of lysates, 0.67µM Biotin-forward (MWG Biotech), 2 µM reverse (MWG Biotech), 1x Platinum buffer (10X, Invitrogen), 0.32mM dNTP mix (10mM, Roche), 1.5mM Platinum $MgCl_2$ (50mM, Invitrogen) plus 0.04U Platinum *Taq* DNA polymerase (5U/µl, Invitrogen) made up in molecular grade water (Fisher Scientific). PCR cycling was carried out on an ABI 2700 thermocycler (Applied Biosystems) with one cycle of 94°C for 5 minutes; 45 cycles of 55°C for 30 seconds, 72°C for 30 seconds and 92°C for 1 minute; with a final extension step of 10 minutes at 72°C.

2.4.4.2 Pyrosequencing reaction

In a 96 well plate, 3µl Streptavidin-coated Sepharose beads (Biotage) and 37µl binding buffer (Biotage) were added to each 40µl PCR product and agitated for five minutes at room temperature to allow binding of the biotin-labelled DNA to the beads. The beads were then processed using the customised sample preparation tool (Biotage) attached to a vacuum line i.e. samples were washed in 70% ethanol, denatured in 0.1M NaOH, washed to remove traces of NaOH (Wash buffer, Biotage) and dispensed into the assay plate (Biotage) containing 40µl of 0.4µM sequencing primer per well. Primer annealing was carried out at 80°C on an ABI 2700 thermocycler (Applied Biosystems) for three minutes and the samples were allowed to cool to room temperature before the assay was started. The onboard computer calculated the peak heights and interpreted the data as genotypes. Each well was given a pass, check or fail certificate depending on the signal *vs.* background light absorbed by the charge coupled device (CCD camera). There were a number of controls included in each assay: a positive heterozygote control where possible (it had to be previously determined either by a test-run Pyrosequencing assay or by allele-specific PCR), between three and six negative PCR controls (reaction mix, no template) and one control containing sequencing primer alone.

2.4.4.3 F200Y (and E198A) isotype I β-tubulin assay

The primers used to generate the biotinylated PCR products were: forward, 5'Biotin- ACC TTA CAA TGC CAC TCT TTC TG -3' and reverse primer: 5'- GCG GAA GCA GAT ATC GTA CAG -3'. A degenerate sequencing primer was used: 5'- (A/G)GA GC(C/T) TCA TTA TCG AT(A/G) -3' as the *MTci5* isolate was known to be variable in this region*. The 'sequence to analyse' as determined by the software was: 5'- AG(A/T) A(C/T)G TTT CA. Two positions with potential SNPs are shown in the latter sequence. The first position is the F200Y isotype I β-tubulin SNP and the second is a commonly-occurring SNP in codon 199 of the *MTci5* isolate. This sequence is the reverse compliment of the isotype I β-tubulin gene. Therefore, if the Pyrosequencer read AA, the sequence was actually TT (susceptible homozygote), AT was TA (susceptible heterozygote) and TT represented AA (resistant homozygote). The second position is not an important SNP, in that it does not cause an amino acid substitution, however, the Pyrosequencer must be informed of such discrepancies or the sample would fail. All samples were analysed at position 1 (F200Y) and these results were compared to the AS-PCR data. There have been reports of a SNP, which causes the amino acid substitution E198A (Ghisi *et al.,* 2007). This assay included codon 198 in the sequence to analyse, therefore, if there were any unexpected bases encountered then the computer would flag up that sample as a fail. All fails were investigated and repeated and thus any mutations in codon 198 would have been spotted by this assay.

2.4.4.4 F167Y assay

Following reports of another SNP, F167Y, in association with BZ resistance in *T. circumcincta* (Silvestre & Humbert, 2002), a Pyrosequencing assay was designed to find it in *MTci5*. The primers used to generate the biotinylated PCR products were: forward, 5'-CCG GAT AGA ATC ATG GCT TCA T -3' and reverse primer: 5'Biotin- AGT GGC ATT GTA AGG TTC CAC AAC -3'. A degenerate sequencing primer was used: 5'- CAT GGC TTC ATT CTC (A/C)GT -3'. The sequence to analyse as determined by the assay software was: 5' - TGT (T/A)C CAT CA - 3'. A heterozygote positive control was not used in this assay as there was no resistant isolate with this mutation available.

* A number of *MTci5* worms had been sequenced to generate enough information regarding this region.

2.4.5 Analysis of F200Y isotype I β-tubulin data

2.4.5.1 Estimation of the differences between two genotype proportions test and confidence intervals

The 'estimation of the differences between two genotype proportions' test and confidence intervals* were calculated manually in Microsoft Excel using the equation:

$$95\%\ CI = (a-b) \pm 1.96 * \sqrt{(a * (1-a)/Ta) + (b * (1-b)/Tb)}$$
$$99\%\ CI = (a-b) \pm 2.5758 * \sqrt{(a * (1-a)/Ta) + (b * (1-b)/Tb)}$$

This test was used to compare genotype proportions between the unselected and drug-selected *MTci5* populations. Significant results were determined when the interval did not contain zero (i.e. it rejects the null hypothesis that there is no difference between proportions). Results were examined at the 95% and 99% confidence intervals and the CI estimates are displayed in brackets.

2.4.5.2 Hardy-Weinberg Equilibrium analysis of F200Y isotype I β-tubulin genotype

Genotype frequencies were tested for deviation from HWE using the GenAlEx add-in software (Peakall & Smouse, 2006) for Microsoft Excel. This analysis computes the expected genotype frequencies based on the allele frequencies and compares them to the observed genotype frequencies. A probability estimate of deviation from HWE is also calculated.

2.4.5.3 Pairwise Fst test for genetic differentiation at the F200Y isotype I β-tubulin locus

Pairwise F_{ST} values were calculated in GDA version 1.1 (Lewis & Zaykin, 2001) as described previously (Section 2.4.2.7) to test for differentiation between *MTci5* populations as a consequence of drug selection.

*British Columbia Institute of Technology, probability and statistics tutorials
http://commons.bcit.ca/math/faculty/david_sabo/apples/math2441/toc2003.htm

2.4.6 Isolation of isotype II β-tubulin from *T. circumcincta*

2.4.6.1 Primer design

Initial experiments involved designing primer pairs based on the consensus β-tubulin isotype II sequences from *Haemonchus contortus*, *Cooperia oncophora*, and some cyathostome species (see Table 2.8). Latterly, degenerate primers were designed based on the translated protein sequences of the isotype II β-tubulin gene from some nematode species (this is described in greater detail in Section 4.5). The amino acid homology is high between species belonging to different subphyla (i.e. trichostrongylids and cyathostomins), however, there are 11 positions where the amino acids are consistently different between isotype I and isotype II β-tubulin genes. There were a further 24 polymorphisms that were not isotype II-specific in every species, hence, these regions were not considered for primer design. Three forward and three reverse primers were designed so that the 3' end was specific to the amino acid which encoded the isotype II β-tubulin genes (see Table 2.9).

2.4.6.2 PCR, cloning and sequencing of isotype II β-tubulin gene

Three forward and three reverse primers (see Table 2.9) were synthesised (MWG Biotech) and all combinations were tested upon the *MTci2* cDNA prepared earlier (see Section 2.3.5). Primer controls were included (i.e. every primer combination with no template added) to ensure that primer-dimer bands were not influencing the result. The PCR conditions used were not very stringent, i.e. one cycle of ten minutes at 94°C; 40 cycles of one minute at 94°C, two minutes at 50°C and three minutes at 72°C; followed by an extension step of ten minutes at 72°C. The PCR products were visualised on a 3% agarose gel with Gel Red staining (1:3v/v, Biotium). The most promising primer combinations were the ones which gave the strongest products with the least amount of non-specific priming. These products were selected for cloning in PGEM-T easy vector and sequencing (MWG Biotech).

2.4.6.3 PCR assay for the presence of isotype II β-tubulin in single worms

New 3' isotype II β-tubulin-specific primers (two forward and two reverse primers) were designed based on the sequence information gained from the *MTci2* cDNA (see Table 2.10). All combinations of the primers were tested on cDNA and a few single adults from the unselected *MTci5* population. All four combinations produced clean strong bands. The pair selected for testing was the one which created the largest product and would thus reveal more

information about the isotype II β-tubulin fragments. These were: forward, 5' – GC(A/T) CT(A/G) TT(C/T) CGT CCA GAC AAC TTT – 3' and reverse, 5'- GGT GAC GGT ACC ACC GAG AAG - 3'. All 72 unselected and 72 BZ-selected *MTci5* worms were tested with these primers. It was not deemed necessary to test the IVM and LEV-selected worms since the deletion of isotype II β-tubulin locus is only associated with BZ resistance. Each 15µl reaction contained 1.5µl single worm lysates, 1x Platinum Buffer (Invitrogen), 1.5mM Platinum $MgCl_2$ (Invitrogen), 0.32mM dNTP mix (Roche), 1µM of each primer (MWG Biotech) and 0.04 units/µl Platinum *Taq* DNA polymerase (Invitrogen). There were six negative controls for each population (one of which contained isotype I β-tubulin plasmid and the rest were PCR mix without template) and one positive control (*T. circumcincta* adult worm cDNA). PCR cycling conditions were one cycle of ten minutes at 94°C; 40 cycles of one minute at 94°C, two minutes at 60°C and three minutes at 72°C; followed by an extension step of ten minutes at 72°C.

2.4.7 Single Strand Conformational Polymorphism (SSCP) analysis

2.4.7.1 Analysis of the diversity of the isotype I β-tubulin gene amongst UK T. circumcincta isolates

SSCP analysis was employed to investigate the genetic diversity of the isotype I β-tubulin gene in the *MTci5* isolate and amongst a number of other UK *T. circumcincta* populations. This analysis was also employed to investigate the origins and diversity of BZ resistance alleles, as defined by the presence of the F200Y isotype I β-tubulin allele.

2.4.7.2 Preparation of samples for SSCP

A small region of isotype I β-tubulin gene was amplified using primers: Forward 5'-CCAAAATTCGCGAGGAGTA-3' and Reverse 5'-TTTCAAGGTGCGGAAGCAGA-3' creating a 276bp product. A 20µl reaction contained: 2µl of lysates, 1x Platinum Buffer (Invitrogen), 1.5mM Platinum $MgCl_2$ (Invitrogen), 0.32mM dNTP mix (Roche), 1µM of each primer (MWG Biotech) and 0.04 units/µl Platinum *Taq* DNA polymerase (Invitrogen). PCR conditions comprised an initial melting phase of five minutes at 95°C , followed by 40 cycles of 30s, 30s and one minute with melting, annealing and extension temperatures of 95°C, 55°C and 72°C respectively. A final extension period of ten minutes at 72°C completed the process.

PCR products were mixed with a formalin-based loading buffer (see Appendix) at a ratio of 1:2 and subsequently denatured at 95°C for five minutes. Samples were then transferred directly from the 95°C hotplate onto ice and stored on ice whilst the gels were loaded with 10µl of each denatured product. A 20% (37.5:1) polyacrylamide: bisacrylamide gel mixture (see Appendix) was selected after evaluating the resolution of banding patterns from several different strength gels. Gels were subjected to vertical electrophoresis at a constant voltage of 120V for 24 hours. Following electrophoresis, gels were soaked in 1µg/ml ethidium bromide stain in preparation for scanning. Image enhancement techniques (brightness, contrast etc) were conducted using Quantity One software (v 4.5, Bio Rad laboratories).

2.4.7.2 Classification of alleles

A three step process was used to identify alleles from the *MTci5* isolate. This is described in detail in Section 5.2.2, however, to summarise, this involved initially characterising all novel genotypes in the *MTci5* isolate by assigning each an arbitrary identity. Subsequently, representatives of each novel genotype (or banding pattern) were cloned in PGEM-T Easy vector to isolate individual alleles, which were in turn assigned an arbitrary identity. These alleles were then used as standards to decode other genotypes in the *MTci5* population and from other isolates.

2.4.7.3 Sequencing of alleles

Representatives of each allele type were sequenced from the *MTci5* isolate. Between two and four sequences per allele, per worm were generated from a total of 28 *MTci5* worms. Multiple sequences per allele were required to identify any *Taq*-induced or sequencing errors to ensure that the sequence information was entirely accurate.

2.4.8 SSCP data analysis

2.4.8.1 Analysis of allele and genotype frequencies

Allele and genotype frequencies were calculated in Microsoft Excel. Tests of two proportions were carried out using Minitab v14 (2006) to determine if there were any effects of drug selection upon allele and genotype frequencies.

2.4.8.1 Hardy-Weinberg Equilibrium analysis of SSCP genotypes

The *MTci5* SSCP genotype data were analysed for deviation from HWE using GenAlEx add-in software (Peakall & Smouse, 2006) for Microsoft Excel (as described in Section 2.4.5.2).

2.4.9 Analysis of the isotype I β-tubulin sequence data from the *MTci5* isolate

2.4.9.1 Generating sequence alignments

Sequences were aligned using GeneDoc software (v 2.6003, Nicholas & Nicholas, 1997) into the allele classes that were assigned by SSCP. The sequences representing an individual allele were aligned to give a consensus sequence with any errors having been removed. Up to four sequences per allele were generated in order to correctly identify sequence errors.

2.4.9.2 Descriptive statistics

The program DnaSP (version 4.0, Rozas *et al.,* 2003) was used to generate some descriptive analysis of all sequences in order to identify the similarities and differences between alleles.

2.4.9.3 Nucleotide diversity estimates

Nucleotide diversity (π) is an estimate of the average number of nucleotide substitutions per site between two sequences (Nei, 1987). However, this is not always an appropriate measure since divergence does not follow a linear pattern. This is because mutations occurring at random could occur repeatedly at the same sites, and a simulation of such a model always follows a curve. Jukes and Cantor (1969) accounted for this phenomenon by applying a correction to π, which states that divergence is a logarithmic function of time. Thus, the logarithmic transformation linearises the rate of divergence. Hence nucleotide diversity estimates were calculated both with and without the Jukes Cantor correction using DnaSP software (version 4.0, Rozas *et al.,* 2003). Estimates of variance and standard deviation are also provided by this program.

2.4.9.4 Haplotype diversity estimates

Haplotype diversity (Hd) is a measure of the extent to which alleles (or haplotypes) have diverged from a common ancestor. The longer ago that this divergence began, the greater the divergence between the current alleles will be, and thus, the higher the Hd estimate. It follows that if the Hd estimate is low this suggests that either these alleles have diverged very recently

or that a sudden expansion in diversity has occurred relatively recently. Haplotype diversity estimates were calculated using DnaSP software (version 4.0, Rozas *et al.,* 2003).

2.4.9.5 Haplotype neutrality tests

Tests of neutrality are used to investigate the hypothesis that all mutations are selectively neutral (Kimura, 1983). These tests are all related to the equation: $\theta = 4Ne\mu$ which is the essential parameter of the theory of neutral evolution, where θ represents the rate of mutation amongst a group of alleles, *Ne* is effective population size and μ is the mutation rate per sequence per generation. Haplotype neutrality tests were conducted using DnaSP software (version 4.0, Rozas *et al.,* 2003) and include four different estimates: Tajima's D statistic (1989); Fu & Li's D statistic (1993); Fu & Li's F statistic (1993) and Fu's F Test (1997). Tajima's D statistic is calculated by comparing two unbiased estimates of θ which is the rate of mutation amongst a group of alleles. These estimates are: θ_π which is based on the average number of pairwise nucleotide differences and θ_S which is based on the number of segregating sites. Its expectation under the neutral model is zero. When D is positive it suggests that balancing selection has created an excess of alleles in intermediate frequencies. Fu & Li's D statistic (1993) is calculated using the number of singletons in the dataset (i.e. mutations which appear only once amongst all sequences) as well as the total number of mutations. In contrast, Fu & Li's F test (1993) is calculated using the number of singletons and the average number of nucleotide differences between pairs of sequences. Fu's F test (1997) is based on the haplotype frequency distribution and the estimate of θ, which is given by $4Ne\mu$. An estimate of Strobeck's S statistic is also generated by this program and it is based on Ewens's sampling formula (1972). In this case it gives the probability of obtaining a sample with less than or equal to the number of haplotypes observed.

2.4.9.6 Clustal W analysis

The consensus sequences generated for each allele in each worm (see Section 2.4.8.1) were aligned via the Clustal W method (Higgins, 1994) using MegAlign software (DNA star v5.08, 2004). This analysis was also used to align the *MTci5* sequences with published sequences (Silvestre & Humbert, 2002) using consensus sequences of each novel haplotype.

2.4.9.7 Phylogenetic network estimation using statistical parsimony

Phylogenetic network estimation was performed by V. Hypsa (University of Glasgow, Scotland) on our behalf. This was done using TCS software (version 1.21, Clement *et al.,* 2000)*, a program that can estimate gene genealogies based on statistical parsimony. Data can be entered in NEXUS or PHYLIP formats and the program collapses the sequences into haplotypes. This program calculates the frequencies of haplotypes in a sample and these are used to generate haplotype outgroup probabilities. The latter is correlated with the age of each haplotype, i.e. how ancient each haplotype is. The program then calculates a distance matrix based on all pairwise comparisons for each haplotype in the sample and parsimony estimates are calculated for all pairwise differences until the probability level reaches 95%. Hence, the maximum number of pairwise differences between all sequence pairs is estimated under the assumption of maximum parsimony. This means that the program is estimating the number of evolutionary steps separating each pair of sequences, i.e. the 'connection limit', based on the number of mutational differences between them. The output shows the most parsimonious arrangement, which is generally more accurate than traditional maximum likelihood trees. This shows the genealogy of all sequences including the number of proposed 'missing sequences' or 'steps' separating each haplotype.

* TCS software can be downloaded from http://darwin.uvigo.es/software/tcs.html

2.5 Anthelmintic selection experiments of the MTci5 isolate

2.5.1 Animals

Two trials were carried out, in two successive summers with Dorset-cross, worm-naïve lambs, aged less than 18 months. All sheep donors were male to allow continual collection of faeces into a specially designed harness without contamination with urine. Despite the fact that all lambs were reared indoors under conditions designed to exclude natural infection with GI nematodes all subjects were drenched with ivermectin ('Oramec', Merial, 0.2mg/kg) and levamisole ('Chanaverm', Chanelle Animal Health, 7.5mg/kg) ten days before the study was initiated. Post treatment egg counts were used to confirm that there were no patent infections which might influence the experiments or contaminate the isolate being passaged. Only those animals infected with the *MTci5* isolate were housed in the same pen to prevent cross-contamination. Re-infection (autoinfection) was extremely unlikely since the total faecal output was collected during 5 out of 7 days and the deep straw bedding was replaced weekly. A ration of pellets was provided daily and hay and water were available *ad libitum*, therefore, floor grazing was minimal. Cross-contamination of isolates/ sub-populations was prevented by disinfection of boots before and after entering each pen and hand washing and glove changing after dealing with each animal. The equipment used for coproculture was also washed carefully to prevent cross-contamination.

2.5.2 Trial design

The experimental design is shown in Figure 2.2. Five animals were involved in Trial One (August 2003) and four animals in Trial Two (September 2004). The same experimental design was used for both Trials, except where indicated. On day one of each Trial, sheep were infected with 15,000 L_3 from the *MTci5* isolate. Following patency on day 19 animals were harnessed for the continual collection of faeces for coproculture. L_3 were collected between days 19 and 26 represented the unselected population. On day 26 one animal from each Trial was given one dose of either fenbendazole (FBZ, 'Panacur', Intervet 5mg/kg), ivermectin (IVM, 'Oramec', Merial 0.2mg/kg) or levamisole (LEV, 'Chanaverm', Chanelle Animal Health, 7.5mg/kg) at the manufacturers recommended dose rate. One untreated control animal was killed on day 26 and one on day 42 in Trial One to provide untreated adult worms. Since

subsequent analysis showed there to be no differences between these two control worm populations, this practice was not repeated in Trial Two, and hence, a total of only four sheep were used in Trial Two. Between days 31 and 41* faeces were collected from all animals to provide unselected (control) and drug-selected populations. Larval cultures obtained during this period were pooled per donor, but stored separately as sub-populations of the *MTci5* isolate. On day 42 all of the sheep were killed to allow collection of the adult worms. Adult male *T. circumcincta* worms were cryopreserved for use in molecular assays.

* NB Eggs were not collected for coproculture for five days following drug treatment. This was to prevent eggs being misrepresented as resistant if released by a dead/dying female which was expelled due to the action of the anthelmintic.

Chapter 2 Tables & figures

Table 2.1 Information regarding the *Teladorsagia circumcincta* isolates used in this study.

Code	Origin	BZ resistance	LEV resistance	IVM resistance
MTci1	Moredun Institute farm, Central Scotland	None	none	none
MTci2	Central Veterinary Laboratories, Weybridge, England	suspected	none	none
MTci3	Firth Mains farm, Central Scotland	positive	none	none
MTci4	Sourhope Farm, Scottish borders (formerly Aberdeenshire, Scotland)	positive	none	positive
MTci5	Glencorse Farm, Central Scotland (formerly Scottish Agricultural College)	positive	positive	positive
ScKiTc	Soay sheep, St Kilda, Scotland	none	none	none

Table 2.2 Microsatellite primer information.

Microsatellite locus	Repeat type	Forward primer	Reverse primer	Tann (°C)
MTG15	(CA)n	5'FAM-tgcaaggaaactgctaagaaggag	5'-atcatggaaccttgataccgcaag	58
MTG67	(CT)n	5'HEX-caagtcgtttaggcacgtctgg	5'-cagggcggaacccaattgatcg	58
MTG73	(TCA)n	5'HEX-ccttgtataaattcgaagc	5'-gtagtagtgattaacttccg	45
MTG74	(ACA)n	5'FAM-gatggactcgtggtacagcg	5'-tcaataacgaactaaatatg	45
HCMS28	(CA)n	5'FAM-agtgtggagatgagagagagca	5'-ccgactaatcacttcttgtttg	55

Table 2.3 Locus HCMS28 allele binning information.

Allele Bin	Minimum size	Maximum size	Average size
155	155.02	155.02	155.02
159	158.75	159.92	159.07
161	160.48	161.70	160.87
163	162.57	163.33	162.89
165	164.22	165.85	164.84
167	166.43	167.11	166.80
169	168.43	169.84	168.80
171	170.48	171.13	170.77
173	172.50	173.02	172.74
175	174.43	175.07	174.74
177	176.49	177.00	176.73
179	178.62	178.82	178.70
181	180.57	181.06	180.82
183	182.41	183.44	182.98
185	184.47	184.69	184.61
189	188.62	-	188.62

Table 2.4 Locus MTG15 allele binning information.

Allele Bin	Minimum size	Maximum size	Average size
227	227.64	-	227.34
233	232.81	234.71	233.98
236	236.49	236.95	236.82
237	237.67	238.38	237.91
239	238.55	240.00	239.17
241	240.77	241.20	240.99
243	242.02	244.17	243.11
245	244.79	245.63	245.09
247	246.84	247.36	247.14
249	249.13	249.48	249.28
251	250.49	251.60	251.24
253	252.40	253.47	253.29
255	254.09	255.52	255.24
257	257.26	257.59	257.36
259	258.17	259.50	258.95
261	260.00	261.86	261.17
263	263.22	264.13	263.68
265	265.51	265.62	265.57
267	267.41	267.85	267.56
269	269.51	269.81	269.60
271	271.61	271.86	271.69
273	273.70	-	273.70
275	275.70	-	275.70
279	279.13	-	279.13
286	285.92	286	285.96
290	290.00	290.15	290.08

Table 2.5 Locus MTG67 allele binning information.

Allele Bin	Minimum size	Maximum size	Average size
172	172.00	173.80	173.38
174	174.00	175.80	174.98
176	176.00	177.80	176.54
178	178.00	179.89	178.99
180	180.00	181.87	181.05
182	182.00	183.80	183.11
184	184.00	185.80	184.64
186	186.00	187.80	186.84
188	188.00	189.80	188.59
190	190.00	191.80	190.85
192	192.00	193.80	192.94
194	194.00	195.96	195.06
196	196.00	197.80	197.62
198	198.00	199.80	198.61
200	200.00	201.80	200.62
202	202.00	203.80	201.02
204	204.00	205.80	202.62
206	206.00	207.80	205.04
208	208.00	209.80	206.84
210	210.00	211.80	Only 2 sizes observed
212	212.00	213.80	211.6
214	214.00	215.80	Only 2 sizes observed

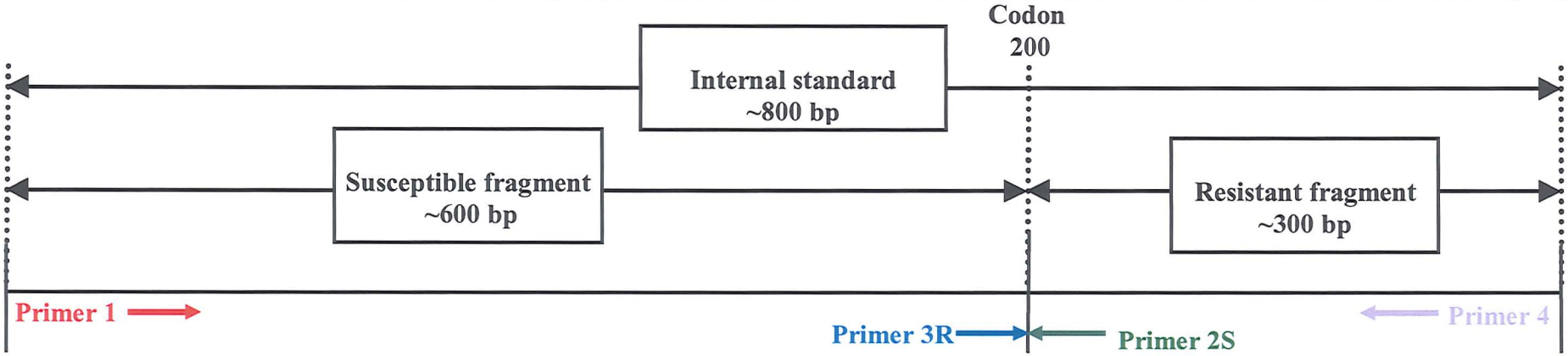
Table 2.6 Locus MTG73 allele binning information.

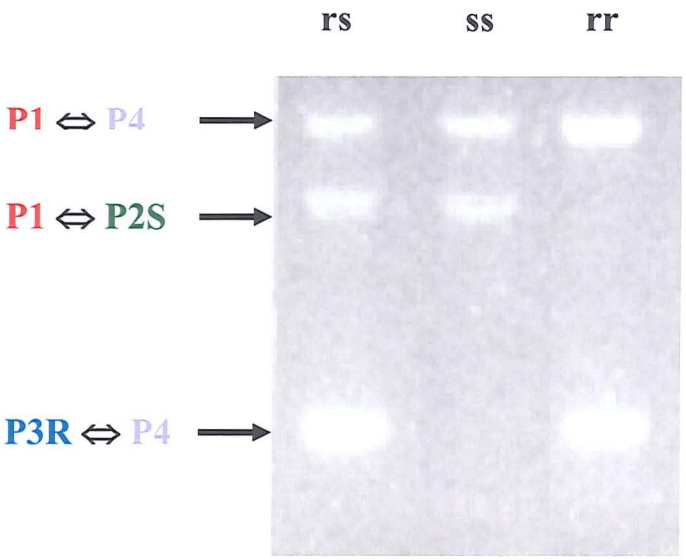
Allele Bin	Minimum size	Maximum size	Average size
148	147.03	149.80	148.47
151	150.08	151.90	151.33
154	153.05	154.98	154.19
157	155.88	157.62	156.86

Table 2.7 Locus MTG74 allele binning information.

Allele Bin	Minimum size	Maximum size	Average size
153	152.93	152.93	152.93
156	156.29	156.99	156.54
159	158.50	160.35	159.59
162	161.20	163.35	162.53
165	164.29	166.19	165.50
168	168.25	169.04	168.55
186	186.33	186.33	183.33
153	152.93	152.93	152.93
156	156.29	156.99	156.54
159	158.50	160.35	159.59
162	161.20	163.35	162.53
165	164.29	166.19	165.50
168	168.25	169.04	168.55
186	186.33	186.33	183.33

Figure 2.1: Allele-specific PCR (after Elard *et al.*, 1999). Primer 1 and Primer 4 are the forward and reverse primers. Primers Primer 2S and Primer 3R flank the F200Y mutation and determine whether the susceptible and/or resistant alleles are present. The letters rr, rs and ss refer to $P200^{Tyr/Tyr}$, $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes, respectively.





β-tubulin isotype I gene partial cDNA with F200Y and primer positions highlighted

GAACAATGGACTCTGTTCGTTCTGGACCGTATGGACAACTTTTCCGTCCAGATAATTACG

TGTTTGGCCAGTCAGGAGCGGGTAACAACTGGGCGAAGGGCCACTATACCGAGGGAGC

TGAGCTTGTTGACAACGTCTTAGATGTTGTTCGTAAAGAGGCAGAGGGTTGCGATTGCC

TTCAGGGCTTCCAACTGACGCATTCTTTGGGAGGAGGTACTGGTTCGGGTATGGGCACT

TTGCTCATCTCCAAAATTCGCGAGGAGTATCCGGATAGAATCATGGCTTCATTCTCCGTT

GTTCCATCACCTAAGGTATCCGACACCGTTGTGGAACCTTACAATGCCACTCTTTCTGTA

CACCAATTGGTAGAAAACACCGATGAAACATTCTGCATCGATAATGAAGCTCTCTGCAT

CGATAATGAAGCTCTGTACGATATCTGCTTCCGCACCTTGAAACTCACAAATCCAACTT

ATGGCGATCTCAATCACTTAGTGTCTGTCACAATGTCTGGAGTCAGACCTGCCTTCGATT

CCCTGGACAGCTGAATGCTGATCTTCGCAAGTTAGCCG

Table 2.8 Primer sequences used in first attempts to isolate of isotype II β-tubulin from pooled *MTci2* mixed adult cDNA. Pairs A and B were based on published isotype II β-tubulin sequences of *H. contortus* and *C. oncophora*, whereas pair C were based on those published by Clark *et al.* (2006) for cyathostome species.

Name	Sequence	Tann
A for	5′-CGT GAG ATY GTC CAC GTT CA-3′	58.3°C
B for	5′-CCT GGC CAC GGA CGC TAT CTG-3′	65.7°C
C for	5′-CGT GAR ATY GTY CAY GTD CAR GC-3′	62.1°C
A rev	5′-TAG GCT TGA GCT CCC TTT GCT GA-3′	62.4°C
B rev	5′-TCC TCG GGG TAC GGC TCT TCT G-3′	65.8°C
C rev	5′-CCA TTT CRT CCA TAC CYT SDC-3′	58.5°C

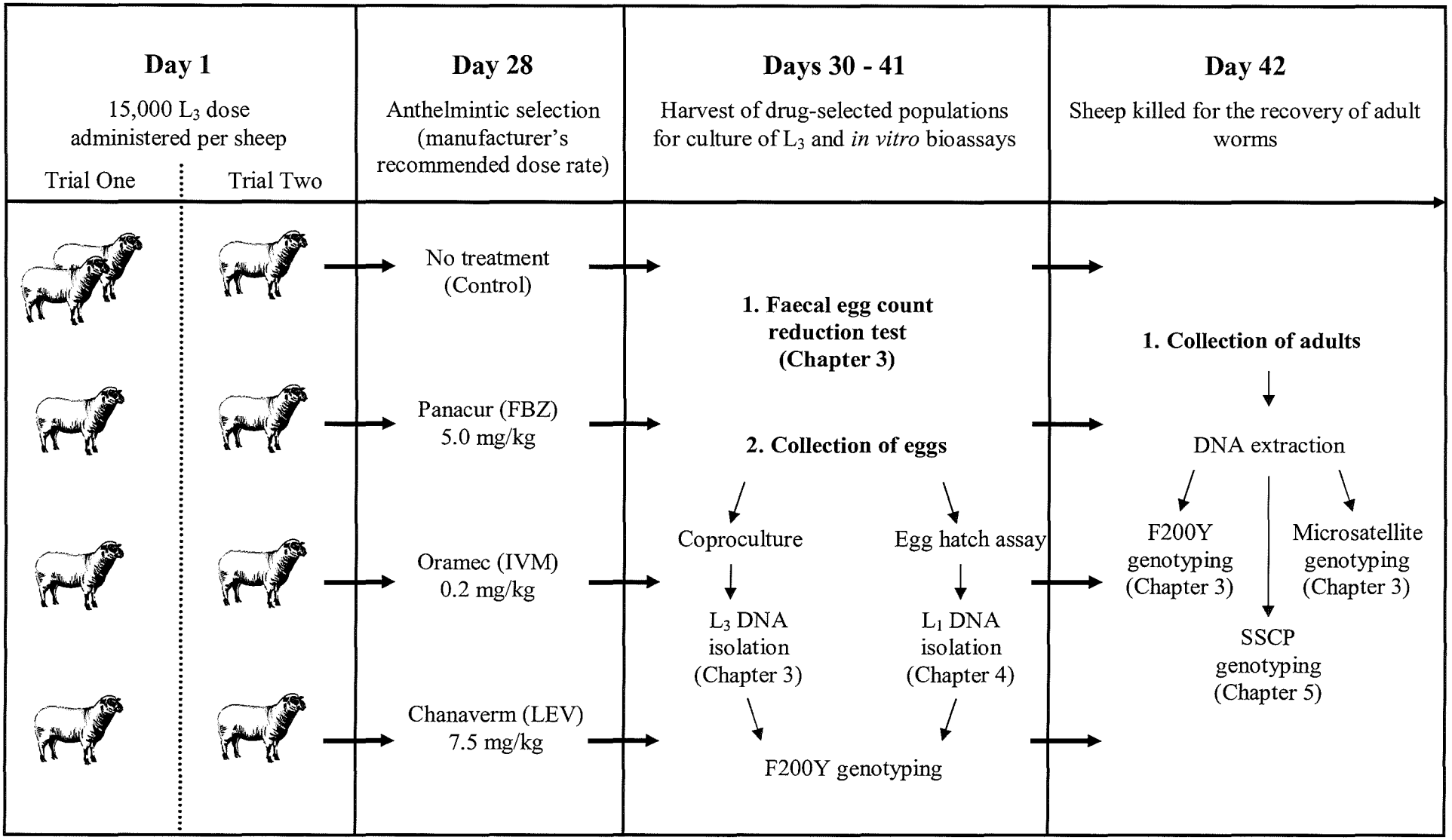
Table 2.9 Degenerate primer sequences based on the protein alignments for published isotype II β-tubulin sequences from a number of trichostrongylid species. These primers were tested upon the *MTci2* cDNA template.

Name	Sequence	Tann
A for	5′- GAY WMN GTN MGN WSN GGN CCN TT - 3′	63.3°C
B for	5′- YTN TTY MGN CCN GAY AAY TT - 3′	53.2°C
C for	5′- GAR GGN GCN GAR YTN GTN GAY WS - 3′	64.2°C
1 rev	5′- GGN CAN CAN SWR AAN SWN SW - 3′	57.3°C
2 rev	5′- AAC ATY TGY TGN GTN ARY TCN SW - 3′	58.0°C
3 rev	5′- TGR TAY TGY TGR TAY TCN SWN AC - 3′	58.0°C

Table 2.10 Primer sequences used in isolation of isotype II β- tubulin from individual adult *MTci5* worms.

Name	Sequence	Tann
B for	5′ - GCW CTR TTY CGT CCA GAC AAC TTT - 3′	68.0 °C
C for	5′ - AGG AGC CGA GCT CGT CGA TAG - 3′	63.7 °C
1 rev	5′ - GGT GAC GGT ACC ACC GAG AAG GAA GA - 3′	68.0 °C
4 rev	5′ - TCA GGR TAT TCT TCR CGG ATC TTA GC - 3′	63.2 °C

Figure 2.2: Experimental design of *in vivo* anthelmintic selection of *MTci5* isolate. Some experiments performed upon the parasite material are described in other Chapters (where indicated). Note that two control sheep were used in Trial One (one was killed on day 28 for recovery of adult worms) and only one control animal was necessary in Trial Two. All egg, L_3 and adult sub-populations were maintained separately. 'F200Y genotyping' refers to the estimation of the genotype frequencies at the F200Y isotype I β-tubulin locus using one or both of the allele-specific PCR or Pyrosequencing techniques. SSCP refers to the Single Strand Conformational Polymorphism technique.


Day 1
15,000 L_3 dose administered per sheep
Trial One
Trial Two
Day 28
Anthelmintic selection (manufacturer's recommended dose rate)
Days 30 - 41
Harvest of drug-selected populations for culture of L_3 and *in vitro* bioassays
Day 42
Sheep killed for the recovery of adult worms
No treatment (Control)
Panacur (FBZ) 5.0 mg/kg
Oramec (IVM) 0.2 mg/kg
Chanaverm (LEV) 7.5 mg/kg
1. Faecal egg count reduction test (Chapter 3)
2. Collection of eggs
Coproculture
Egg hatch assay
L_3 DNA isolation (Chapter 3)
L_1 DNA isolation (Chapter 4)
F200Y genotyping
1. Collection of adults
DNA extraction
F200Y genotyping (Chapter 3)
Microsatellite genotyping (Chapter 3)
SSCP genotyping (Chapter 5)

3.0 Chapter 3 Characterisation studies of a multiple drug resistant field isolate of *Teladorsagia circumcincta*

3.1 Introduction

Anthelmintic resistance in the UK is predominantly associated with *T. circumcincta* and to a lesser extent *Haemonchus contortus* (Jackson & Coop, 2000), with the latter species having a more restricted locale (Southern parts of the UK). In Scotland alone, multiple drug resistance (MDR) has been reported from four sheep farms (Sargison *et al.*, 2001; Bartley *et al.*, 2004; Sargison *et al.*, 2004), and one goat farm (Jackson *et al.*, 1992a,b,c). Multiple resistance has also been recorded on one sheep flock (Yue *et al.*, 2003) and one Cashmere goat herd (Coles *et al.*, 1996) in England. However, it is almost certain that this problem is underestimated due to a lack of resistance surveys.

3.1.1 History and origin of the *MTci5* isolate

As one of the first known cases of ovine MDR in *T. circumcincta* in the UK, and one in which resistance was so rapidly selected, it was deemed necessary to characterise the *MTci5* field isolate in some detail. The history of the *MTci5* isolate has been documented by Sargison *et al.* (2007). This isolate originates from Glencorse Farm (a Scottish lowland sheep farm), which was established in 1997 as a Trial plot under the auspices of the Scottish Agricultural College (SAC), with approximately 70 breeding Suffolk ewes. Animals were sourced primarily from existing SAC flocks and ewes and lambs were grazed on 11 Ha pasture between the months of April and October until 2001. There had been no animals grazing on the land for around 20 years prior to establishment of the farm, at which time there were goats. The flock was considered to be susceptible to all broad spectrum anthelmintics when established, and was treated with BZ in 1997 and 1999, and with LEV in 1998, following advice to rotate anthelmintics to preserve efficacy. BZ resistance was detected in 1999 and treatment with IVM began thereafter. In 2001, IVM resistance was diagnosed and the farm

was closed in 2002 after MOX resistance also became apparent (Sargison *et al.*, 2007). Whilst lambs had been drenched at three to four week intervals with the aim of suppressive control, the ewes were only treated (with MOX) at the post-parturient stage and this was after they had been moved onto clean pasture. This meant that the clean pasture would be seeded only with resistant worms. The authors concluded that the high frequency of anthelmintic treatment in lambs and the cultivation of a primarily resistant worm population *in refugia* were the main reasons for the rapid development of MDR in this population. This farm was said to be a 'closed flock', meaning that no new stock were introduced from elsewhere. This is important from a population genetics point of view, since resistance would have developed *in situ,* as opposed to being introduced from multiple sources. However, this is unrealistic as the arrival of tups for breeding could introduce resistant nematodes particularly if effective quarantine measures (i.e. 'dose on arrival') were not employed. Furthermore, it is unlikely that the original flock would have carried wholly susceptible worms, given the widespread nature of BZ resistance at that time; the insensitivity of the widely used faecal egg count reduction test (FECRT); and the likely pre-exposure to IVM and LEV anthelmintics. Therefore, it seems likely that the founding worm population already carried resistance alleles to multiple anthelmintics at a low frequency and that the subsequent drug selection increased their frequency in a relatively short period of time.

3.1.2 Previous characterisation studies of the *MTci5* isolate

Bartley *et al.* (2005) initially characterised this isolate by examining the effect of various anthelmintic treatments upon worm survival *in vivo*. The predominant species prior to treatment was *T. circumcincta* and this was the only surviving species found post-treatment. This study employed the controlled efficacy test (CET), whereby donor animals received a dose of an anthelmintic or combination drench and subsequently the remaining adults and immature worms (mostly L_4s) were recovered from the abomasum for enumeration. Efficacies of 59%, 60% and 88% were observed for FBZ, IVM and LEV anthelmintics, respectively, confirming the triple resistant status of this isolate. The combination drenches, which were administered at the manufacturer's recommended dose rate (FBZ+ IVM, FBZ+ LEV and FBZ+IVM+LEV), had efficacies of between 92 and 94%, and MOX had the greatest efficacy removing 98% of the worm burden. The CET method is more accurate than the

faecal egg count reduction test (FECRT), however, preservation of the abomasal digesta and worms with formalin prohibits their use in genetic assays. A FECRT for each treatment/ combination was also conducted in this study and the findings were broadly in agreement with the results of the CET. However, in some cases, egg counts were zero (implying 100% efficacy), whilst a proportion of the worms were known to survive every treatment from the CET data. This is important since the FECRT is one of the standard tests for anthelmintic efficacy on farms (for financial reasons the CET is not used other than for research purposes). If it suggests that a drug or combination drench is 100% effective, then the survival of resistant worms will go unnoticed, assuming that worms are able to recover their fecundity after the period of residual anthelmintic activity, as has been described previously (Tyrell *et al.,* 2002; Jackson, 1993). This is a clear indicator of the need for more sensitive assays for resistance, and the molecular approach is one that is becoming increasingly attractive.

3.1.3 Use of microsatellites in population genetics analysis

Although there have been very few population genetics studies on nematodes and fewer still which involve *T. circumcincta*, the information this type of study can provide about population structure and genetic variability is invaluable. Three of the most recent studies were conducted in the UK and France, one of which provided the microsatellite markers used here (Grillo *et al.,* 2006). Grillo analysed the genetic structure and diversity of 18 *T. circumcincta* populations from Scotland, France and New Zealand using five neutral markers. Her findings revealed that genetic diversity for the most part, was found within and not between populations, which is explained by the consistently high levels of polymorphism in each population. However, there is always the possibility that low genetic differentiation between populations is a feature of gene flow. For instance, there has been extensive movement of cattle in the USA and Blouin *et al.* (1992) found very little differentiation between *Ostertagia ostertagi* populations in analysis of the rapidly evolving mitochondrial DNA (mtDNA) of these parasites. One way of testing this hypothesis is to examine populations which are known to have been geographically isolated for some time. Braisher *et al.* (2004) used mtDNA analysis to compare two 'open' *Teladorsagia* populations (i.e. with regular gene flow) with that of the Soay sheep from St Kilda, which have been isolated since 1932. Despite the opportunity for genetic drift over time and the lack of gene flow, the three

populations were remarkably similar, leading the authors to conclude that these parasites show inherently high levels of genetic variation, which are maintained over long periods of time.

One of the few studies which have highlighted significant genetic variation between *T. circumcincta* populations was that by Leignel *et al.* (2002). This study involved characterisation of three goat and five sheep parasite populations at three different loci: the isotype I β-tubulin gene, the mtDNA ND4 gene and the ITS-2 region (second transcribed spacer of rRNA cistron). The authors found a unique allele, which was only associated with parasites infecting goats. They concluded that two species were in existence, one which could infect both sheep and goats and one cryptic species, which could only persist in goats. A previous study using isoenzyme analysis of the malate dehydrogenase (MDH-2) locus supported these findings (Gasnier & Cabaret, 1996). Furthermore, microsatellite analysis supports the view that a cryptic species of *T. circumcincta* exists in goats from this region (Grillo *et al.,* 2007). This is a rare occurrence, but it provides an excellent example of the way in which neutral markers can be used to predict genetic structure. For instance, the presence of novel, population-specific alleles can be revealed by the lack of expected genotypes (carrying this allele) in equal occurrence from all populations.

The classification of a species has for the most part, been based on morphological features. This is where population genetics fulfils another requirement in characterisation studies. For instance, *T. circumcincta* has been described as being part of a species complex with *T. davtiani* and *T. trifurcata* for many years. This was based upon the spicule characteristics of adult males. However, sequencing of the ITS-2 region showed these putative species to be genetically indistinguishable (Stevenson *et al.,* 1996; Wimmer *et al.,* 2004) and multi-locus allozyme studies failed to show any significant differentiation (Andrews & Beveridge, 1990; Gasnier *et al.,* 1993) implying that these are morphological variants of the same species.

Many of the population genetics studies in the past have been based upon single-locus analysis, but caution should be applied here since it is not always clear if the locus is highly conserved between closely related species. As Anderson *et al.* (1998) explain, when a species splits, the daughter species will diverge, but for a long time there will still be phylogenetically similar alleles at each locus (termed polyphyly). As the daughter species continue to diverge, the alleles will no longer be similar (termed paraphyly) and eventually reciprocal monophyly

results, whereby phylogenetically distinct sequences are found in each species. Single locus analysis cannot be used with confidence in the early stages of divergence (polyphyly or paraphyly) for the simple reason that the locus chosen may be one which is slow to diverge. Single locus analysis can be used where reciprocal monophyly has occurred, but this is difficult to identify. Therefore, multi-locus analysis (as used here), is a much more robust test of relatedness and differentiation and microsatellite markers provide a ubiquitous and consistent source of such information.

3.1.4 Analysing the frequency of the F200Y isotype I β-tubulin mutation

The involvement of the F200Y isotype I β-tubulin locus remains undisputed amongst BZ resistant populations of *T. circumcincta*. Initial studies involving this species showed that only worms which were homozygous for $P200^{Tyr}$ survived BZ treatment at the recommended dose rate, leading the authors to conclude that this mutation was both recessive and necessary for the expression of BZ resistance (Elard & Humbert, 1999). This mutation has also been found in association with BZ resistance in a number of other nematode species, including *H. contortus* (Kwa *et al.,* 1994), *Cooperia oncophora* (Winterrowd *et al.,* 2003, Njue & Prichard, 2003), *Trichostrongylus colubriformis* (Silvestre & Humbert, 2000), in numerous species of equine cyathostome (Pape *et al.,* 1999, von Samson-Himmelstjerna *et al.,* 2001, Drogemuller *et al.,* 2004), and amongst human endoparasite species (Albonico *et al.,* 2004, Melville *et al.,* 2006). It has also been reported in numerous BZ resistant fungal strains including *Aspergillus nidulans* (Jung *et al.,* 1992), *Venturia inaequalis, Venturia pirina, Monilinia fructicola, Sclerotinia homoeocarpa, Neospora crassa* and six *Penicillium spp.* (Koenraadt *et al.,* 1992); as well as in some protozoa (Edlind *et al.,* 1994). Given its clear importance, this study aimed to identify first, whether this mutation was present in the *MTci5* field isolate and secondly, to examine the effect of drug selection at this locus. Numerous methods have been described for the genotyping of the F200Y isotype I β-tubulin locus and two methods are described here. The allele-specific PCR was first adopted in this study and when the Pyrosequencing technology became available, it was decided to compare the performance of each. The conventional method was pioneered by Kwa *et al.* (1994) when they designed the first allele-specific PCR to determine the F200Y isotype I β-tubulin genotype in *H. contortus*. A multiplex PCR was subsequently designed to improve upon this assay and this included an

internal control to allow confidence in the efficiency of the PCR (Elard *et al.,* 1999). One of the more advanced methodologies is a mutation scanning technique called Pyrosequencing, based on an automated, high-throughput *de novo* gene sequencing strategy (Biotage). Both methods are described in detail in Chapters 1 and 2, (Sections 1.4.2 and 2.4.3).

3.1.5 Assessing the resistance phenotype

Phenotypic assays have been routinely employed for laboratory and field diagnosis of anthelmintic resistance for many years. Those used in detection of BZ resistance include the tubulin binding assay (Lacey & Snowdon, 1988) and the *in vitro* egg hatch assay (Le Jambre, 1976). Two more universal tests are available, which are the *in vivo* anthelmintic efficacy (or controlled efficacy) test and the faecal egg count reduction test (FECRT). For the reasons explained in Section 3.1.2, the controlled efficacy test was not used here. The technically demanding tubulin binding assay was not employed either, since previous studies have indicated that it is no more sensitive than the egg hatch assay (Martin *et al.,* 1989).

3.1.5.1 Faecal egg count reduction test (FECRT)

In the previous characterisation study of *MTci5*, the FECRT values were found to vary depending on the number of days post-treatment that the samples were obtained (Bartley *et al.,* 2005). After 24 hours, none of the treatments (single drenches and combinations of FBZ, IVM, LEV and MOX) showed greater than 90% efficacy, although this could be affected by the release of eggs from dead/dying females. After 48 hours, MOX and all of the combination drenches used showed efficacies of at least 95%. Throughout the next 5 days, efficacies of the single drench doses (FBZ, IVM and LEV) progressively decreased. This is an important consideration when carrying out this assay, since the female worm population appear to recover their fecundity after an initial depression due to the action of the drug. In this study, FECRT efficacies were calculated after ten days to ensure the recovery in fecundity of resistant worms.

3.1.5.2 Egg hatch assay (EHA)

The egg hatch assay was conceived to measure the effect of BZ activity upon embryonic development (Le Jambre, 1976). When eggs are passed in the faeces, they must be collected and extracted immediately for this assay before the developmental process has reached a

certain stage. There is a small time frame in which the egg shells are permeable to the drug and beyond this, maturity to the L_1 stage will occur irrespective of drug concentration (F. Jackson, personal communication), and thus, care should be taken to prevent false diagnosis of resistance. Another problem with this assay is that it only informs us about the number of eggs which hatch successfully, and this could be dependent upon other factors besides drug concentration. For instance, it is frequently observed that a proportion of eggs only exposed to water (control samples) fail to hatch, suggesting that there are unknown genetic fitness issues involved (Le Jambre, 1976). However, the results of the egg hatch assays have been corrected in this study for the natural mortality rate.

3.1.5.3 Reliability of the FECRT and EHA

Martin *et al.* (1989) proposed that the frequency of resistance alleles would be somewhere in the region of 25 to 50% in a population before resistance was detectable by any of these tests. Despite the lack of knowledge of the resistance mechanisms involved at the time of this study, the authors compared composite isolates consisting of different ratios of phenotypically resistant and susceptible parent isolates. They found that none of the *in vitro* bioassays was able to diagnose resistance in composite isolates containing 25% resistant worms, however, all were successful in resistance diagnosis of isolates containing 50% resistant worms. Obviously this is also dependent upon the dominance or recessiveness of a resistance mechanism and BZ resistance may not be caused (in all populations) by the same single mechanism. For these reasons, care should be taken when conducting a phenotypic test in isolation, to prevent the wrong conclusions being drawn. This is why molecular techniques need to be used in conjunction with phenotypic measures of resistance in order to fully understand the mechanisms involved.

3.1.6 Aims of molecular characterisation studies

There are two basic aims of this Chapter. Firstly, to undertake a molecular genetic characterisation of the *MTci5* isolate using neutral genetic markers (microsatellites) and F200Y isotype I β-tubulin genotyping. Secondly, to study the genotypic and phenotypic consequences of treatment with broad spectrum anthelmintics on the *MTci5* isolate. This will provide important baseline data to allow this isolate to be used in experimental work to identify the molecular mechanisms underlying multiple anthelmintic resistance.

A number of specific questions regarding the isolate and the effects of anthelmintic selection are addressed in this Chapter.

1. *Is the MTci5 isolate composed of a single interbreeding worm population or is there evidence of genetic sub-structuring?*
2. *What is the frequency of the F200Y isotype I β-tubulin mutation in the MTci5 isolate and is does this reflect the BZ resistance phenotype?*
3. *Are there any hitchhiking effects of selection with BZ, IVM and LEV anthelmintics as detected by a panel of microsatellite markers e.g. generalised loss of polymorphism?*
4. *How does the BZ resistance phenotype change following selection with BZ, IVM and LEV anthelmintics?*
5. *How does the frequency of the F200Y isotype I β-tubulin mutation respond to selection with BZ, IVM and LEV anthelmintics?*
6. *Are the changes in BZ resistance phenotype following anthelmintic selection correlated with changes in the F200Y isotype I β-tubulin genotype?*

These questions are interrelated and will help address a number of key issues:

- Questions 1, 3, 4 and 5 will help determine whether the *MTci5* isolate is a single population of worms with multiple resistance or is comprised of separate populations of worms with resistance to each anthelmintic.
- Questions 2, 4, 5 and 6 will help determine whether the F200Y isotype I β-tubulin mutation is likely to be the sole, or major, determinant of BZ resistance in this isolate or whether other mechanisms may be involved.
- The answers to questions 3, 4 and 5 will help determine if there is any evidence of genetic linkage between the loci responsible for resistance to the three separate anthelmintic classes.
- The answer to question 3 is also critical for interpretation of future selection experiments and candidate gene association studies using this isolate. These issues will be expanded upon in the discussion Section of this Chapter.

3.2 Use of microsatellite markers to determine population genetic structure of the MTci5 isolate

3.2.1 Microsatellite genotyping of *MTci5* populations

A panel of five microsatellites (MTG15, MTG67, MTG73, MTG74 and HCMS28) was used to investigate the population genetic structure of the *MTci5* isolate and to examine the effect of drug selection upon these neutral markers. Twenty nine adult male worms from each population from Trial One were genotyped with these microsatellites according to the methods and binning ranges described previously (Sections 2.4.1 and 2.4.2). These worms were identified as *T. circumcincta* based on the spicule characteristics (as described in Section 2.1.2).

3.2.1.1 Occurrence of null alleles

The MTG15 and HCMS28 loci displayed missing data so these data points were automatically excluded from the analysis by statistical programs. The missing data is summarised in Table 3.1. It was necessary to determine whether the missing data represented null alleles or genotyping errors. True null alleles are non-amplifying alleles, whereas genotyping errors can result from three possibilities: short allele dominance, poor template quality and sequence variation at the primer sites. Short allele dominance refers to a situation whereby smaller PCR products (hence shorter alleles) are amplified more efficiently by PCR than larger ones. This leads to misrepresentation of allele frequencies (Wattier, 1998). Poor template quality, where the DNA is incomplete, perhaps allows amplification of some loci, but not others. This is known as 'allelic dropout' (Miller, 2002). Allelic dropout was unlikely in this case, given the stringent DNA extraction and storage methods employed, and the fact that some markers amplified correctly in all worms. Furthermore, short allele dominance was ruled out in previous studies using these markers in a number of UK and French isolates, including unselected *MTci5* (Grillo *et al.*, 2007). The last possibility is that sequence variation at the primer sites leads to inefficient amplification of PCR products (Callen *et al.*, 1993). It is likely from these results and from the work of Grillo *et al.* (2007) that *T. circumcincta* carries a number of 'true' null alleles and this is most likely to occur as a result of sequence variation at

the primer sites. *T. circumcincta* is known to be a highly polymorphic species in this respect (Grillo *et al.*, 2007).

The presence of null alleles leads to an overestimation of the frequency of homozygous genotypes (heterozygote deficiency) (Shaw *et al.*, 1999) and this could be potentially confused with other causes of low heterozygosity such as population admixture or inbreeding due to geographical isolation, or anthelmintic selection. Consequently, all data are presented both inclusive and exclusive of locus MTG15, since the frequency of null alleles was particularly high for this locus.

3.2.2 Linkage Disequilibrium analysis: are these markers genetically linked?

In order to test for evidence of genetic linkage between the markers, these data were analysed for linkage disequilibrium (Table 3.2). This test was performed using Genepop option 2 (version 3.4, Raymond & Rousset, 2004) with Fisher's Exact test. Significant p values (<0.05) provide evidence of linkage between pairs of microsatellite loci. There were no significant results from the Chi-square analysis of all possible locus pairings (all p values were greater than 0.05 and χ^2 values were small). This suggests that there is no genetic linkage between the five microsatellite markers allowing them to be considered as genetically independent markers.

3.2.3 Allele statistics among *MTci5* populations

The allele frequencies observed at each locus are displayed in Figures 3.2 a-e. Across all four populations, loci MTG15, MTG67 and HCMS28 were all highly polymorphic having 18, 12 and 14 alleles, respectively; whereas loci MTG73 and MTG74 displayed moderate polymorphism with four and five alleles, respectively. No alleles were found that were unique to the *MTci5* populations compared to other previously analysed *T. circumcincta* isolates (Tables 2.3 to 2.7, Chapter 2; V. Grillo, personal communication). Generally the frequencies of the different alleles for each marker were very similar between the different populations. The only potential difference was allele 257 of locus MTG15 following levamisole and ivermectin selection (Figure 3.2a). However, this is an extremely polymorphic marker and consequently the numbers in each allele class are very low and so this is likely to a stochastic

effect due to the limitations of sample size. More worms would need to be genotyped with this marker to confirm this suggestion. Hence, with this proviso, there is no significant evidence of selection for any particular allele at any particular locus following anthelmintic selection.

An allelic pattern summary was generated using Microsoft Excel (Windows XP) with GenAlEx version 6 add-in software (Peakall & Smouse, 2006), (Figure 3.3). This analysis summarises allele statistics across all loci and displays this information per population, making any general effects of selection on allelic diversity easier to identify. There were no obvious differences in the number of effective alleles (i.e. those which are most likely to be passed onto the next generation), common, rare or private (unique) alleles between populations. There were also no obvious differences in the level of observed heterozygosity amongst populations. Thus, there were no marked differences between the *MTci5* populations based upon observed allele frequencies. This suggests there is no evidence of drug selection (genetic hitchhiking) upon neutral markers, hence none of these markers are closely linked to a major resistance-conferring locus in the genome.

3.2.4 Hardy-Weinberg equilibrium multiple locus analysis

Probability tests were performed on all four populations and at each locus to determine whether there was any deviation from Hardy-Weinberg Equilibrium (HWE) using Fishers Exact Test (10,000 runs). This included a measure of Wright's F_{IS} statistic. F_{IS} values close to +1 indicate heterozygote deficiency, thus inbreeding, and values close to -1 represent heterozygote excess (outbreeding). Table 3.3a displays the observed (Ho) and expected (He) heterozygosities (Nei, 1987), as well as Wright's F_{IS} statistic and accompanying p-values across each population and each locus (calculated in GDA version 1.1, Lewis & Zaykin, 2001). These results indicated a significant deviation from HWE: in all populations at locus MTG15; in the BZ-selected population at MTG67 and in the BZ, IVM and LEV-selected populations at locus MTG74. These deviations were due to an apparent reduction in heterozygosity (which was demonstrated by dramatic differences in Ho versus He, and with high positive F_{IS} values); this was indicative of inbreeding. However, the data in Tables 3.3b and 3.3c show the analysis summarised by population and by locus when locus MTG15 data were both included and excluded from the analysis. As predicted, inclusion of the MTG15

locus corrupted the global result, however, when these data were removed, there were still significant reductions in heterozygosity at loci MTG67 and MTG74. The results by population showed that loss of heterozygosity was greatest in the BZ, followed by LEV and IVM-selected populations, which all showed statistical significance (p values of <0.05).

A Bonferroni correction was performed upon both the results by population (Table 3.3b) and the results by locus (Table 3.3c) to test the significance of these data. In the results by population, the correction revealed that only the BZ and LEV-selected populations (with p values of < 0.001) can be regarded as showing significant heterozygote deficiency with confidence (i.e. standard p value of 0.05 / 3 populations = 0.0167, which becomes the new significance threshold and 0.0167 is less than the p value of the IVM-selected population: 0.037, thus the IVM-selected population loses significance). Whereas the Bonferroni correction applied to the results by locus does not reduce the significance of heterozygote deficiency at either locus (MTG67 or MTG74).

3.2.5 Genetic diversity of *MTci5* populations

The population diversity statistics were calculated in Microsoft Excel (Windows XP) using the add-in software 'Microsatellite Toolkit' (Park, 2001) and are summarised in Table 3.4. All populations were reasonably polymorphic and there were no striking differences between them. Although, there were a few instances where unique alleles were observed, however, these were are at a very low frequency and so were likely to be due to stochastic effects as a consequence of small sample size. For example, four unique alleles were observed at the MTG67 locus in the BZ-selected population (Table 3.4) and all had frequencies of less than 4%.

3.2.6 Genetic differentiation between *MTci5* populations

Three different types of analysis were used to study the level of genetic differentiation within and between *MTci5* populations as a further approach to investigate the possible effects of drug selection on the *MTci5* isolate. There were: Principle Coordinates Analysis (PCA); Pairwise Fst and AMOVA.

3.2.6.1 Principle coordinates analysis of multi-locus genotypes

Principle coordinates analysis (PCA) was performed on the data using Microsoft Excel (Windows XP) with GenAlEx version 6 add-in software (version 6, Peakall & Smouse, 2006) to produce a scatter plot based upon each individual's multi-locus genotype. This provides a measure of the level of similarity between individuals based on their proximity on the plot. To first test that the sample of worms we have genotyped is representative of the *MTci5* population, the multi-locus genotypes of the unselected worm population from Trial One were compared with that from a previous study by V. Grillo (Grillo *et al.,* 2007). The previous study had genotyped thirty worms from an earlier passage of the same unselected *MTci5* isolate using female adult worm heads as a source of DNA with the same microsatellite markers. The PCA plots comparing the Trial One unselected population data with the data of Grillo *et al.* (2007) showed no differentiation between the two populations, both inclusive and exclusive of the MTG15 locus data (Figures 3.4a, b). This suggests that the unselected Trial One population data was representative of the *MTci5* isolate and showed that genetic diversity had not been affected by interim passage through sheep. Furthermore, it is worth noting that the male and female data are indistinguishable, suggesting that none of these markers are sex-linked and that either male or female worms can be used for the analysis.

In order to determine whether anthelmintic treatment affected the population structure of the isolate, this analysis was performed upon the four *MTci5* populations from Trial One (Figure 3.5a). A pattern where individual multi-locus genotypes of worms from the same population tended to cluster together, would have suggested genetic differentiation between the populations (Grillo *et al.,* 2007). However, the results clearly show an evenly scattered distribution where individuals from the four populations are intermingled indicating there was no detectable between-population variation (Figure 3.5a). Jackknifing these data (repeating the analysis with sequential removal of a single locus to determine the effect of each upon the global result) confirmed this result (Figures 3.5b-f). The cumulative variance given by the first two coordinates in each plot explains over half of the total variance estimated between individuals, which give confidence in the analysis. This analysis provides further evidence to suggest there is no obvious sub-structuring within the *MTci5* isolate and no effect of anthelmintic selection upon the population genetic structure.

3.2.6.2 Pairwise F_{ST}

Pairwise F_{ST} values were calculated in GDA version 1.1 (Lewis & Zaykin, 2001) (Table 3.5a). All values were very low (<0.05), indicating little genetic differentiation between populations when all loci were considered. The results were not greatly influenced by the inclusion of locus MTG15 data as the F_{ST} values were reduced when these data were removed (Table 3.5b). Again, this test implies that there was no effect of drug selection upon the genetic structure of the *MTci5* isolate.

3.2.6.3 Analysis of molecular variance (AMOVA)

Analysis of molecular variance (AMOVA) was carried out using Arlequin version 2.0 (Schneider *et al.,* 2000) (Table 3.6). There was no effect of removing locus MTG15 data from the analysis (data not shown). The F_{ST} statistic was very low and was in agreement with the outcome of the previous test (Pairwise F_{ST}), i.e. that there is very little genetic differentiation between the populations. The 'percentage of variation' statistics revealed that very little of the variance (0.41%) occurs between or 'among' populations and most of the variance is found (99.59%) within populations. This is further evidence that the genetic structure of the *MTci5* isolate is unaffected by drug selection.

3.3 Effect of drug selection upon the F200Y isotype I β-tubulin locus in the MTci5 isolate

3.3.1 Determination of the frequency of the F200Y isotype I β-tubulin mutation by allele-specific PCR and Pyrosequencing

The frequency of the F200Y isotype I β-tubulin mutation was determined first by allele-specific PCR and subsequently, when the technique became available, by Pyrosequencing (see Chapter 1, Section 1.4.2). This dual analysis provided an accurate measure of allele frequency and allowed a comparison of the two techniques. In this Section, the methods are compared first using only the data from the unselected *MTci5* population. Thereafter the data from the drug-selected populations are summarised.

3.3.1.1 Optimisation of multiplex (allele-specific) PCR

The four-primer reaction (A. Silvestre, personal communication; Figure 3.6) was found to be unreliable in my hands. As is typical in PCR, smaller fragments are amplified more readily than larger fragments, and this often led to the internal standard (IS) fragment (~800bp) and/or the susceptible fragment (~600bp) failing to amplify. In cases where the small resistant fragment (~300bp) had been amplified and the IS fragment was not visible, the genotype could not be reliably determined as the susceptible fragment may also have been too large to be amplified efficiently. Therefore, the assay was adapted by undertaking two separate three-primer reactions. In each reaction, the forward and reverse primers (primer 1 and 4 in Figure 3.6) and just one of either the resistant allele-specific (primer 3R in Figure 3.6) or susceptible allele-specific primers (primer 2S in Figure 3.6) were used. This meant that as well as the susceptible or resistant fragment, the IS band was amplified in each reaction. This improved reaction efficiency, however, there were still occasions where the IS band failed to amplify sufficiently. In these cases, a repeat was carried out and if there was still no IS band, but the allele-specific band was present then the worms could be genotyped. It was expected that this method could introduce some error, however, it was assumed that these errors were minimal as the genotype ratios appeared to be consistent between L_3 and adult data, and between Trials.

3.3.1.2 F200Y isotype I β-tubulin genotypes of unselected MTci5 as determined by allele-specific PCR

Approximately 30 adult worms from Trial One and 40 adult worms from Trial Two unselected *MTci5* populations were analysed by allele-specific PCR to determine their F200Y isotype I β-tubulin genotype ratios (Table 3.7). One single worm could not be genotyped reliably by this method after two attempts and was excluded. Since the datasets between the two Trials were not statistically different at the 95% confidence interval ($p>0.05$) the results were pooled to give ratios of 42.3%, 50.7% and 7% of $P200^{Tyr/Tyr}$, $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes, respectively (Table 3.7). The L_3 parasites obtained from faecal samples in both Trials were also genotyped by this method (Table 3.8). Similarly the results were consistent ($p<0.05$) between Trials and also consistent with the adult data ($p<0.05$). From a pool of 109 individuals, the ratios were 43% $P200^{Tyr/Tyr}$, 45% $P200^{Phe/Tyr}$ & 12% $P200^{Phe/Phe}$ (Table 3.8).

3.3.1.3 F200Y isotype I β-tubulin genotypes of unselected MTci5 as determined by Pyrosequencing assay

The same unselected *MTci5* adult male worms from the two Trials were genotyped by Pyrosequencing to check the accuracy of the allele-specific PCR results. The Pyrosequencer is assumed to be completely accurate given that it is based upon direct analysis of sequence. The Pyrosequencer was able to genotype the one worm that failed the allele-specific PCR analysis; therefore, 72 individuals were genotyped in total. Table 3.9 displays the genotype ratios as determined by the Pyrosequencer and Figure 3.7 compares this data with the allele-specific PCR results. These results are broadly in agreement, however, there was an error rate associated with the allele-specific PCR method. There were nine worms (out of a total of 72) from both Trials which had been genotyped incorrectly (12.5%). In particular, the allele-specific PCR had under-estimated the number of $P200^{Phe/Phe}$ individuals and overestimated the number of $P200^{Tyr/Tyr}$ individuals. It is possible that contamination was an issue as the resistant fragment does amplify more readily than the susceptible fragment and despite the inclusion of negative controls, contamination may have occasionally occurred. Contamination does seem to be more of a problem with conventional PCR than it does with Pyrosequencing, the possibility of spill-over between wells when loading gels is one problem avoided with the Pyrosequencer. Furthermore, the PCR set-up and gel-running areas were not separated during the period when allele-specific PCR was conducted, which provided ample opportunity for contamination.

From the Pyrosequencing data, it can be said with confidence that the frequency of the resistant allele in the unselected *MTci5* isolate was 62.5% with the susceptible allele at 37.5%. The majority of worms carried the resistant allele (88%) with around half of those being homozygous resistant ($P200^{Tyr/Tyr}$ 37.5%). The true proportion of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes was found to be 50% and 12.5%, respectively.

3.3.2 Effect of drug selection upon F200Y isotype I β-tubulin genotype of adult *MTci5* worms

As demonstrated in Section 3.3.1, it is clear that the F200Y isotype I β-tubulin mutation is present at high frequency in the unselected *MTci5* isolate. A key question is whether 100% of the worms that survive a full therapeutic dose of BZ are homozygous for the resistant allele ($P200^{Tyr/Tyr}$). If this were the case, it would be consistent with the hypothesis that the F200Y isotype I β-tubulin mutation was the sole determinant of resistance and that it behaved in a genetically recessive fashion in the *MTci5* isolate. Conversely, if there were some $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ survivors of BZ treatment, it might suggest that the resistant allele is not completely recessive, or that there may be other mechanisms of BZ resistance in the *MTci5* isolate. The effects of IVM and LEV selection upon the F200Y isotype I β-tubulin locus are also of interest, as this will indicate whether there is evidence of genetic linkage between this locus and other loci conferring resistance to different anthelmintics. Consequently, the drug-selected *MTci5* populations from Trials one and two were genotyped at the F200Y isotype I β-tubulin locus.

Approximately 70 adult male worms (pooled total from both Trials) were chosen from each drug-selected *MTci5* population and genotyped using both the allele-specific PCR and the Pyrosequencing assays. The allele-specific PCR results are displayed in Table 3.10a and the Pyrosequencing data are displayed in Table 3.10b and Figure 3.8. Out of all 571 worms genotyped from both Trials, there were 50 worms incorrectly genotyped by the allele-specific PCR method. This amounted to an 8.8% error. However, if one compares the difference in genotypes at the population level, there were only 15 inconsistencies, equating to a 2.6% error.

Therefore, at the population level, the allele-specific PCR is not too inaccurate to be employed as a diagnostic measure of BZ resistance.

An 'estimation of the difference between genotype proportions' test (with 95% and 99% confidence intervals) was used to compare the data for each population between Trials. There were no significant differences between the results of the Trials ($p<0.01$, see Table 3.11); thus the results were pooled (per population) for analysis. The same statistical test (with 95 and 99% confidence intervals) was again used to compare each drug-selected population with the unselected group to analyse the effects of treatment. The significant changes in genotype frequencies were only found in the BZ-selected population. There was a highly significant increase ($p<0.01$) in the proportion of $P200^{Tyr/Tyr}$ worms from 38 to 79% (99% CI estimates: -0.225,-0.608) and a concomitant decrease ($p<0.01$) in the proportion of $P200^{Phe/Tyr}$ individuals from 50 to 19% (99% CI estimates: 0.499, 0.112) and $P200^{Phe/Phe}$ individuals from 13 to 1% ($p<0.01$) (99% CI estimates: 0.218, 0.005). These results confirm that the F200Y isotype I β-tubulin mutation is a major determinant of BZ resistance. However, the survival of significant numbers of $P200^{Phe/Tyr}$ individuals and a small number of $P200^{Phe/Phe}$ individuals casts doubt over the recessiveness of the resistant allele, or suggests that this mutation is not the sole determinant of BZ resistance in this isolate. The lack of a change in frequency of the F200Y isotype I β-tubulin mutation provides no evidence of selection at this locus as a consequence of IVM or LEV treatment.

3.3.2.1 Pairwise F_{ST} analysis of adult data

The Pairwise F_{ST} test was employed to further investigate the level of genetic differentiation between populations at this locus. The results confirmed that there is great genetic differentiation between the BZ-selected population and each of the other populations, however, there is no indication of an effect of IVM or LEV selection (Table 3.12).

3.3.2.2 Hardy-Weinberg Equilibrium analysis of adult F200Y isotype I β-tubulin genotypes

Tests were performed to examine whether the F200Y genotypes of each *MTci5* population were in HWE. This analysis was conducted using the GenAlEx add-in software (Version 6, Peakall & Smouse, 2006) for Microsoft Excel (see Figure 3.9a-d). The analysis showed no significant deviation from HWE ($p>0.05$) in the unselected, BZ-selected and LEV-selected

populations. However, the IVM-selected population did show a significant deviation from HWE at this locus ($p<0.05$). This was further investigated by HWE analysis of the L_3 F200Y genotype data.

3.3.3 Effect of drug selection upon F200Y isotype I β-tubulin genotypes of L_3

Approximately 40 L_3 were genotyped by allele-specific PCR from each Trial (Table 3.13) and the results were tested for significant differences between Trials (at the 99% CI, see Table 3.14). All genotype ratios were in agreement except for the $P200^{Phe/Tyr}$ proportion from the IVM-selected population, which was significantly greater ($p<0.01$) in Trial One than in Trial Two (0.592, 0.057). This could be due to genotyping errors associated with the allele-specific PCR, which was known to reach a level of 12.5% from the adult data. When this 12.5% error was applied to the data, the difference was no longer significant (data not shown). The L_3 data from both Trials were thus pooled and the results are displayed in Figure 3.10. The 'estimation of the differences between genotype proportions' test (with 95% and 99% confidence intervals) performed on these data revealed that a significant increase ($p<0.01$) in $P200^{Tyr/Tyr}$ genotypes occurred following BZ selection from 43 to 75% (99% CI estimates: -0.155, -0.488). A significant decrease ($p<0.01$) in the proportion of $P200^{Phe/Tyr}$ from 45 to 22% (99% CI estimates: 0.396, 0.070) was apparent in the same group and the decrease in $P200^{Phe/Phe}$ genotypes from 12 to 3% was also significant ($p<0.05$) (95% CI estimates: 0.158, 0.018). Again, survival of significant numbers of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes suggests that either the recessiveness of the F200Y isotype I β-tubulin mutation is questionable or there may be other BZ resistance mechanisms involved. There were no significant effects of IVM or LEV selection upon F200Y isotype I β-tubulin genotypes.

These results are in agreement with the adult data, which implies that the error rate associated with the allele-specific PCR is consistently low (i.e. 8.8% as described in Section 3.4.2). These results were not analysed further by Pyrosequencing, since they were in agreement with the adult data. HWE analysis was conducted upon these data (as described in Section 3.4.2.2) and in contrast to the adult data analysis, there was no significant deviation from HWE in any of the *MTci5* populations ($p>0.05$, data not shown).

3.4 Phenotypic assessment of anthelmintic resistance

The aim of the research outlined in this Section was to investigate the effect of selection of the *MTci5* isolate by different anthelmintics on the phenotypic expression of BZ resistance. As well as forming part of the basic characterisation of the isolate there are two particular questions to which these experiments have relevance.

1. Are the genotypic changes described in the previous Section consistent with, and do they potentially account for, changes in the BZ resistance phenotype? This has relevance for whether the F200Y isotype I β-tubulin mutation is the sole, or major, determinant of BZ resistance in this isolate.

2. Does selection by LEV and/or IVM alter the BZ resistance phenotype? This has relevance for two issues. Firstly, is there linkage between the BZ resistance-determining loci and IVM or LEV resistance-determining loci? Secondly, is the *MTci5* isolate a single population with multiple resistance or does it consist of distinct sub-populations each resistant to a different anthelmintic class? That is, does the phenotypic data support or conflict with the microsatellite data presented earlier.

3.4.1 *In vitro* egg hatch assay as a phenotypic assay of BZ resistance

The egg hatch assay for BZ resistance is the most reliable and consistent test of all the *in vitro* bioassays used in this study. However, it is important to ensure that the thiabendazole (TBZ) solution is prepared and stored appropriately or unreliable results can be obtained. Subsequent to undertaking some of the egg hatch assays presented in this thesis, it was discovered that TBZ does not remain entirely in solution in dimethyl sulphoxide (DMSO) after a period of 24-48 hours. Hence the egg development (ED) values obtained for some of the first egg hatch assays conducted (i.e. all EHA data displayed in Chapter 3 and in Sections 4.2 and 4.3) are now known to be inaccurate in terms of the absolute concentrations of TBZ. Hence the results of these assays cannot be compared with each other. However, the experiments are internally controlled and so the differences between the samples tested concurrently are still valid. Therefore, analysis has been confined to the trends observed by the egg hatch assay,

specifically the resistance factors, as opposed to analysis of the actual ED values. This problem was overcome in later experiments (Section 4.6) as explained in the materials and methods Chapter (Section 2.2.1). Note that these assays were conducted upon the eggs obtained from the drug selection experiments described at the start of this Chapter, thus Trials one and two refer to the same experiments.

3.4.1.1 Comparison of the unselected MTci5 isolate with a susceptible laboratory isolate of T. circumcincta (MTci1)

MTci1 is susceptible to all three classes of broad spectrum anthelmintic and is the standard laboratory isolate used at the Moredun Research Institute. Egg hatch assays were performed on eggs harvested from donor sheep infected with the *MTci5* versus the *MTci1* isolates to compare their relative sensitivity to BZ (Table 3.15 and Figure 3.11). The ED values were calculated in Minitab version 14, using reliability/survival analysis. In order to obtain the best estimate of ED values, means and standard deviations were calculated from six separate assays carried out over 15 or 16 days. In Trial One, data was obtained between days 24 to 40 post-infection and the Trial Two data were taken between days 21 and 36 post-infection. The standard deviations are relatively high for the *MTci5* data and a possible reason for this may be that the level of resistance changed over the course of the infection, as has been reported in previous studies (Borgsteede & Couwenberg, 1987; Kerboeuf & Hubert, 1987). This is further examined in Chapter 4 (Section 4.2). Note that the *MTci1* data for Trial Two was not conducted during the same period, due to the availability of donor animals. Instead, the nearest assays to that date (and age of infection) are shown. The resistance factor calculations show that the *MTci5* isolate is around five times (between 4.4 and 5.6) more resistant than the susceptible (*MTci1*) isolate. A one-way ANOVA was used to analyse the significance of the difference in ED_{50} values between these isolates. Two ED_{50} values were obtained for each isolate (from replicate experiments) and these were: 0.281 and 0.314μg/ml TBZ for the *MTci5* isolate, and 0.096 and 0.099μg/ml TBZ for the *MTci1* isolate. The one-way ANOVA showed the difference between these isolates to be highly significant ($F_{1,3} = 140.64$, $p = 0.007$).

3.4.1.2 Effects of drug selection upon egg development in the MTci5 isolate

Following the drug selection Trials, the egg hatch assay was used as a measure of the BZ resistance phenotype. Since it is known that the TBZ solutions used in these assays were subject to dissolution over time, more attention has been directed towards the trend between

assays than to the actual concentrations and egg development (ED values). Assays were performed prior to and following all anthelmintic treatments in an effort to determine the effects of each treatment upon BZ resistance phenotype. These data were then considered in relation to the genotypic expression of BZ resistance in terms of the F200Y isotype I β-tubulin genotype ratios. The data shown in Figures 3.12 and 3.13 summarise the trend of ED_{50} values over time for each *MTci5* population in each Trial.

Trial One Data

In Trial One, the unselected *MTci5* population (which was analysed as a pooled sample of eggs from all donors before treatment) had an ED_{50} value of 0.341μg/ml (a mean of the first two data points at days 24 & 26, see Figure 3.12). A comparable susceptible isolate (*MTci1*) showed an ED_{50} value of 0.118μg/ml at the same stage of infection (a mean of the first two data points at days 24 & 26). The dataset was not complete for all groups from Trial One as there was some difficulty in recovering sufficient numbers of eggs after treatment with IVM and LEV. The egg hatch assay was conducted from days 24 to 40 post-infection. The BZ-selected group provided the most data due to the higher level of BZ resistance displayed by this worm population. The ED_{50} value post-BZ selection increased steadily between days 26 and 37 and then suddenly dropped (ED_{50} = 0.51μg/ml) to almost the same level of resistance as seen in the unselected group by day 40. The data points for the unselected, LEV- and IVM-selected populations on day 37 displayed a very low level of resistance (ED_{50} = 0.446, 0.302 and 0.388μg/ml, respectively) compared with the BZ-selected population (ED_{50} = 1.53μg/ml).

Trial Two Data

The data obtained from Trial Two were complete due to the success in recovering sufficient numbers of eggs from all of the donor animals. The time course extended from days 21 to 36 post-infection. Despite the pattern of the Trial Two egg hatch assay data being somewhat different to that of the Trial One data, the overall result that BZ (but not IVM or LEV) treatment increased the BZ resistant phenotype was confirmed (see Figure 3.13). At day 21 (post-infection), the unselected *MTci5* population had an ED_{50} value of ~ 0.3μg/ml, which rose to 0.701μg/ml at day 24. After treatment (day 28), all populations experienced a decline in BZ resistance; ED_{50} values for the LEV and BZ treated groups on Day 30 were 0.333μg/ml and 0.200μg/ml, respectively. The unselected population experienced a smaller decline on day 30 showing an ED_{50} value of 0.532μg/ml. By day 35, resistance had recovered to roughly the

same level as day 28 (the day of treatment) in all populations except the BZ-selected group, which had risen to 1.203ug/ml (a 1.6- fold increase).

Statistical relevance of data

Despite the egg hatch assay data being incomplete due to a number of missing data points and due to the fact that there were differences in the pattern of BZ resistance over time between the two Trials, the increase in resistance observed as a result of BZ selection was very consistent between the Trials. Given that different TBZ solutions were used for each Trial and the same process of dissociation is expected to have occurred, it is interesting that these results are broadly in agreement over the starting and end points for resistance. As Table 3.16 demonstrates, the overall resistance factors for the BZ-selected population *vs.* the unselected control were 2.0 and 1.6 for Trials One and Two, respectively. A post-treatment mean ED_{50} value was taken from each population and each Trial and a one-way ANOVA (Minitab v14, 2006) was conducted to determine the statistical relevance of these data (see Table 3.17). As the boxplot in Figure 3.14 demonstrates, the effect of BZ treatment in increasing the BZ resistance phenotype was significant at the 95% confidence interval ($F_{3,7} = 9.58$, $p = 0.027$) over both Trials, whilst the other populations were not significantly affected by IVM or LEV selection. Furthermore, a one-way ANOVA was performed to show that there was no significant effect of the Trial upon the ED_{50} data ($F_{1,7} = 0.54$, $p = 0.492$), suggesting that the assay was repeatable between consecutive summers, despite the gaps in the datasets and the partial dissolution of TBZ.

3.4.2 Faecal egg count reduction test as a phenotypic measure of resistance

There are currently two methods, which are used to estimate the efficacy of an anthelmintic treatment; the first is the controlled efficacy test (CET). The CET was not employed here due to the requirement for worm recovery without the use of the fixative, formalin. The second, less accurate method, is the faecal egg count reduction test (FECRT). Two analyses were performed on the faecal egg count data. The standard FECRT data are summarised in Figure 3.15. The FECRT provided mean BZ, IVM and LEV efficacies of 32 ± 7%, 73 ± 24% and 88 ± 3%, respectively.

A paired T-test was performed upon these data using Minitab v14 (2006). This test compared the means of the raw FEC data before and after treatment and estimated the probability that these means were different by chance (using 95% confidence intervals). The test for equal variances was performed first on the data as this is an important parameter for the pairwise T-test. The test results are summarised in Table 3.18. These data indicate that there was no significant reduction in egg counts in the unselected populations (as expected) or the BZ-selected populations (unexpected). However, there were highly significant reductions in the IVM and LEV-selected populations' egg counts. These results were consistent across both Trials. Therefore, in contrast with the standard FECRT method, the statistics imply that BZ treatment had no effect upon faecal egg output. This is probably due to the variance in the FEC data, which is removed when an average is taken for FECRT. Thus a one-way ANOVA was also performed using the mean FECRT percentages calculated from each Trial (as displayed in Table 3.19). The results of this test indicated that there was a significant effect of treatment upon reduction in faecal egg count at the 99% confidence interval ($F_{3,7} = 16.42$, $p = 0.010$). As the boxplot in Figure 3.16 shows, the variance was highest in the IVM-selected populations. Again, a one-way ANOVA of FECRT by Trial showed that there was no effect of the Trial upon the results ($F_{3,7} = 0.06$, $p = 0.809$), suggesting that the results are reliable and consistent. From these results, we can conclude that *MTci5* shows very high levels of resistance to BZ, and moderate to low resistance against the IVM and LEV anthelmintics, respectively.

Chapter 3 Tables & Figures

Table 3.1: Missing data due to null alleles, calculated in GDA version 1.1 (Lewis & Zaykin, 2001). There were no missing data from the LEV-selected population at any locus.

Population	Locus	Missing data
Unselected	MTG15	6%
BZ-selected	MTG15	17%
IVM-selected	MTG15	13%
Unselected	HCMS28	6%
BZ-selected	HCMS28	6%
IVM-selected	HCMS28	17%

Table 3.2: Linkage disequilibrium analysis. Chi-square values and P-values are shown for each possible locus pairing across all populations (Fisher's method). Genepop version 3.4 (Raymond & Rousset, 2004) option 2 used.

Locus pair	Chi-square value	Degrees freedom	P-value
MTG15 & MTG67	5.581	8	0.694
MTG15 & MTG73	5.248	8	0.731
MTG15 & MTG74	8.427	8	0.393
MTG15 & HCMS28	2.360	8	0.968
MTG67 & MTG73	7.396	8	0.495
MTG67 & MTG74	10.507	8	0.231
MTG67 & HCMS28	1.642	8	0.990
MTG73 & MTG74	13.792	8	0.087
MTG73 & HCMS28	5.282	8	0.727
MTG74 & HCMS28	4.659	8	0.793

Figure 3.2a: Chart displaying the allele frequencies at locus MTG15 of the *MTci5* populations.

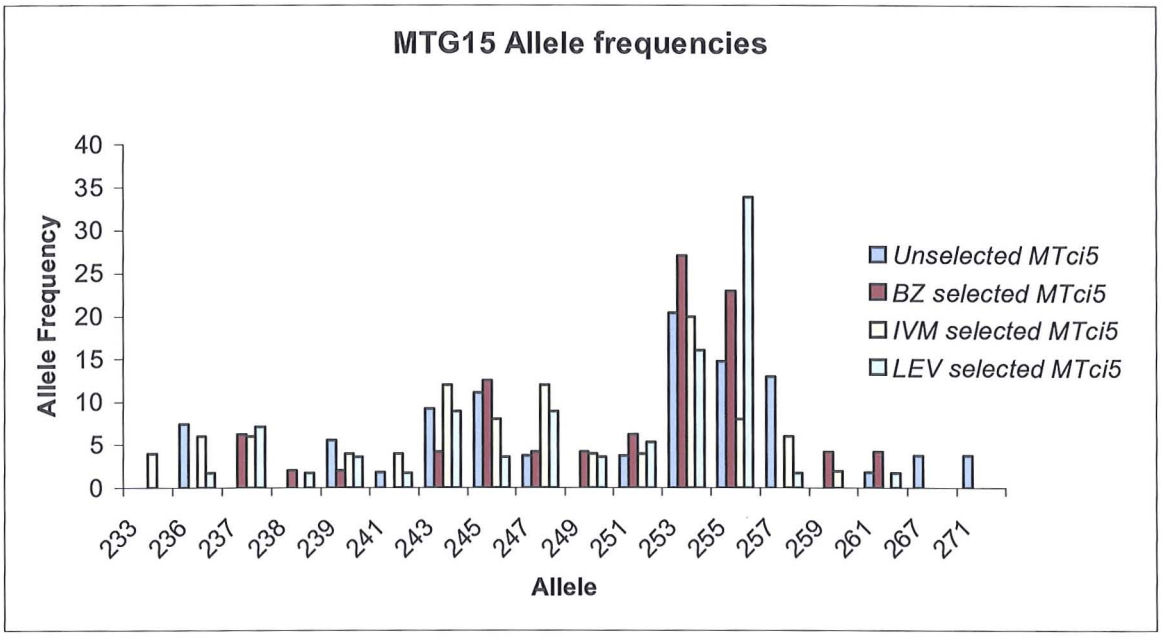


Figure 3.2b: Chart displaying the allele frequencies at locus MTG67 of the *MTci5* populations.

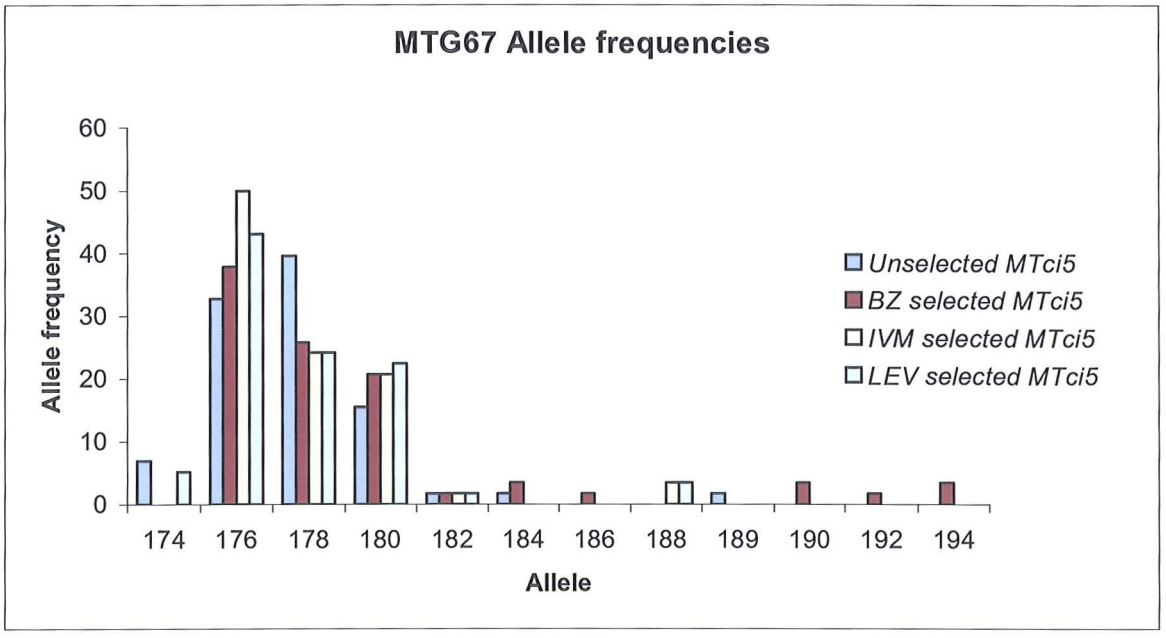


Figure 3.2c: Chart displaying the allele frequencies at locus MTG73 of the *MTci5* populations.

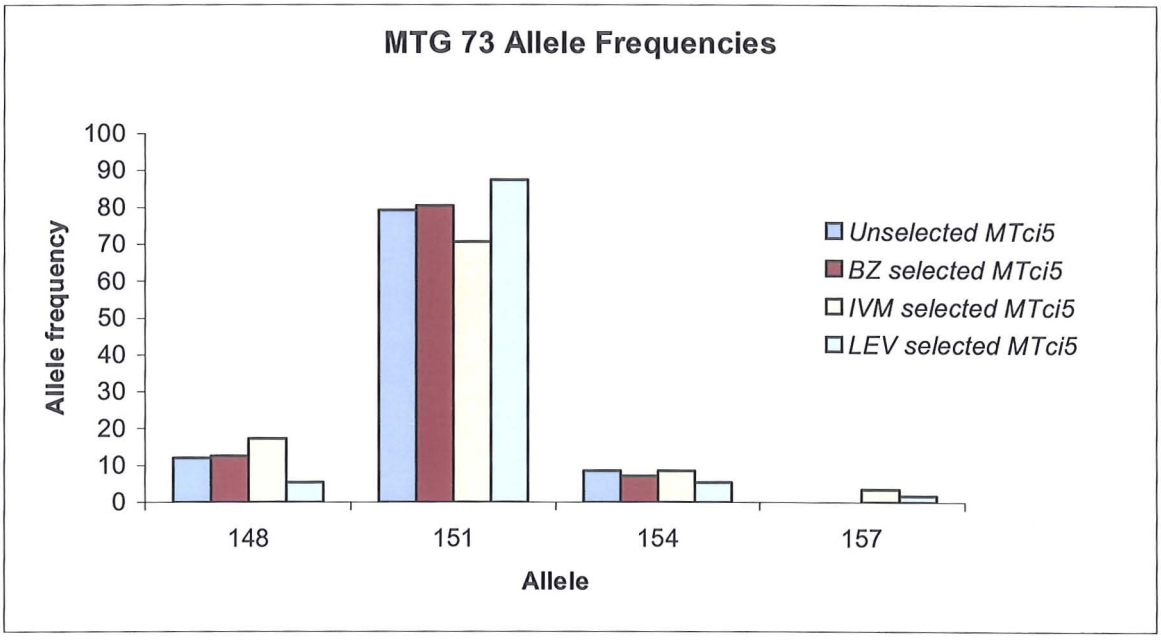


Figure 3.2d: Chart displaying the allele frequencies at locus MTG74 of the *MTci5* populations.

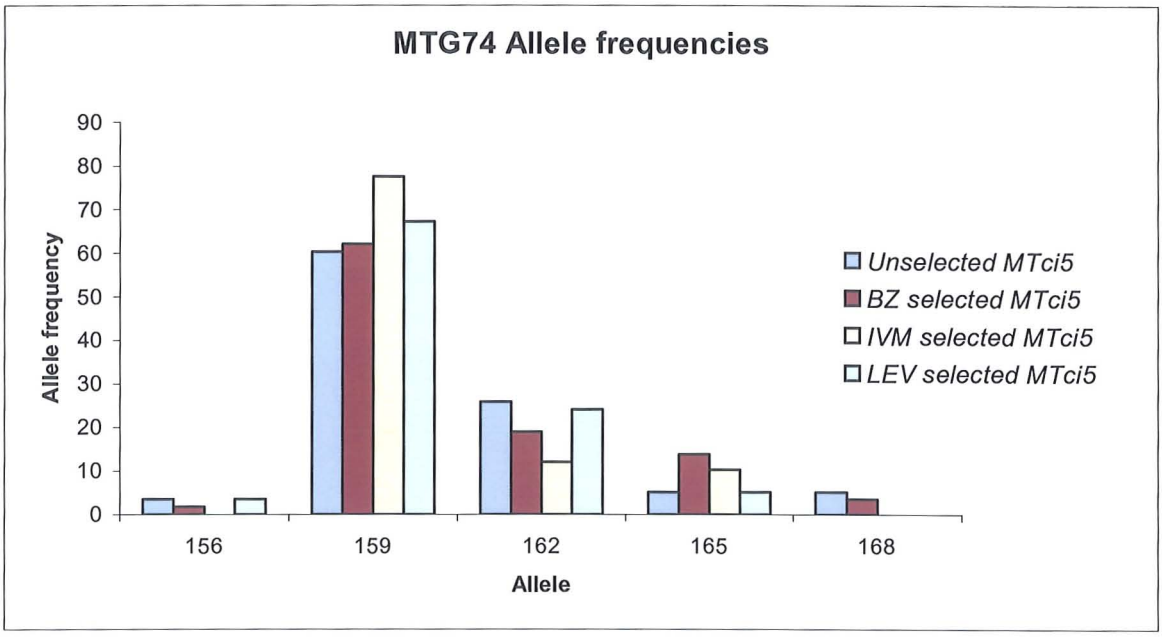


Figure 3.2e: Chart displaying the allele frequencies at locus HCMS28 of the *MTci5* populations.

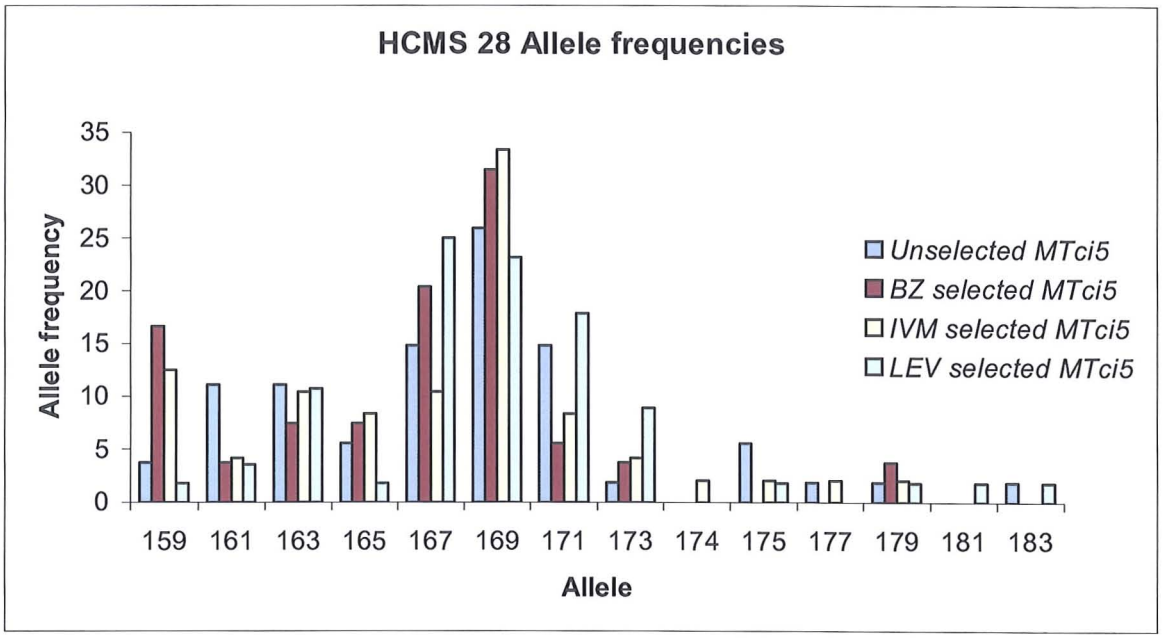


Figure 3.3: Summary of allelic patterns across all loci calculated in GenAlEx, version 6 (Peakall & Smouse, 2006). Most of the legends are self-explanatory however, the 'number of effective alleles' is the quantity which will be most likely be passed on to the next generation and the 'number of private alleles' refers to the number which are unique to each population. Expected heterozygosity is displayed on the secondary y axis.

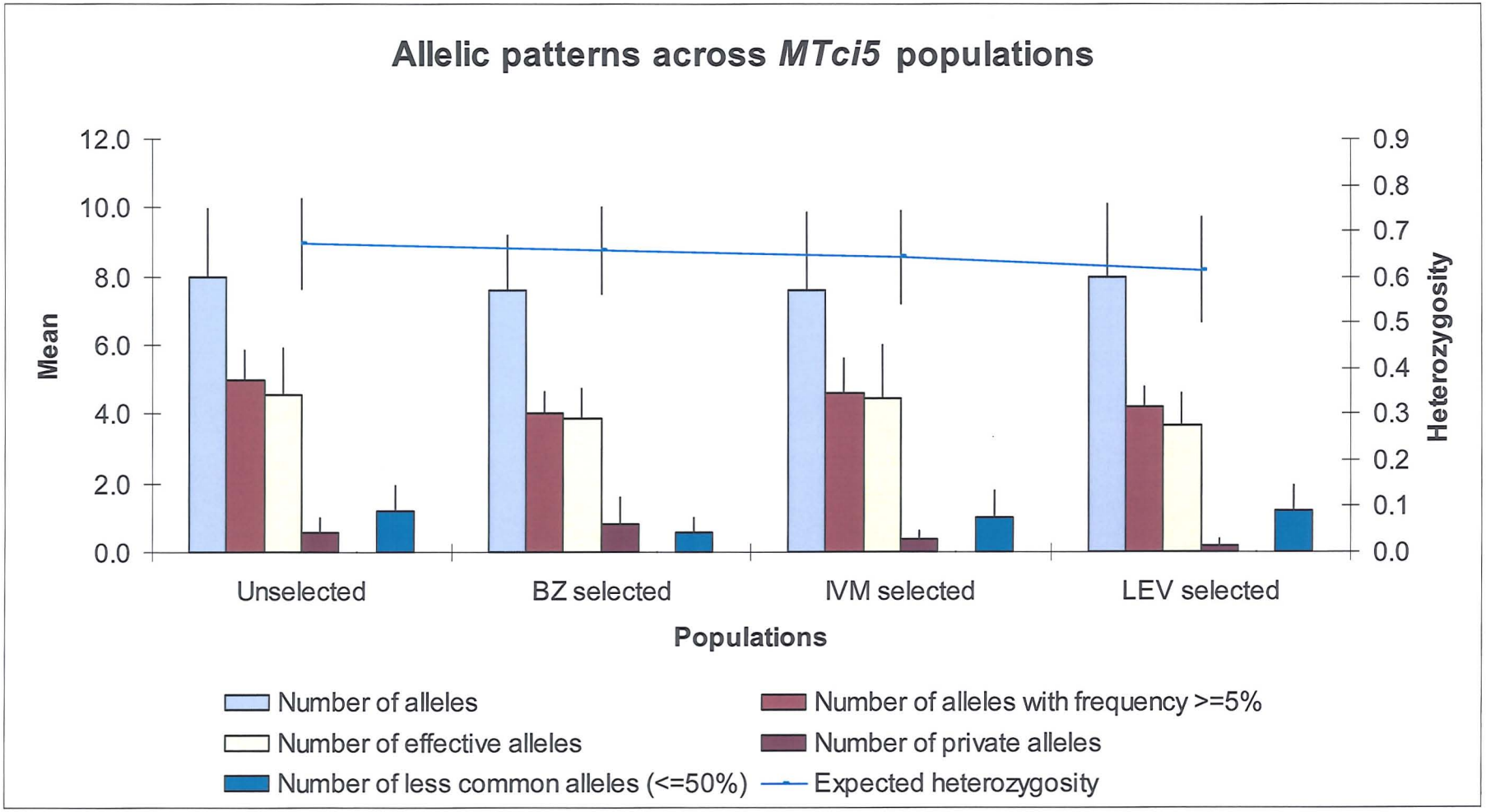


Table 3.3a: Tests of Hardy-Weinberg Equilibrium. Expected and observed heterozygosity statistics across all loci per *MTci5* population as well as Wright's F_{IS} (Weir & Cockerham method) statistic and p-values were calculated in GDA version 1.1 (Lewis & Zaykin, 2001). Significant p values (at the 5% level or less) are shown in bold.

Population	MTG15				MTG67				MTG73				MTG74				HCMS28			
	Ho	He	F_{IS}	P value	Ho	He	F_{IS}	p value	Ho	He	F_{IS}	p value	Ho	He	F_{IS}	p value	Ho	He	F_{IS}	**p value**
Unselected	0.296	0.901	0.675	**0.000**	0.759	0.718	-0.06	0.559	0.310	0.355	0.128	0.391	0.517	0.572	0.098	0.066	0.889	0.871	-0.02	0.383
BZ-selected	0.250	0.859	0.713	**0.000**	0.552	0.755	0.273	**0.008**	0.321	0.340	0.054	0.475	0.310	0.568	0.458	**0.003**	0.703	0.829	0.153	0.121
IVM-selected	0.480	0.918	0.482	**0.000**	0.552	0.659	0.165	0.305	0.448	0.470	0.047	0.469	0.172	0.379	0.550	**0.002**	0.875	0.850	-0.03	0.602
LEV-selected	0.321	0.845	0.624	**0.000**	0.552	0.714	0.230	**0.016**	0.250	0.232	-0.08	1.000	0.207	0.494	0.586	**0.000**	0.786	0.844	0.070	0.856
Overall	0.337	0.880	0.624	**0.000**	0.603	0.711	0.155	**0.006**	0.332	0.349	0.037	0.770	0.301	0.503	0.423	**0.000**	0.813	0.849	0.043	0.487

Table 3.3b: HWE Statistics by population when locus MTG15 data are included in the analysis. Significant p values (at the 5% level or less) are shown in bold.

Population	All loci				All loci excluding MTG15			
	Ho	He	F_{IS}	p value	Ho	He	F_{IS}	**p value**
Unselected	0.554	0.684	0.192	**0.000**	0.619	0.629	0.017	0.551
BZ-selected	0.427	0.670	0.366	**0.000**	0.472	0.623	0.246	**0.000**
IVM-selected	0.505	0.655	0.232	**0.000**	0.512	0.590	0.134	**0.037**
LEV-selected	0.423	0.626	0.328	**0.000**	0.449	0.571	0.218	**0.000**
Overall	0.478	0.659	0.279	**0.000**	0.513	0.603	0.152	0.147

Table 3.3c: HWE Statistics by locus (i.e. all populations are treated as one) when locus MTG15 data are excluded from the analysis. Significant p values (at the 5% level or less) are shown in bold.

Locus	All populations			
	Ho	He	F_{IS}	p value
MTG67	0.711	0.603	0.154	**0.008**
MTG73	0.332	0.349	0.049	0.771
MTG74	0.302	0.503	0.405	**0.000**
HCMS28	0.813	0.849	0.042	0.438
Overall	0.512	0.606	0.154	**0.000**

Table 3.4: Population diversity information. N_T denotes the number of worms genotyped per population and N_A gives the number of worms included in analysis. An asterisk* indicates the number of unique alleles found per locus and per population. The numbers in brackets indicate the total number of alleles possible at each locus (determined by microsatellite author: V. Grillo, personal communication).

Population	N_T	N_A	Number of alleles observed per locus				
			MTG15 (26)	MTG67 (19)	MTG73 (6)	MTG74 (8)	HCMS28 (18)
Unselected	29	27	13*[2]	7*[1]	3	5	12
BZ-selected	29	24	12	9*[4]	3	5	9
IVM-selected	29	25	14*[1]	5	4	3	12*[1]
LEV-selected	29	28	14	6	4	4	12*[1]
Overall	116	104	18	12	4	5	14

Figure 3.4a: Principle Coordinate Analysis of unselected *MTci5* isolate data vs. V. Grillo's data of female adult *MTci5* worms (population name: 'VG *MTci5*') using all loci.

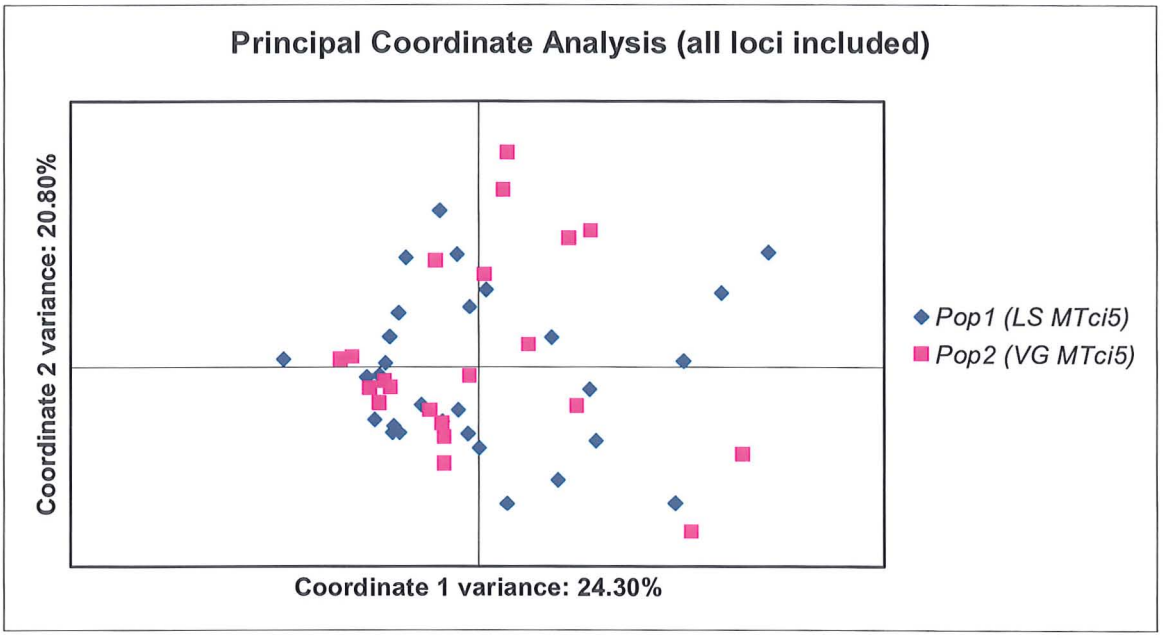


Figure 3.4b: Principle Coordinate Analysis of unselected *MTci5* isolate data vs. V. Grillo's data of female adult *MTci5* worms (population name: 'VG *MTci5*') excluding MTG15 data.

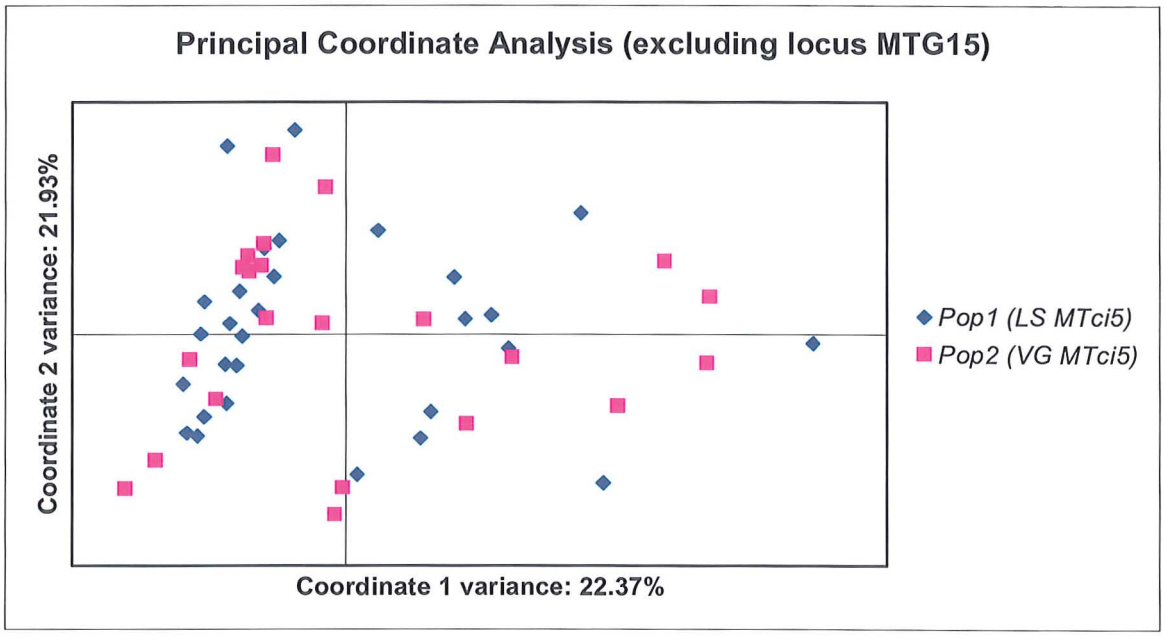


Figure 3.5a: PCA analysis: all populations, all loci.

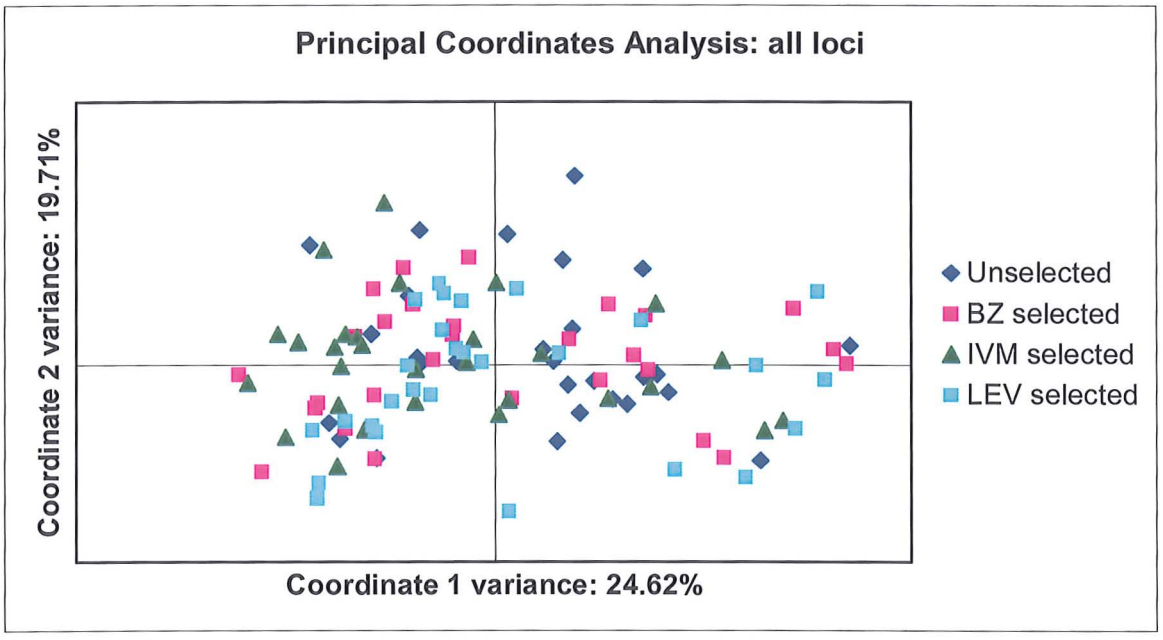


Figure 3.5b: PCA analysis: all populations, excluding locus MTG15.

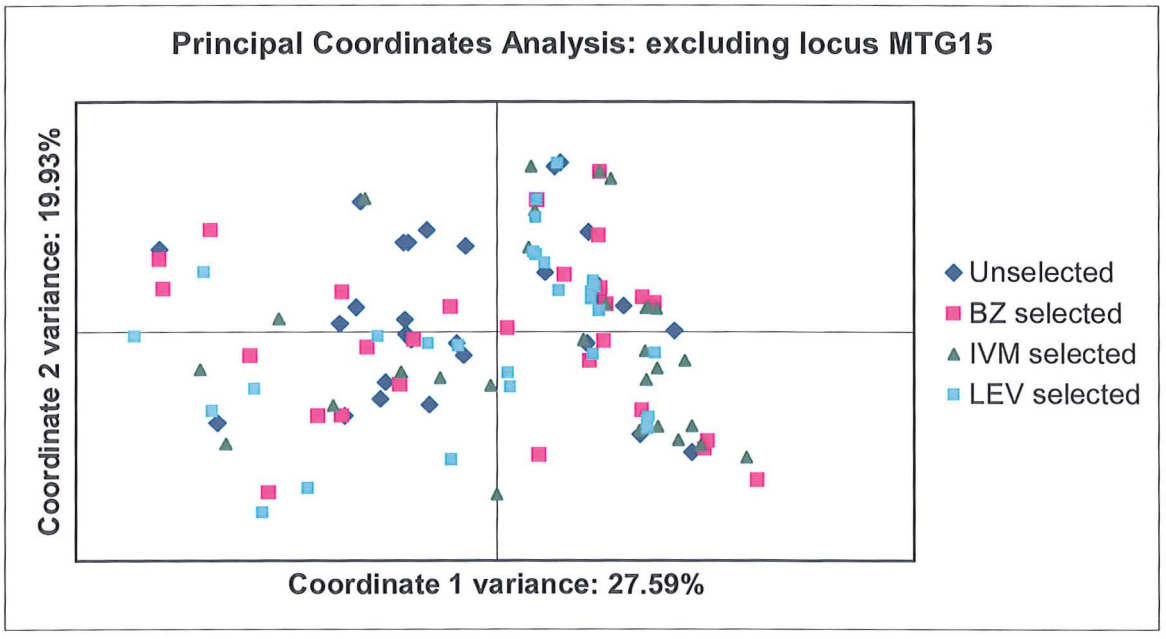


Figure 3.5c: PCA analysis: all populations, excluding locus MTG67.

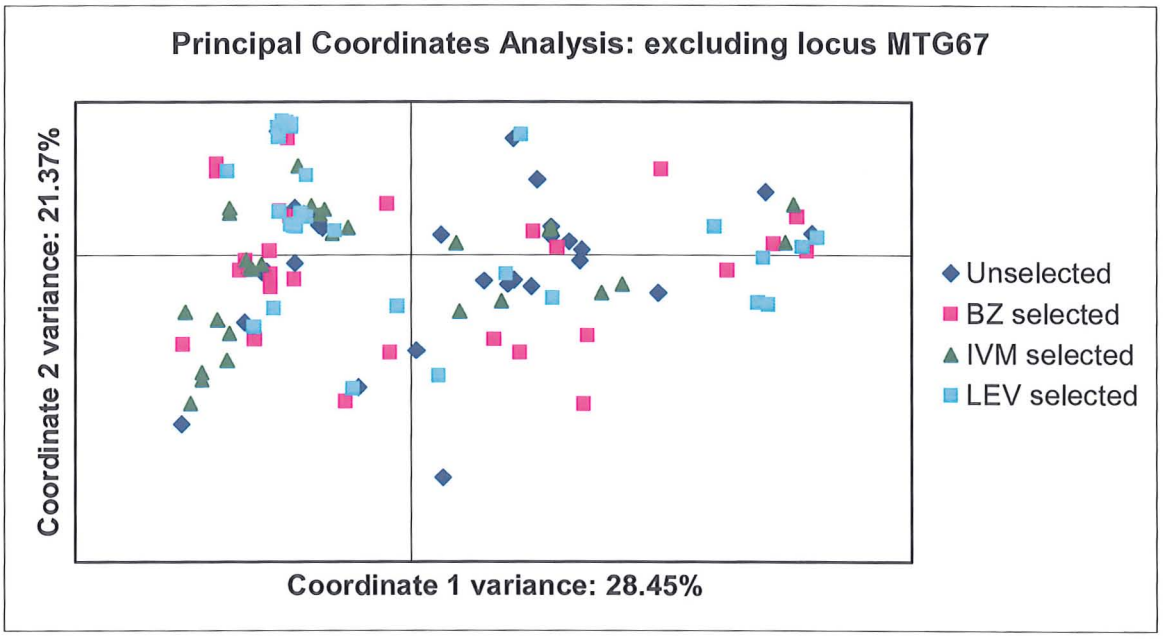


Figure 3.5d: PCA analysis: all populations, excluding locus MTG73.

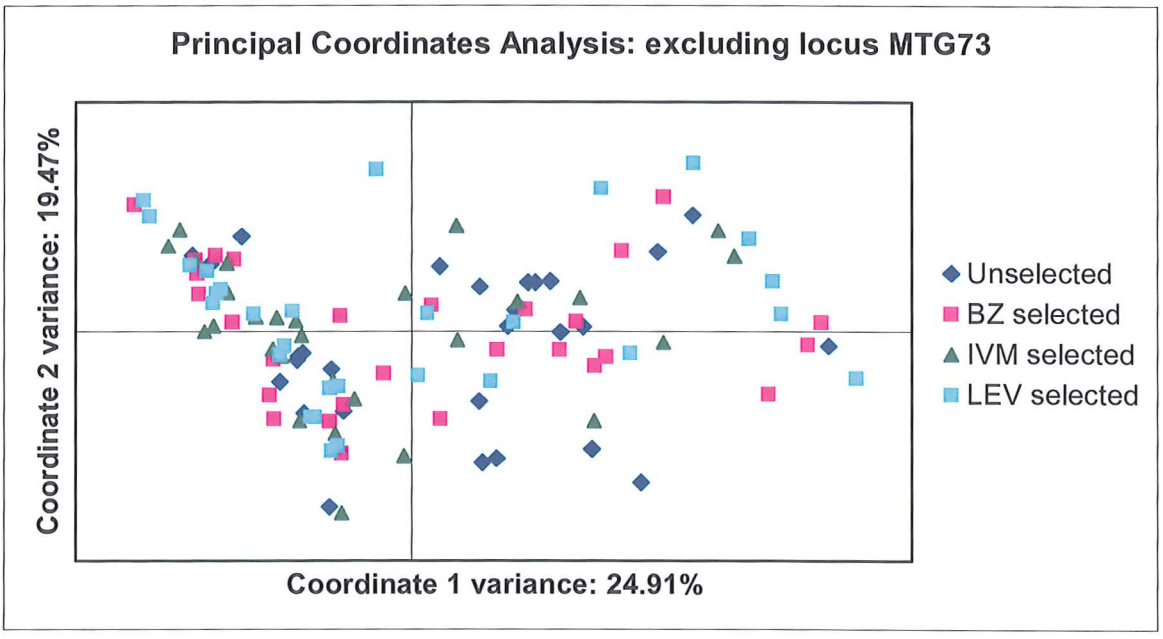


Figure 3.5e: PCA analysis: all populations, excluding locus MTG74.

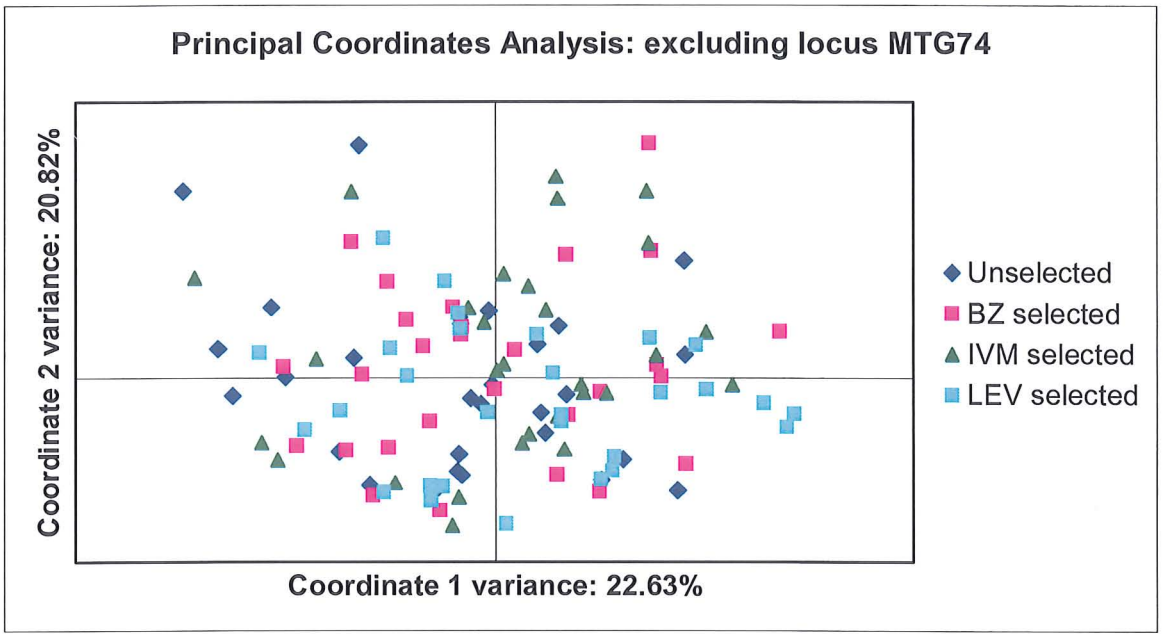


Figure 3.5f: PCA analysis: all populations, excluding locus HCMS28.

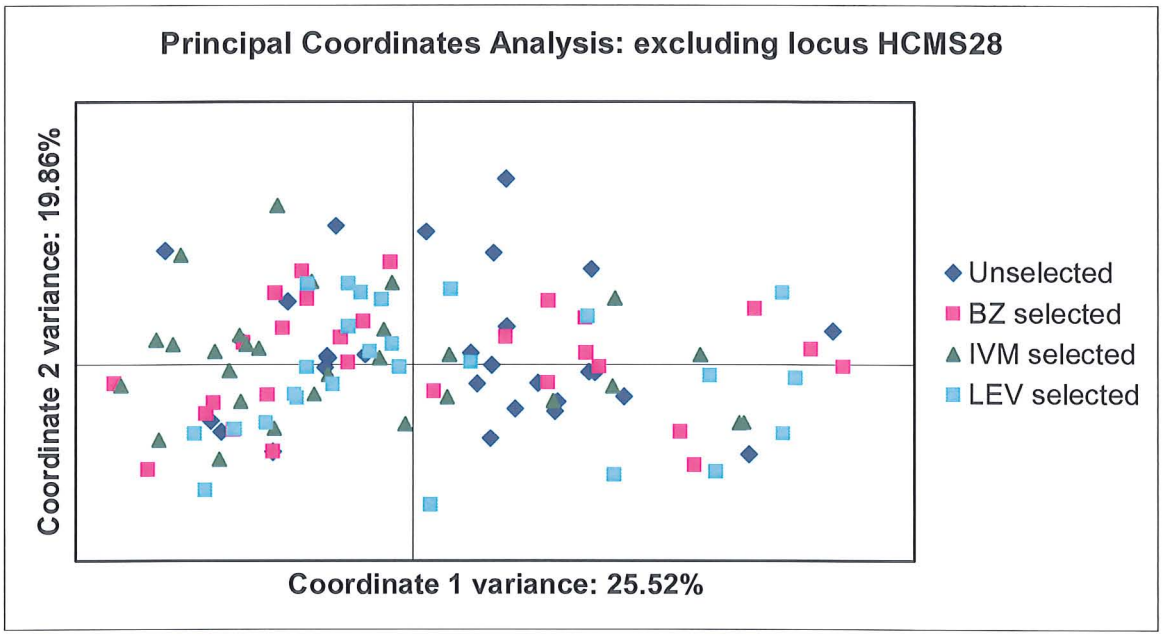


Table 3.5a: Pairwise F_{ST} values of *MTci5* populations calculated using GDA version 1.1 (Lewis & Zaykin, 2001).

	Unselected	BZ-selected	IVM-selected	LEV-selected
Unselected				
BZ-selected	0.002266			
IVM-selected	0.009347	0.001702		
LEV-selected	0.006792	0.002185	0.015863	

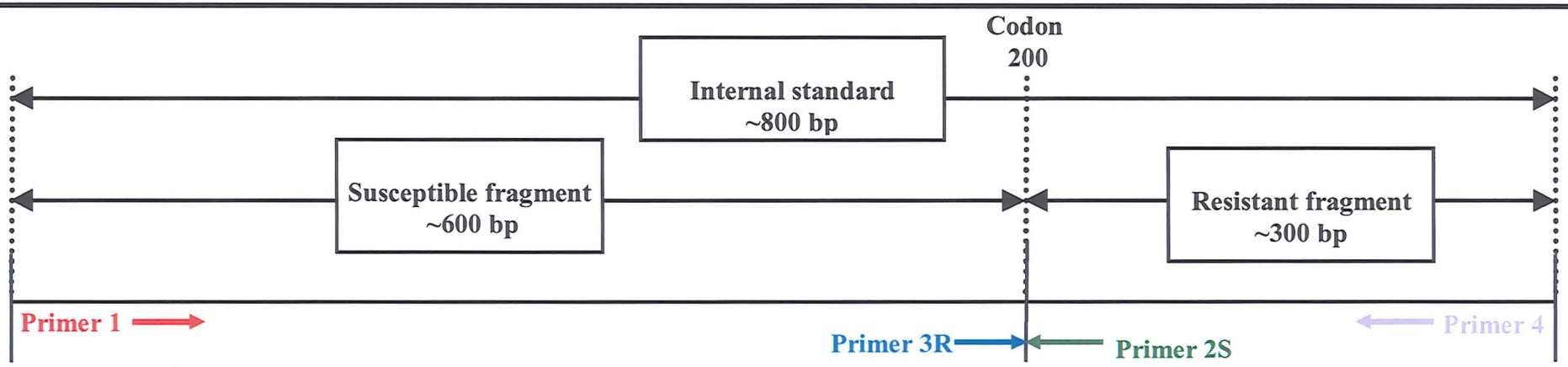
Table 3.5b: Pairwise F_{ST} values of *MTci5* populations calculated using GDA version 1.1 (Lewis & Zaykin, 2001) when locus MTG15 data are excluded from analysis.

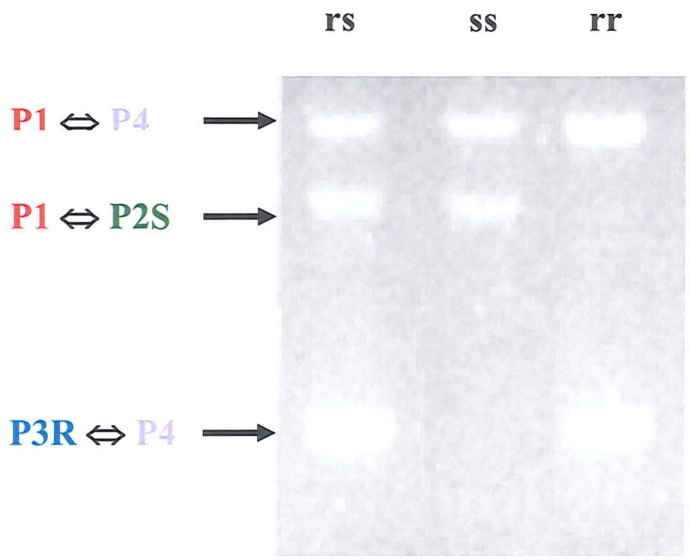
	Unselected	BZ-selected	IVM-selected	LEV-selected
Unselected				
BZ-selected	0.000119			
IVM-selected	0.013925	-0.001629		
LEV-selected	0.000819	0.001397	0.012089	

Table 3.6: Analysis of molecular variance test conducted using Arlequin version 2.0 (Schneider *et al*, 2000). There was no effect of removing locus MTG15 data from the analysis. The F_{ST} statistic was 0.004 and the probability that the random value is equal to the observed value was very low (<0.01).

Source of variation	Degrees of freedom	Sum of Squares	Variance components	Percentage of variation
Among populations	3	2.884	0.003	0.41
Within populations	228	177.207	0.777	99.59
Total	231	180.091	0.780	100.00

Figure 3.6: Allele-specific PCR (after Elard *et al*, 1999). Primer 1 and Primer 4 are the forward and reverse primers. Primers Primer 2S and Primer 3R flank the $P200^{Phe\text{-}Tyr}$ mutation and determine whether the susceptible and/or resistant alleles are present.





β-tubulin isotype I gene partial cDNA with F200Y and primer positions highlighted

GAACAATGGACTCTGTTCGTTCTGGACCGTATGGACAACTTTTCCGTCCAGATAATTACG

TGTTTGGCCAGTCAGGAGCGGGTAACAACTGGGCGAAGGGCCACTATACCGAGGGAGC

TGAGCTTGTTGACAACGTCTTAGATGTTGTTCGTAAAGAGGCAGAGGGTTGCGATTGCC

TTCAGGGCTTCCAACTGACGCATTCTTTGGGAGGAGGTACTGGTTCGGGTATGGGCACT

TTGCTCATCTCCAAAATTCGCGAGGAGTATCCGGATAGAATCATGGCTTCATTCTCCGTT

GTTCCATCACCTAAGGTATCCGACACCGTTGTGGAACCTTACAATGCCACTCTTTCTGTA

CACCAATTGGTAGAAAACACCGATGAAACATTCTGCATCGATAATGAAGCTCTCTGCAT

CGATAATGAAGCTCTGTACGATATCTGCTTCCGCACCTTGAAACTCACAAATCCAACTT

ATGGCGATCTCAATCACTTAGTGTCTGTCACAATGTCTGGAGTCAGACCTGCCTTCGATT

CCCTGGACAGCTGAATGCTGATCTTCGCAAGTTAGCCG

Table 3.7: F200Y isotype I β-tubulin genotypes of adult worms from the unselected *MTci5* population as determined by allele-specific PCR. Trial One worms were collected on 31/08/2003, Trial Two worms were collected on 13/09/2004. The actual counts for each genotype are shown in brackets (n).

Trial	%P200$^{Tyr/Tyr}$ genotypes (n)	%P200$^{Phe/Tyr}$ genotypes (n)	%P200$^{Phe/Phe}$ genotypes (n)	Number genotyped
One	48.4% (15)	41.9% (13)	9.7% (3)	31
Two	37.5% (15)	57.5% (23)	5.0% (2)	40
Overall	**42.3% (30)**	**50.7% (36)**	**7.0% (5)**	**71**

Table 3.8: F200Y isotype I β-tubulin genotypes of L_3 worms from the unselected *MTci5* population as determined by allele-specific PCR. The actual counts for each genotype are shown in brackets (n).

Trial	%P200$^{Tyr/Tyr}$ genotypes (n)	%P200$^{Phe/Tyr}$ genotypes (n)	%P200$^{Phe/Phe}$ genotypes (n)	Number genotyped
One	47.3% (35)	39.2% (29)	13.5% (10)	74
Two	34.3% (12)	57.1% (20)	8.6% (3)	35
Overall	**43.1% (47)**	**45.0% (49)**	**11.9% (13)**	**109**

Table 3.9: F200Y isotype I β-tubulin genotypes of adult worms from the unselected *MTci5* population as determined by Pyrosequencing. Trial One worms were collected on 31/08/2003, Trial Two worms were collected on 13/09/2004. The actual counts for each genotype are shown in brackets (n).

Trial	%P200$^{Tyr/Tyr}$ genotypes (n)	%P200$^{Phe/Tyr}$ genotypes (n)	%P200$^{Phe/Phe}$ genotypes (n)	Number genotyped
One	32.2% (10)	58.1% (18)	9.7% (3)	**31**
Two	41.5% (17)	43.9% (18)	14.6% (6)	**41**
Overall	**37.5% (27)**	**50.0% (36)**	**12.5% (9)**	**72**

Figure 3.7: F200Y isotype I β-tubulin genotyping results from both techniques: allele-specific PCR vs. Pyrosequencing (adult male worm data from both Trials).

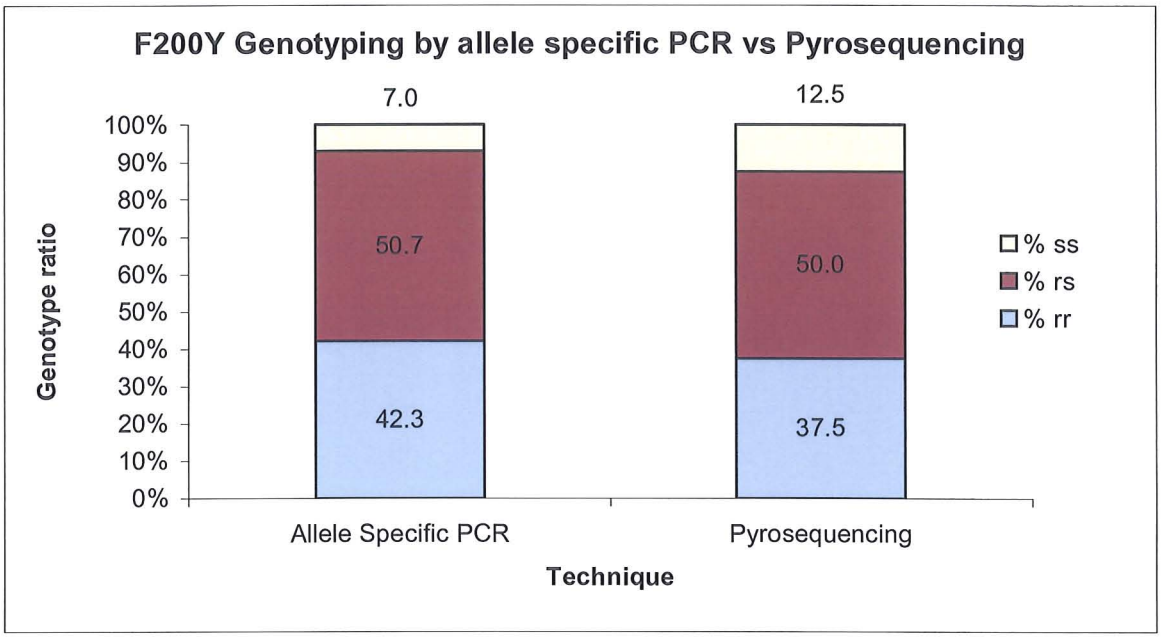


Table 3.10a: Allele-specific PCR results are shown below for adult worms from each Trial and each *MTci5* population. The actual counts for each genotype are shown in brackets (n).

Population	Trial	%P200$^{Tyr/Tyr}$ genotypes (n)	%P200$^{Phe/Tyr}$ genotypes (n)	%P200$^{Phe/Phe}$ genotypes (n)	Number genotyped
Unselected	One	48% (15)	42% (13)	10% (3)	31
	Two	37% (15)	58% (23)	5% (2)	40
BZ-selected	One	81% (25)	19% (6)	0% (0)	31
	Two	77% (30)	21% (8)	3% (1)	39
IVM-selected	One	47% (17)	42% (15)	11% (4)	36
	Two	44% (17)	49% (19)	8% (3)	39
LEV-selected	One	50% (14)	36% (10)	14% (4)	28
	Two	40% (16)	48% (19)	13% (5)	40

Table 3.10b: Pyrosequencing results are shown below for adult worms from each Trial and each *MTci5* population. The actual counts for each genotype are shown in brackets (n).

Population	Trial	%P200$^{Tyr/Tyr}$ genotypes (n)	%P200$^{Phe/Tyr}$ genotypes (n)	%P200$^{Phe/Phe}$ genotypes (n)	Number genotyped
Unselected	One	32% (10)	58% (18)	10% (3)	31
	Two	41% (17)	44% (18)	15% (6)	41
BZ-selected	One	74% (23)	26% (8)	0% (0)	31
	Two	83% (34)	15% (6)	2% (1)	41
IVM-selected	One	44% (16)	50% (18)	6% (2)	36
	Two	38% (11)	58% (14)	5% (3)	40
LEV-selected	One	39% (14)	50% (10)	11% (4)	28
	Two	39% (16)	39% (19)	22% (9)	41

Figure 3.8: Chart displaying pooled adult genotypes of each *MTci5* population (Pyrosequencing data). The error bars are standard deviations calculated in excel based on Trial One vs. Trial Two percentage genotypes.

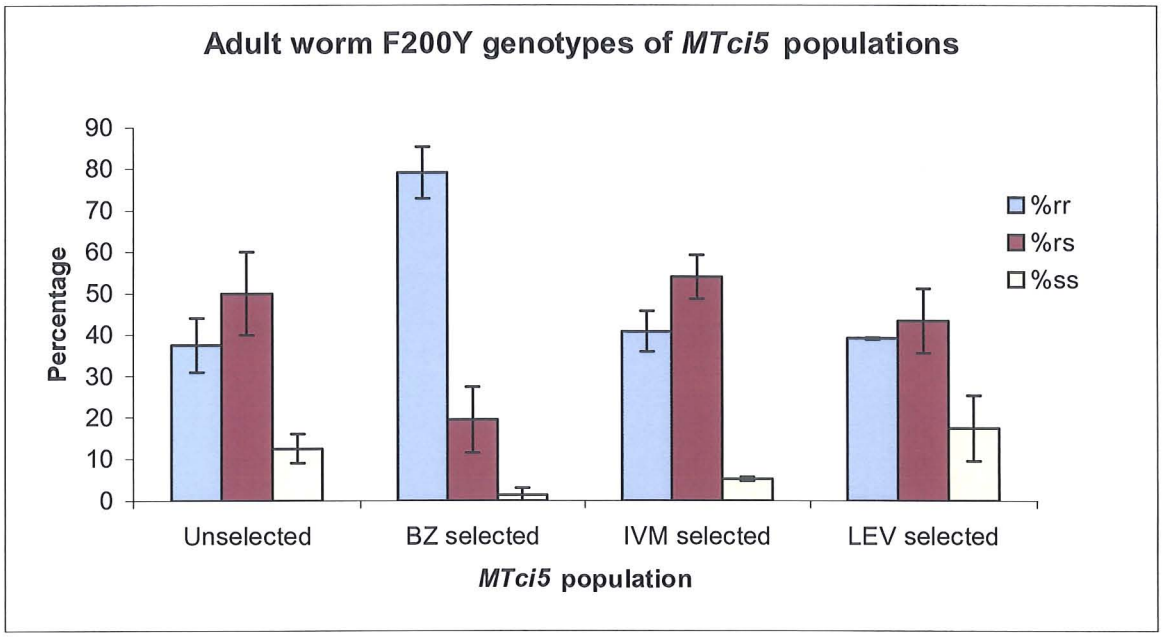


Table 3.11: Results of an 'estimation of the differences between genotype proportions' test (showing 95% confidence interval estimates) conducted upon adult F200Y isotype I β-tubulin genotype data obtained by Pyrosequencing. T1 and T2 refer to Trials 1 and 2. All have p values of > 0.05.

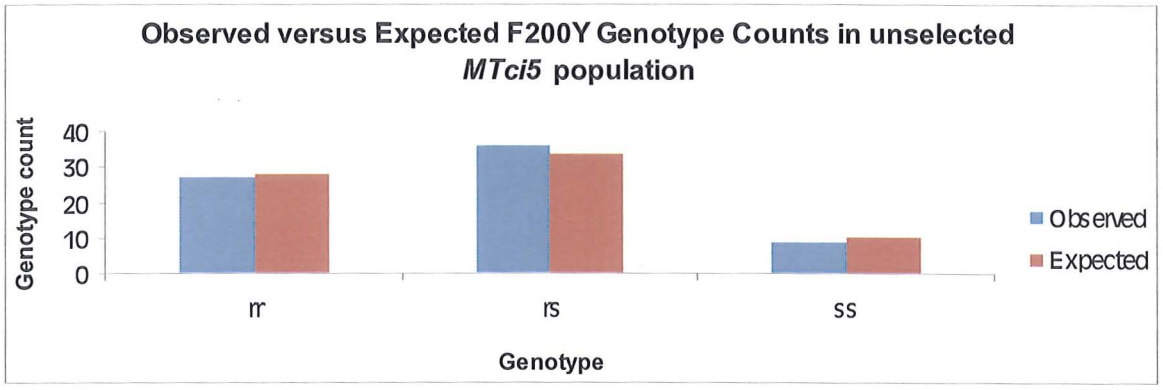
	T1 vs. T2 $P200^{Tyr/Tyr}$	T1 vs. T2 $P200^{Phe/Tyr}$	T1 vs. T2 $P200^{Phe/Phe}$
Unselected	0.201, -0.385	0.445, -0.162	0.148, -0.247
BZ-selected	0.165, -0.340	0.359, -0.136	0.038, -0.086
IVM-selected	0.360, -0.221	0.219, -0.369	0.138, -0.127
LEV-selected	0.311, -0.306	0.422, -0.203	0.112, -0.337

Table 3.12: Pairwise Fst conducted in GDA at locus F200Y isotype I β-tubulin locus. Values highlighted in red indicate great genetic differentiation according to Wright's guidelines. All other values indicate little differentiation.

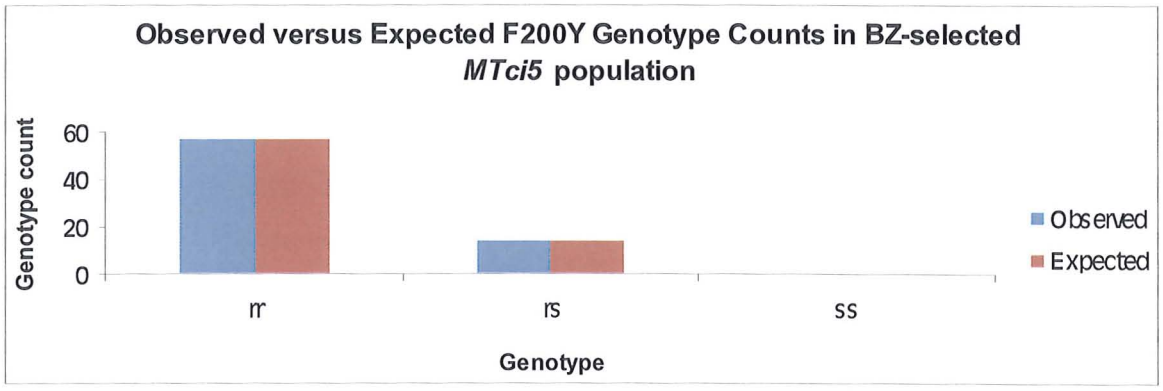
	Unselected	BZ-selected	IVM-selected	LEV-selected
Unselected				
BZ-selected	0.167109			
IVM-selected	-0.000680	0.116272		
LEV-selected	-0.006580	0.184541	0.003432	

Figure 3.9 Chi-square (X^2) analysis (of Hardy Weinberg Equilibrium) calculated in GenAlEx version 6 (Peakall & Smouse, 2006) add-in for Microsoft Excel. The graphs show observed versus expected F200Y isotype I β-tubulin genotype counts in each *MTci5* population. The Chi-square statistics are shown below. Df = degrees of freedom, X^2 = Chi-square statistic and p = probability value. The letters rr, rs and ss refer to $P200^{Tyr/Tyr}$, $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes, respectively.

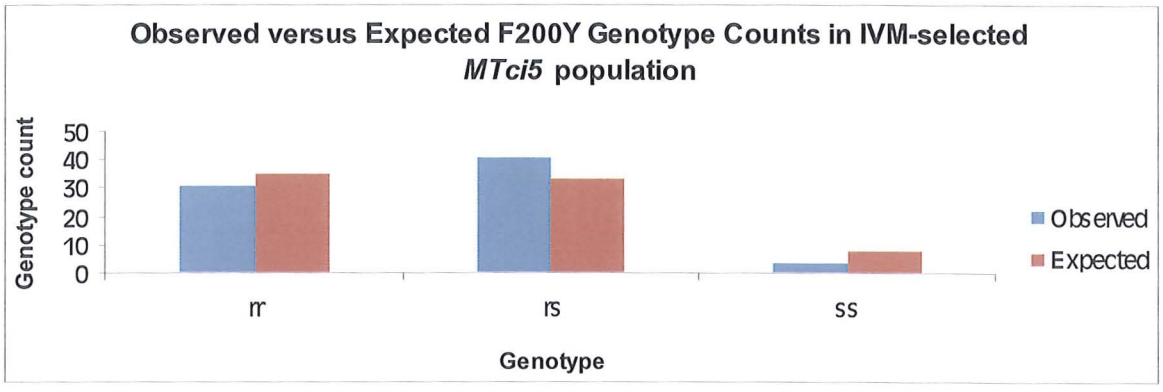
3.9a Chi-square result for unselected *MTci5* population: Df = 1, X^2 = 0.320, p = 0.572.



3.9b Chi-square result for BZ-selected *MTci5* population: Df = 1, X^2 = 0.018, p = 0.895.



3.9c Chi-square result for IVM-selected *MTci5* population: Df = 1, X^2 = 4.190, p = 0.041.



3.9d Chi-square result for LEV-selected *MTci5* population: Df = 1, X^2 = 0.526, p = 0.468.

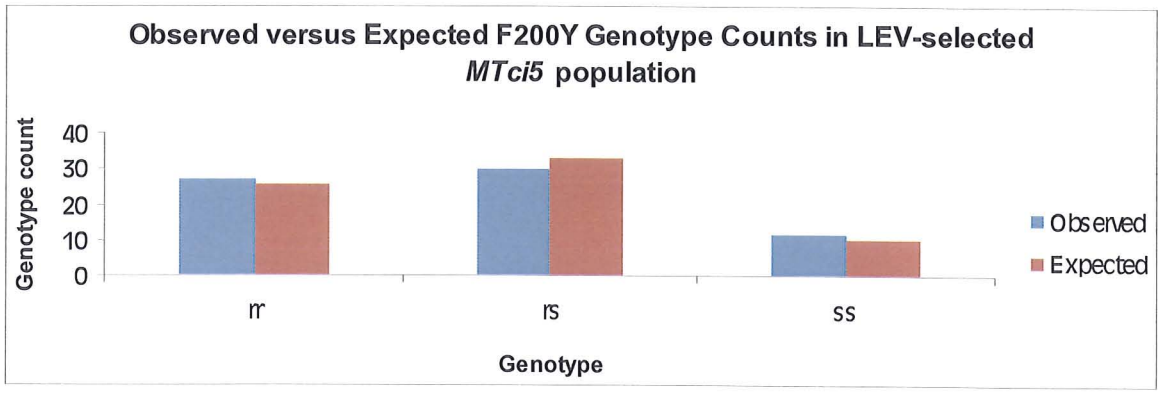


Table 3.13: Allele-specific PCR results are shown below for L_3 worms from each Trial and each *MTci5* population. The actual counts for each genotype are shown in brackets (n).

Population	Trial	%P200$^{Tyr/Tyr}$ genotypes (n)	%P200$^{Phe/Tyr}$ genotypes (n)	%P200$^{Phe/Phe}$ genotypes (n)	Number genotyped
BZ-selected	One	72% (47)	23% (15)	5% (3)	65
	Two	81% (26)	19% (6)	0% (0)	32
IVM-selected	One	28% (12)	67% (29)	5% (2)	43
	Two	43% (17)	35% (14)	23% (9)	40
LEV-selected	One	40% (20)	46% (23)	14% (7)	50
	Two	35% (14)	45% (18)	20% (8)	40

Table 3.14: Results of an 'estimation of the differences between genotype proportions' test (with 95% confidence interval estimates) conducted upon L_3 F200Y isotype I β-tubulin genotype data obtained by allele-specific PCR. T1 and T2 refer to Trials 1 and 2. All have p values of > 0.05 except the IVM-selected P200$^{Phe/Tyr}$ group, which was significantly higher in Trial One than in Trial Two (shown in bold, 99% CI estimates).

MTci5 population	T1 vs. T2 P200$^{Tyr/Tyr}$	T1 vs. T2 P200$^{Phe/Tyr}$	T1 vs. T2 P200$^{Phe/Phe}$
Unselected	0.385, -0.125	0.081, -0.440	0.209, -0.110
BZ-selected	0.139, -0.318	0.266, -0.180	0.113, -0.021
IVM-selected	0.122, -0.413	**0.592, 0.057**	0.011, -0.368
LEV-selected	0.314, -0.214	0.282, -0.262	0.146, -0.266

Figure 3.10: Chart displaying pooled L_3 genotypes from each *MTci5* population (allele-specific PCR data). The error bars are standard deviations calculated in excel based on Trial One vs. Trial Two percentage genotypes.

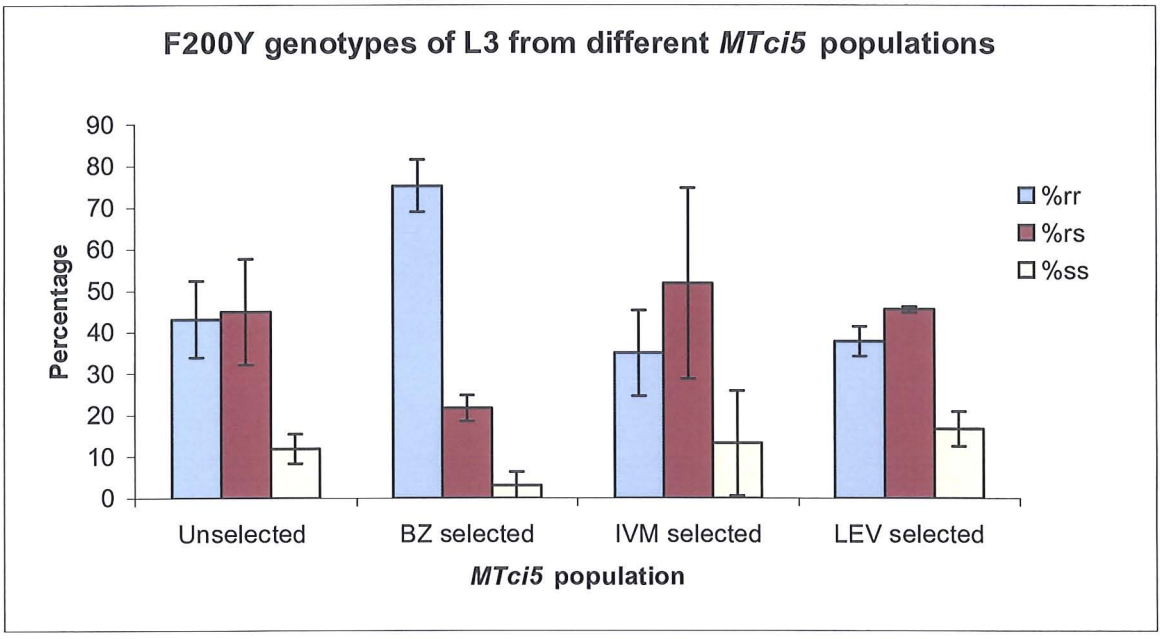


Table 3.15: Summary of egg hatch assay data obtained for *MTci5* field isolate compared to that of a susceptible isolate (*MTci1*). ED values are defined as the amount of TBZ required (μg/ml) to reduce the egg hatch rate by 50, 96 and 99%. The stage of infection is indicated by the number of days PI (post-infection). All Trials include data from at least four independent assays carried out on different days, with each drug concentration performed in duplicate. The resistance factor was obtained by dividing the mean ED value of the *MTci5* data by that of the susceptible isolate (*MTci1*). Note that the Trial Two *MTci1* data were not performed at the same time of year as the *MTci5* isolate.

Data		Mean ED_{50} ± SD	Mean ED_{96} ± SD	Mean ED_{99} ± SD
MTci5 isolate	Trial One (24 - 40 days PI)	0.424 ± 0.114	1.099 ± 0.454	1.322 ± 0.568
	Trial Two (21 - 36 days PI)	0.525 ± 0.167	1.559 ± 0.474	1.899 ± 0.583
	Mean over both Trials	**0.488 ± 0.152**	**1.392 ± 0.501**	**1.689 ± 0.621**
MTci1 isolate	Trial One (24 - 31 days PI))	0.118 ± 0.029	0.290 ± 0.081	0.346 ± 0.098
	Trial Two (21 - 27 days PI)	0.106 ± 0.029	0.208 ± 0.059	0.268 ± 0.107
	Mean over both Trials	**0.112 ± 0.024**	**0.249 ± 0.074**	**0.307 ± 0.095**
Resistance factor based on means of *MTci5* vs. *MTci1* isolate		**4.362**	**5.598**	**5.502**

Figure 3.11: Summary of egg hatch assay data from *MTci5* vs. *MTci1* isolate. Error bars are standard deviations calculated in Excel.

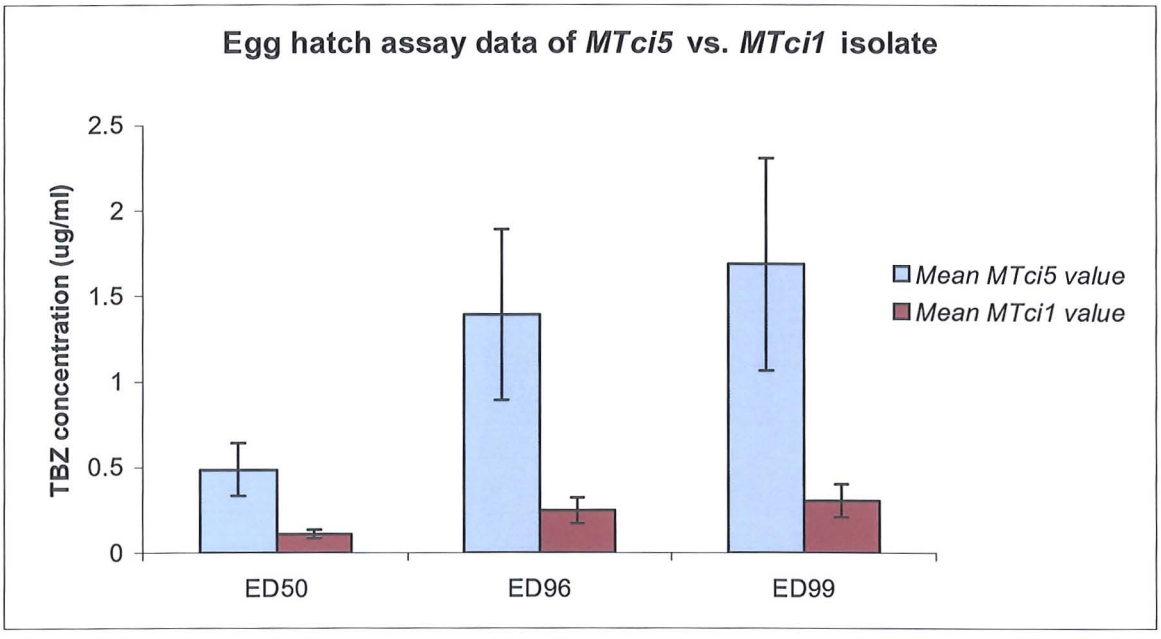


Figure 3.12: Egg hatch assay data from Trial One (*MTci5* isolate). Data points are fewer for some populations as indicated in the graph. Treatment was carried out on day 26.

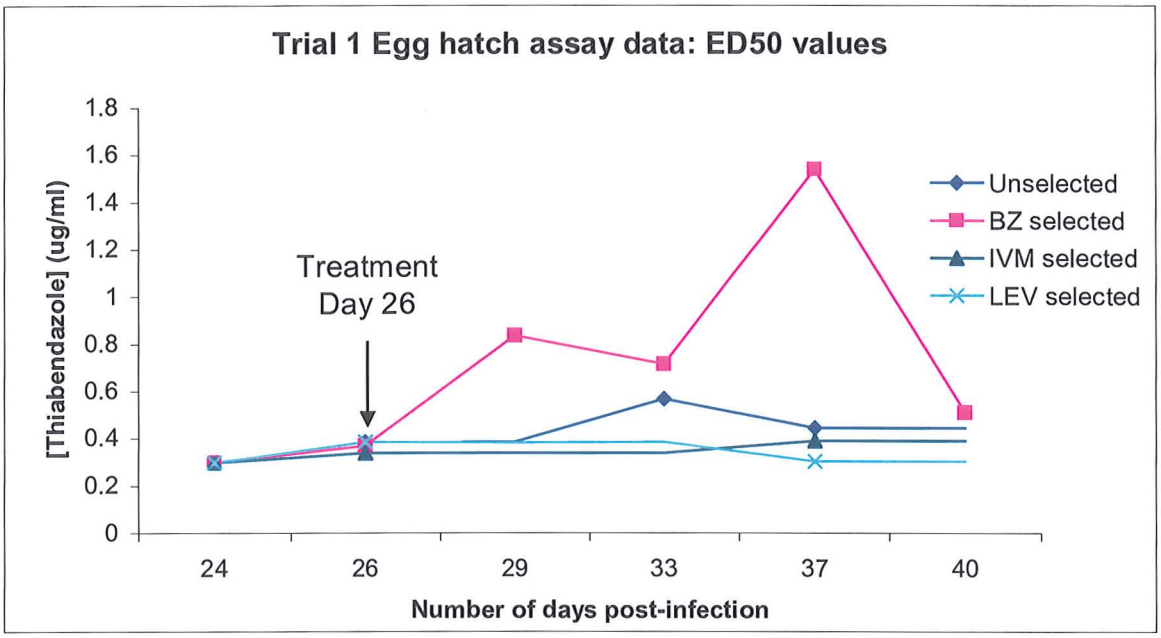


Figure 3.13: Egg hatch assay data from Trial Two (*MTci5* isolate). This dataset was complete. Treatment was carried out on day 28.

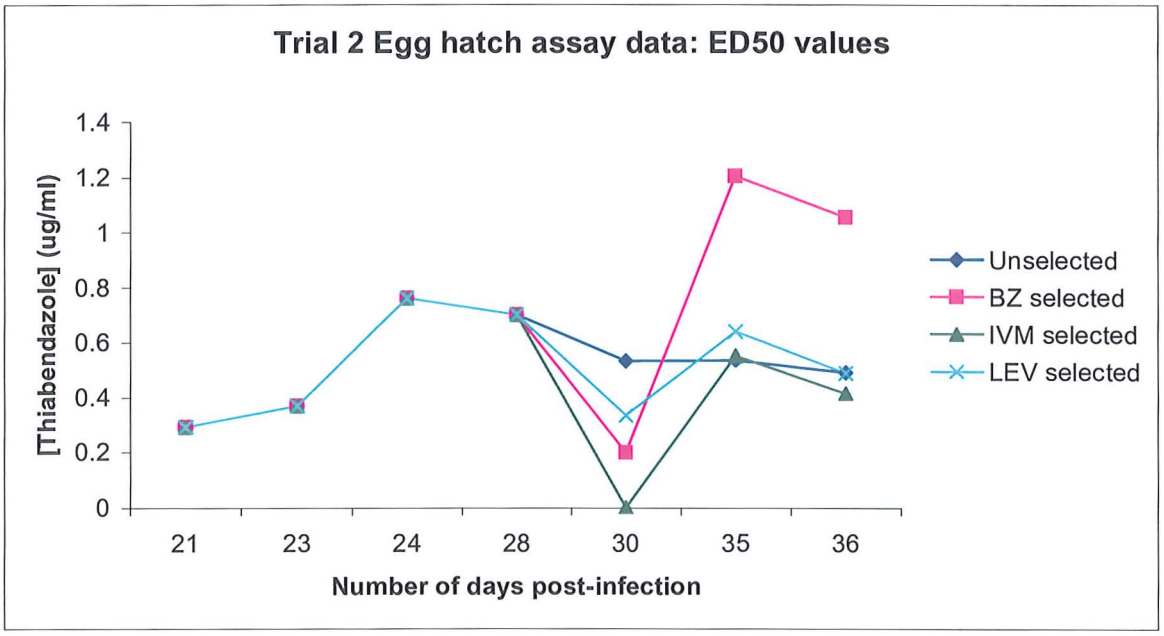


Table 3.16: Comparison of egg hatch assay data from Trials one and two. The resistance factor (RF) was calculated as the increase in resistance observed (Trial One: days 29-40 mean ED_{50} value divided by days 24-26 mean ED_{50} value; Trial Two: days 30-36 mean ED_{50} value divided by days 21-28 mean ED_{50} value). The overall RF was achieved by dividing the RFs of BZ-selected RF by unselected RF.

	Trial One			Trial Two		
	Mean ED_{50} over days 24-26 (μg/ml TBZ)	Mean ED_{50} over days 29-40 (μg/ml TBZ)	RF	Mean ED_{50} over days 21-28 (μg/ml TBZ)	Mean ED_{50} over days 30-36 (μg/ml TBZ)	RF
Unselected	0.341	0.461	1.353	0.530	0.530	0.978
BZ-selected	0.333	0.899	2.701	0.519	0.819	1.545
Overall RF			**1.997**			**1.580**

Table 3.17: Input and results of one-way ANOVA performed upon egg hatch assay data (conducted in Minitab v14, 2006).

MTci5 population	Mean post treatment ED_{50}		$F_{(3,7)}$ statistic	P value
	Trial One	Trial Two		
Unselected	0.507	0.512		
BZ-selected	0.899	1.129		
IVM-selected	0.388	0.482		
LEV-selected	0.303	0.564	9.58	**0.027**

Figure 3.14: Boxplot of ED_{50} values from each population (created in Minitab v14, 2006).

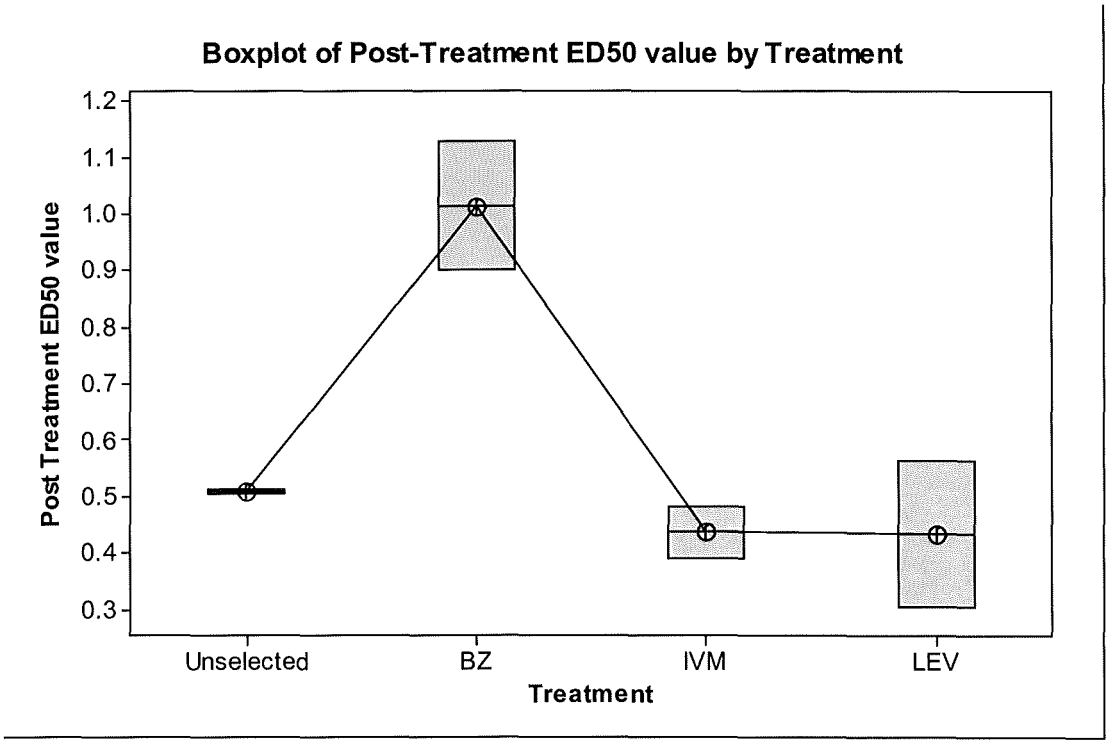


Figure 3.15: Graph showing the % reduction in faecal egg counts in each population and in each Trial.

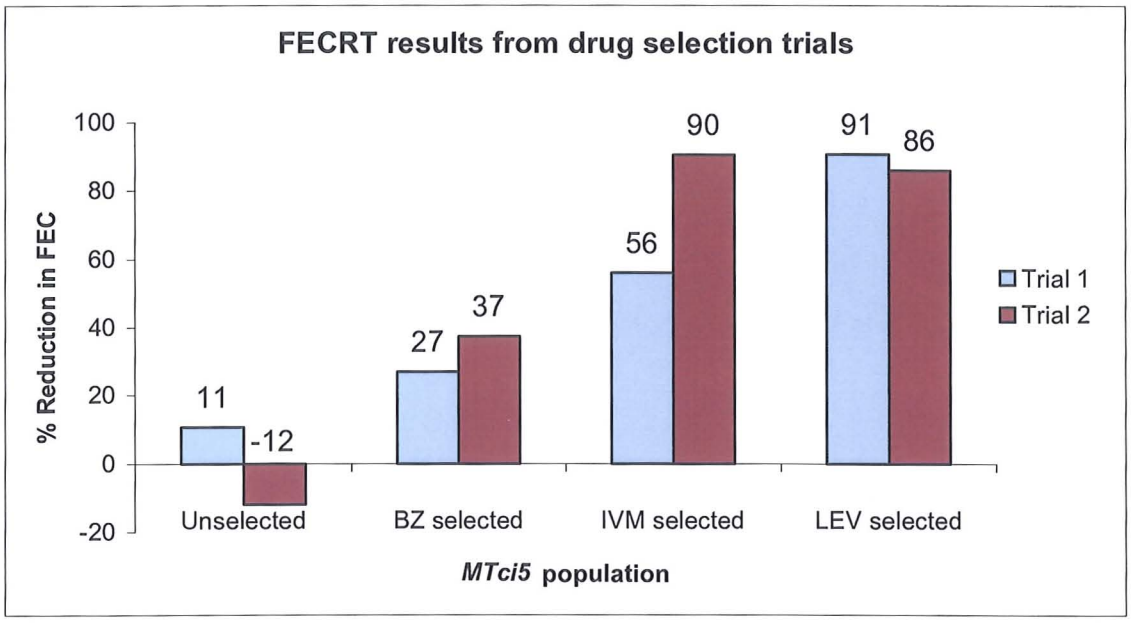


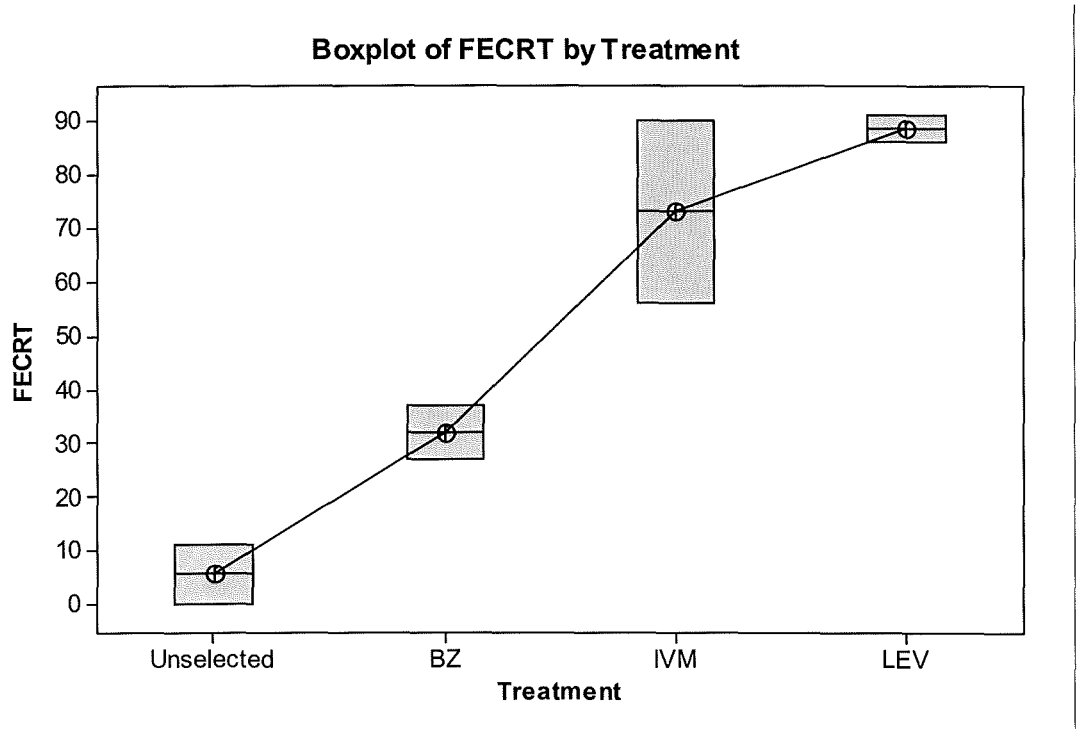
Table 3.18: Summary of statistical analysis performed on faecal egg counts. Degrees of freedom are given in subscript. T is the T statistic used in a paired t-test (Minitab v14, 2006) and F is the F statistic used in a test of equal variances following a normal distribution. The equal variances test had to be performed first to specify whether to assume equal variances in the paired t-test.

Trial	*MTci5* population	Equal variances test (F test)	Two sample T-test	Conclusion (95% CI)
1	**Unselected**	Assumed equal (F= 2.292, p= 0.384)	T_{10}= 0.45, p= 0.661	No reduction
	BZ-selected	Assumed equal (F= 2.070, p= 0.444)	T_{10}= 1.66, p= 0.127	No reduction
	IVM-selected	Assumed equal (F= 7.009, p= 0.052)	T_{10}= 2.65, p= 0.025	Significant reduction
	LEV-selected	Assumed unequal (F= 153.4, p= 0.000)	T_5= 5.97, p= 0.002	Significant reduction
2	**Unselected**	Assumed equal (F= 2.160, p= 0.474)	T_8= 0.01, p= 0.989	No reduction
	BZ-selected	Assumed equal (F= 0.300, p= 0.271)	T_8= 1.79, p= 0.112	No reduction
	IVM-selected	Assumed unequal (F= 20.03, p= 0.013)	T_4= 4.92, p= 0.008	Significant reduction
	LEV-selected	Assumed equal (F= 7.338, p= 0.079)	T_8= 10.16, p< 0.001	Significant reduction

Table 3.19: Input and results of a one-way ANOVA performed on faecal egg count reduction data (conducted in Minitab v 14, 2006).

MTci5 population	Mean FECRT value (%)		$F_{(3,7)}$ statistic	P value
	Trial One	Trial Two		
Unselected	11	0		
BZ-selected	27	37		
IVM-selected	56	90		
LEV-selected	91	86	16.42	**0.010**

Figure 3.16: Boxplot of FECRT value for each population (created in Minitab v14, 2006).



3.7 Discussion

The phenotypic and genotypic characterisation of MDR nematode populations, such as the *MTci5* isolate, provides a much needed insight into the nature of the resistance expressed within these populations and is a pre-requisite for the development of effective anthelmintic-based strategies for the control of nematode pathogens. The need for more sensitive and reliable molecular assays for resistance diagnosis drives such research. The work described in this chapter has provided valuable information on the basic population structure, the BZ resistance phenotype and genotype (F200Y isotype I β-tubulin locus) of a MDR isolate and has analysed the response of these parameters to anthelmintic selection.

3.7.1 Population genetic analysis of the *MTci5* isolate

The molecular and genetic basis of anthelmintic resistance is poorly understood and there has been little detailed characterisation of MDR isolates of parasitic nematodes of any species. The *MTci5* isolate has been characterised using general parasitological techniques (Bartley *et al.,* 2005) and is currently being used to investigate a number of candidate resistance genes (P. Skuce, personal communication). The population genetic structure of this isolate must be understood in order for detailed molecular studies to be meaningfully interpreted. The first, most basic question to address is whether the *MTci5* isolate is comprised of subpopulations each resistant to a different drug, or drugs (admixture), or whether these parasites are a single, freely interbreeding population with triple resistance. If the former is true, it is likely that there will be a number of drug-specific resistance mechanisms (genes) involved and the combination drench approach may be worthwhile, and moreover may be sustainable, at least in the short to medium term. If, however, the *MTci5* isolate is a single, freely interbreeding population, with either one or several mutations contributing to the MDR phenotype, then the combination drench strategy will probably have a limited value.

The purpose of this Section was to characterise the *MTci5* isolate using neutral genetic markers, specifically to determine whether there was sub-structuring (admixture) in the *MTci5* isolate. Five well-characterised polymorphic microsatellite markers were already available for population genetics analysis (Grillo *et al.,* 2006). These markers were used to genotype the unselected *MTci5* population and the populations generated through selection of this isolate,

using full therapeutic doses of each broad spectrum anthelmintic. These microsatellites showed no linkage disequilibrium on pairwise comparisons, implying that they were not genetically linked and so can be regarded as independent markers. The level of polymorphism observed was similar to that described by Grillo *et al.* (2006, 2007) whereby the MTG15, HCMS28 and MTG67 loci were the most polymorphic (displaying 18, 14 and 12 alleles, respectively). This is consistent with previous studies (Anderson *et al.,* 2000, Rohrer *et al.,* 2002), which have shown that dinucleotide repeats, such as these, tend to exhibit greater polymorphism than trinucleotide repeats (e.g. MTG73 and MTG74 displayed 4 and 5 alleles, respectively) or larger repeat sequences. In contrast to previous work, perfect microsatellites (i.e. MTG67) are, generally speaking, more polymorphic than imperfect ones (Hutter *et al.,* 1998), however, in the *MTci5* isolate, the MTG15 and HCMS28 loci displayed a greater number of alleles.

Two inter-related approaches were used to investigate whether there was sub-structuring in the *MTci5* isolate.

- Firstly, the data from the unselected population were examined and compared to the results of a previous study on an earlier passage of the same isolate (V. Grillo, personal communication).
- Secondly, the effect of anthelmintic selection upon the *MTci5* isolate was examined using the same markers. If sub-structuring was present, one might expect each of the drug-selected populations to become more differentiated from one another as well as from the parent population.

3.7.1.1 Population genetics analysis of the unselected MTci5 population

Dealing with the first of these approaches, Hardy Weinberg Equilibrium (HWE) analysis was applied to the allele frequency data. If admixture was present in the unselected population, heterozygote deficiency at each locus would have been expected. This was not the case, except at locus MTG15. Despite the high level of polymorphism displayed at this locus and its potential as a good marker, it is known to have a high frequency of null alleles (Grillo *et al.,* 2006), hence the reason for excluding the MTG15 data from most of the analyses. The other markers did not show a deviation from HWE, indicating a lack of admixture in the *MTci5* isolate. Secondly, the PCA analysis of the multi-locus genotypes might have been

expected to reveal clustering of related individuals within the population. Again, this was not the case, further suggesting a lack of sub-structuring in this isolate.

3.7.1.2 Population genetics analysis of the drug-selected MTci5 populations

The second approach encompassed a number of different analyses to determine the effects, if any, of anthelmintic selection. Allelic diversity, PCA, Pairwise F_{ST} and AMOVA statistics all showed no evidence of genetic differentiation between the drug-selected populations of *MTci5*. This implied that there was no overall change in population genetic structure as might be predicted if the *MTci5* isolate was sub-structured. This was supported by the lack of any significant change in the frequency of alleles at any of the microsatellite loci. These results also indicate that none of the microsatellite markers are genetically linked with resistance genes. Hence these results, based on five independent microsatellite markers, suggest that the *MTci5* population is a single, freely interbreeding population and not an admixture of separate populations with differing drug sensitivities.

This lack of sub-structuring of the *MTci5* isolate is consistent with the population genetics studies that have been conducted upon *T. circumcincta* to date. Several studies have shown no detectable population structure between geographical isolates of *T. circumcincta* in the USA, UK & France (Blouin *et al.,* 1992, 1995; Braisher *et al.,* 2004; Grillo *et al.,* 2007). This has been ascribed to large population sizes and/or high rates of gene flow resulting from host animal movement. However, there is certainly potential for sub-structuring of an isolate such as *MTci5*, due to admixture of genetically divergent populations, since there are two examples of genetically differentiated populations of *T. circumcincta* co-existing in the same country. Firstly, Leignel & Humbert (2001) compared the genetic diversity of the mitochondrial DNA ND4 gene amongst a number of *T. circumcincta* populations from farms in West-Central France, South-West France and Morocco. Significant population subdivision was apparent between the West-Central and South-Western French populations. The same result was achieved previously from isoenzyme studies of five other loci (Gasnier & Cabaret, 1998). Moreover, population subdivision has not just been found between populations in France, but also within populations. For instance, the presence of a cryptic species of *Teladorsagia* was found in co-existence with *T. circumcincta* on a goat farm in Southern France (Gasnier & Cabaret, 1996; Leignel *et al.,* 2002; Grillo *et al.*, 2007). This isolate was from a long-standing closed flock, where lack of gene flow and subsequent genetic drift is thought to have given

rise to a cryptic species (Gasnier & Cabaret, 1996). Experiments using multiple markers (β-tubulin isotype I, ITS-2 of rDNA, MDH-2 locus and the ND4 locus of mtDNA) showed this population to comprise two populations: a *T. circumcincta* population capable of infecting both goats and sheep; and a goat-specific *Teladorsagia spp.* line, which could not persist beyond one generation in sheep (Gasnier & Cabaret, 1996). Grillo *et al.* (2007) also analysed this population using the same panel of microsatellites used here. The results showed great genetic differentiation of this population when compared with 12 other *T. circumcincta* populations from the UK and France. This was consistent with the previous findings regarding this being a cryptic species. This further demonstrates that these markers are ideally suited for the purpose of population genetics analysis of nematodes.

Despite the majority of the statistics performed in this study reaching the same conclusion, the HWE analysis provided a potentially conflicting result. There were heterozygote deficiencies observed overall in each of the drug-selected populations, even following the exclusion of the MTG15 locus (due to null alleles). The BZ- and LEV-selected populations showed heterozygote deficiencies at loci MTG67 and MTG74, even after the Bonferroni correction for multiple samples had been performed. Taken alone, these results could be interpreted as providing some evidence of selection or of admixture. However, since no other aspect of the analysis suggests population sub-structuring or any general hitchhiking effects of selection exists, it is more likely that these heterozygote deficiencies are due to the presence of null alleles. The apparent variability in the extent of these heterozygote deficiencies between the different *MTci5* populations examined may be a function of limited sample size. Similar observations were made by Grillo *et al.* (2007) who reported very little genetic differentiation amongst ten *T. circumcincta* populations from the UK and France (excluding the cryptic goat-specific line), however, they also noted that significant deviations from HWE were observed in many populations across a number of loci, particularly at locus MTG15.

It must be noted, that only the first generation of each drug-selected line was examined in this study. Further selection with this experimental design whereby reintroduction of genetic diversity (gene flow) is precluded by the removal of the metapopulation, (continual faecal collection) would presumably lead to some tangible differentiation over time. However, it is well documented that the serial passage of isolates in the laboratory does not reduce the genetic diversity of the population (Roos *et al.,* 1990; Otsen *et al.,* 2000) and this is thought to

be a feature of large effective population size *(Ne)* (Anderson *et al.*, 1998). Furthermore, the lack of variation observed between the adult female *MTci5* sample analysed by V. Grillo (personal communication) and the data presented here, suggest that minimal loss of genetic diversity has occurred between these periods.

The proviso of these analyses is the fact that only five microsatellite markers were available and ideally, more markers could be used to investigate the genome-wide effects of selection. However, though five seems like a limited number of markers, some studies have been conducted with fewer. For example, two microsatellites were used to show that adult *Echinococcus multilocularis* engages in both outcrossing and self fertilisation, a matter of long-standing contention in the field (Nakao *et al.,* 2003). Although more analysis would be desirable to confirm the overall result, the data generated within this project strongly support the view that drug selection exerted no specific effects upon the genetic variation of the *MTci5* isolate. This is consistent with the view that this isolate is a single, freely interbreeding population with triple resistance. This conclusion could be tested by using greater sample sizes, more markers and further generations of anthelmintic selection.

3.7.2 Phenotypic assessment of BZ resistance

The widely used FECRT and egg hatch assays were employed in this study to assess the phenotypic expression of resistance in the *MTci5* isolate. The relative accuracy and merits of these tests will now be discussed.

3.7.2.1 In vitro egg hatch assay

Despite the known inaccuracies of the egg hatch assay, the observed trend followed the expected pattern in each of the drug-selected groups. The effect of BZ selection upon the *MTci5* isolate was highly significant from both trials and approximately doubled the phenotypic expression of BZ resistance (resistance factor of 2.0 and 1.6 for Trials one and two, respectively).

3.7.2.2 Faecal egg count reduction test

The FECRT is the most widely used assay for anthelmintic resistance detection worldwide (Taylor *et al.*, 2002). However, it also suffers from inaccuracy (Martin *et al.,* 1989). This was

exemplified by characterisation of resistance in a *T. circumcincta* population, whereby the FECRT diagnosed susceptibility, but F200Y genotyping showed frequencies of $P200^{Tyr/Tyr}$: 3%, $P200^{Phe/Tyr}$: 18% and $P200^{Phe/Phe}$: 79% (Elard & Humbert, 1999). This is crucially important since, as shown in this study, the number of resistant homozygotes can be doubled with every round of BZ selection. The large error margins observed in the present study suggest that the FECRT data are also somewhat imprecise. The anthelmintic efficacies were calculated as 32 ± 7%, 73 ± 24% and 88 ± 3% for FBZ, IVM and LEV, respectively. There was a particularly high error in the efficacy estimate of IVM between the two trials. This may be due in part to the temporary depression in fecundity associated with worms under IVM stress, which later resumes (Jackson, 1993, Tyrell *et al.,* 2002). Despite the relatively large error margins, the statistical analysis showed no significant differences between trials, indicating that even though this technique may underestimate the level of resistance in the *MTci5* population, at least it does so consistently. Moreover, the results do show some agreement with the more reliable CET data from a previous study of *MTci5* (Bartley *et al.,* 2005), where the following efficacies were observed: FBZ 59%, IVM 60%, LEV 88%. The level of BZ efficacy has probably been underestimated in this study, since it is almost half of that estimated previously by CET. A pairwise T-test was also employed to determine whether the reduction in egg count following selection was significant. In the BZ-selected populations, it was not, implying that BZ had little or no effect upon these worms. This result is probably due to the variance in the dataset, which is not accounted for when the standard FECRT method is employed. According to the WAAVP guidelines for evaluating anthelmintic efficacy (Wood *et al.*, 1995), a reduction in parasite burden of: 98% is highly effective; 90-98% is deemed effective; 80-89% is termed moderately effective and below 80% the anthelmintic is said to be insufficiently active. Therefore, in accordance with these guidelines, BZ and IVM have insufficient efficacy, and LEV only shows a moderate efficacy against these parasites. As stated by Coles *et al.* (2006), efficacies of less than 95% for each drug confirm *MTci5* as a triple-resistant isolate. The use of FECRT should be restricted to analysis of whether a population is becoming more or less resistant over time, as an indicator of success in resistance management, however, more sensitive (and similarly cost-effective) techniques are required for resistance diagnosis.

In contrast to these results, whereby the egg hatch assay is in broad agreement with the FECRT data, others have reported the egg hatch assay to be far less reliable. A study was

conducted upon populations of small strongyles from horses in Chile, whereby the effects of BZ selection were estimated using the egg hatch assay, FECRT and F200Y genotyping assay (von Samson-Himmelstjerna *et al.,* 2002). The results of the egg hatch assay were not in agreement with that of the FECRT or allele-specific PCR results. Furthermore, the ED_{50} values failed to indicate that resistance was present when the frequency of the resistant allele was on average 55% amongst the three farms tested. Others have reported similar findings (Craven *et al.,* 1999). Thus, the egg hatch assay should not be relied upon in the absence of other assays; moreover, the bioactivity of the TBZ stock solution should be ensured. These issues further highlight the requirement for more sensitive molecular assays for resistance detection.

3.7.3 Genotypic assessment of BZ resistance of the *MTci5* isolate and the relative accuracy of the allele-specific PCR and Pyrosequencing assays

It is apparent that all biological assays are, to some extent, imprecise and incorporate some error rate, a feature which requires acknowledgement when interpreting their findings. As a general rule, advances in technology and methodology lead to greater precision and reliability as well as increases in throughput; all of which can reduce the costs of analyses. During the course of this research a novel technology (Pyrosequencing) became available to supplant the existing allele-specific PCR technique which had been used to genotype resistant populations (Elard *et al.,* 1999) in terms of the F200Y isotype I β-tubulin mutation.

The initial findings generated using the allele-specific PCR provided consistent results with regard to genotype frequencies between life stages and between trials and gave confidence in the technique, however, the Pyrosequencing method employed latterly highlighted an error rate amongst those results. There are several reasons for the error of the allele-specific PCR technique. Firstly, the band resolution on the agarose gels was not always clearly defined, particularly between the internal standard and susceptible bands. Occasionally there was some non-specific primer annealing (highlighted by extraneous bands), which may have been mistaken for the diagnostic bands. This could be due to mis-priming from other β-tubulin genes, which led to concerns about whether these data were actually representing the isotype I gene or not. The Pyrosequencing assay also had the considerable advantage of specificity of annealing thus minimising the risk of false genotypes being observed. Furthermore, its greater

stringency comes from the fact that it is a sequencing method and if the sequence of the template does not match that expected (as predetermined by the user), then the sample will fail. Moreover, the isotype II sequence in this region has been shown to be different to that of isotype I (see Chapter 4, Section 4.5) thus, the data are reliable. In any case, the Pyrosequencing method was much more efficient in terms of sensitivity, throughput, time and workload. Other techniques such as amplified fragment length polymorphism (AFLP)-PCR (Tiwari *et al.,* 2006) and real time allele-specific PCR (von Samson-Himmelstjerna *et al.,* 2003; Walsh *et al.,* 2007) have also been reported to be more reliable than the conventional allele-specific PCR.

3.7.4 Frequency of the F200Y isotype I β-tubulin mutation in the *MTci5* isolate and the effects of drug selection at this locus

The F200Y isotype I β-tubulin mutation has been found in association with BZ resistance in many parasite species and populations across the world (reviewed by Gilleard, 2006). One aim of this study was to examine the role of this mutation in determining the BZ resistance phenotype of the MDR *MTci5* isolate. Both allele-specific PCR and Pyrosequencing genotyping found the frequency of the F200Y isotype I β-tubulin mutation to be high in the unselected *MTci5* population, with 93% of the adult worms carrying at least one copy of the allele. The overall frequency of the resistant allele was 62.5% with the susceptible allele at 37.5%. The effect of BZ selection upon this isolate was to increase the frequency of the resistance allele to 89%, and the number of individuals carrying at least one copy of the allele rose to 98%. Hence, these results clearly support the suggestion that this mutation is selected by BZ treatment. Unlike previous studies which have examined the association of the F200Y isotype I β-tubulin mutation with BZ resistance, this work has shown there is no general effect of selection upon the genetic structure of the *MTci5* population. This is important since an apparent increase in the frequency of the resistant allele could arise as a consequence of general changes in polymorphism across the genome in response to selection. This was not the case in this experiment and the fact that no selection was apparent on the five genetically independent microsatellite loci, provides strong evidence that there is specific selection for the F200Y isotype I β-tubulin mutation.

One potentially surprising aspect of these results was that the survivors of BZ selection were not 100% homozygous resistant ($P200^{Tyr/Tyr}$), as would be expected if this mutation was the sole determinant of BZ resistance and was wholly recessive. This expectation comes from previous studies using a French BZ resistant *T. circumcincta* isolate, whereby all $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ worms succumbed to treatment, due to the proposed recessive nature of the resistant allele (Elard *et al.,* 1998; Elard & Humbert, 1999). This was apparent in two resistant populations, which, prior to the full therapeutic dose of BZ, did reveal the presence of all three genotypes.

There are several possible explanations for the survival of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ individuals following BZ selection.

1. The surviving worms are phenotypically susceptible, but have survived due to pharmacokinetic considerations (this is further discussed in Chapter 4).
2. The surviving worms are phenotypically resistant because the F200Y isotype I β-tubulin mutation is not fully recessive.
3. The surviving worms are phenotypically resistant because there are additional (unknown) resistance mechanisms involved (this is further investigated in Chapter 4).

The first point is a potential feature of *in vivo* drug selection, whereby the effects of the drug may be reduced by host metabolism. The drug selection experiments only employed one animal per anthelmintic per trial, and thus, host metabolism was one variable which was not controlled in these experiments. However, the animals were maintained on a concentrate diet, which has been shown to reduce the rate of metabolism of the BZ derivative compounds by the gut microflora, compared with a diet of hay (Virkel *et al.,* 1999). Furthermore, since two animals were used overall, it is unlikely that both happened to show abnormally high rates of drug metabolism. In any case, the numbers of $P200^{Phe/Tyr}$ worms surviving BZ treatment was consistently high in both trials (26 and 15%, respectively); thus, it is unlikely that this high proportion of survivors is a result of variability of drug metabolism between individual hosts. One way of testing this conclusion is to select these parasites *in vitro* with BZ, and assess the survival of each genotype and this is examined in Chapter 4.

The second possibility carries a lot of weight in the literature. Many studies of trichostrongylid nematodes have suggested that BZ resistance is an incompletely dominant

trait controlled by multiple loci (Le Jambre *et al.,* 1979; Herlich *et al.,* 1981; Martin *et al.,* 1988; Kwa *et al.,* 1993; Roos *et al.,* 1995; Sangster *et al.,* 1998). The recessive nature of the resistance allele has not been examined in this thesis, however, if the survival of a $P200^{Phe/Phe}$ worm post-BZ selection had not been observed, it could have been assumed that this trait was incompletely dominant.

Moreover, the survival of a $P200^{Phe/Phe}$ genotype does appear to indicate, as point 3 suggests, that there may be other resistance mechanisms involved. Although, we should examine the significance of this finding by consulting the recent WAAVP guidelines (Coles *et al.,* 2006). These state that if an anthelmintic is less than 95% effective, then resistance should be diagnosed. If we use this as a guide for the proportion of worms expected to survive treatment (5%) due to some reason other than resistance (e.g. the effects of host metabolism), then we say that 5% of each genotype could equally survive treatment. Therefore, a 5% survival of $P200^{Tyr/Tyr}$ worms (in this study of unselected *MTci5* isolate, n = 27 $P200^{Tyr/Tyr}$ worms), $P200^{Phe/Tyr}$ worms (n = 36) and $P200^{Phe/Phe}$ worms (n = 9) from the unselected population would allow 1.35 $P200^{Tyr/Tyr}$ worms, 1.8 $P200^{Phe/Tyr}$ worms and 0.45 $P200^{Phe/Phe}$ worms to survive treatment. Since only one $P200^{Phe/Phe}$ worm survived BZ treatment, it is possible that this individual may have 'slipped through the net'. However, there is other evidence which supports the view that $P200^{Phe/Phe}$ survival following BZ selection may not simply be a chance event. A study of BZ resistant strongyle populations from horses in Chile, showed that whilst BZ treatment did select in favour of the resistant allele, the proportion of $P200^{Phe/Phe}$ genotypes between the field sample and the BZ-selected population did not change significantly, suggesting that there were other resistance mechanisms involved (von Samson-Himmelstjerna *et al.,* 2002). In the current study, there was a significant decrease in the proportion of $P200^{Phe/Phe}$ worms following BZ selection, but the existence of other contributory resistance mechanisms cannot be dismissed. In the case of the heterozygotes, it is most unlikely that the 14 $P200^{Phe/Tyr}$ worms (versus the expectation of 1.8 worms, see above) from the BZ-selected group, survived due to some reason other than resistance. Again, the survival of $P200^{Phe/Phe}$ genotypes will be examined *in vitro* in Chapter 4 and other potential resistance mechanisms will be investigated in the *MTci5* isolate.

In contrast to the effects of BZ, there was no evidence of IVM or LEV selection upon the allele frequencies of the F200Y isotype I β-tubulin locus. However, it is interesting that the

only *MTci5* population to show a deviation from HWE was the IVM-selected population. This was observed from the adult data ($p < 0.05$), however, the fact that this result was not observed from the L_3 data makes this finding less conclusive. This was investigated since there have been previous studies, which implicate a link between the resistance mechanisms of the different drug classes and, in particular, the selection of the β-tubulin locus by IVM. For instance, a study of the allele frequencies of the β-tubulin isotype I gene (and a P-glycoprotein gene) in *Onchocerca volvulus*, revealed significant differences between IVM- susceptible and resistant populations (Eng & Prichard, 2005). Furthermore, three SNPs were highlighted in the H3 helix region of the isotype I β-tubulin gene, which were strongly associated with the development of IVM resistance. For instance, Eng & Prichard (2005) reported three amino acid substitutions (M117L, V120I and V124A). These SNPs were accompanied by a 24bp deletion in the intron I region. Ten polymorphic control genes and three other candidate resistance genes included in the study did not show the same evidence of selection, indicating that these effects were not due to genetic linkage or epistasis. The worms examined were isolated from hosts which had either been treated with IVM on a number of occasions and from those which had never received treatment, and these were located in the same geographical region; therefore, it is unlikely that these results were caused by Founder effects. Although a functional relationship between the IVM and BZ drug targets is difficult to envisage, the authors further suggest that an alteration of microtubule structure (since tubulin is ubiquitous) could feasibly affect the function of neuronal ion channels, upon which IVM is known to act. This theory is based upon evidence of distortion of microtubule bundles in the amphidial neurons of IVM resistant *H. contortus* (Freeman *et al.,* 2003) Here, shortened amphidial sensilla (containing sensory neurons) are associated with an IVM resistant phenotype and may have a role in precluding entry of IVM into the worm. Alternatively, the F200Y isotype I β-tubulin gene could simply be a marker by virtue of being linked to an IVM resistance gene. Whilst an effect of IVM upon tubulin (de)polymerisation was also investigated, it was not deemed likely (Oxberry & Prichard, cited in Eng & Prichard, 2005). However, recent reports of an effect of IVM selection upon this gene in *H. contortus* have also been made (Eng *et al.,* 2006). Using Single Strand Conformational Polymorphism (SSCP) analysis, these authors have demonstrated an effect of IVM selection across the region encoding amino acids 195 to 235 of the isotype I β-tubulin gene of *H. contortus*. Moreover, a significant effect of IVM selection upon the F200Y locus has been observed, whereby IVM appears to favour the survival of $P200^{Phe/Tyr}$, but not $P200^{Tyr/Tyr}$, genotypes. There was no

evidence for an increase in $P200^{Phe/Tyr}$ genotypes following IVM selection in this study, however, these findings clearly indicate that further investigation into potential mechanisms of cross- resistance is required.

It was also suggested in the literature that LEV and BZ resistance mechanisms may be linked, as a LEV-selected population of *T. circumcincta* showed a decreased level of resistance to BZ in the EHA when compared with the same isolate that had been selected with BZ (Donald *et al.,* 1980). However, in this study, there were no significant differences between the control and test groups in β-tubulin F200Y allele frequencies (Tables 3.11 and 3.14) or in resistance phenotype (EHA data, Table 3.17) and so there is no evidence of either a functional association or genetic linkage between BZ and LEV resistance. Hence, the results in this chapter suggest that in *T. circumcincta*, or in the *MTci5* isolate at least, there is no evidence of an effect of IVM or LEV selection at the F200Y isotype I β-tubulin locus. However, it must be remembered that this conclusion is based upon the assay of a single SNP. For instance, there could still be IVM or LEV selection across the isotype I β-tubulin gene that would go undetected by this SNP assay. Thus, selection across a larger region of the isotype I β-tubulin gene is further investigated by SSCP in Chapter 5.

3.7.5 Summary

In summary, the work presented in this Section presents a basic characterisation of the MDR *MTci5* isolate. It provides evidence for this isolate being a freely interbreeding population without genetic sub-structuring. The expression of resistance against BZ was greatest amongst the three drug classes, and this was strongly correlated with the F200Y mutation of the β-tubulin isotype I gene. Furthermore, treatment with BZ clearly produced selection at this locus in the absence of changes in overall polymorphism or population structure as shown by the allele frequencies of five microsatellite markers. This strongly implies an association of the F200Y isotype I β-tubulin mutation with the BZ resistance phenotype. However, the survival of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes following a full therapeutic dose suggests that either the resistance allele is incompletely recessive or that there may be other resistance mechanisms involved. This is further investigated in Chapter 4. Finally, treatment with IVM and LEV does not appear to produce selection upon the F200Y isotype I β-tubulin locus, however, further investigations into the effects of these anthelmintics upon the isotype I β-tubulin gene is presented in Chapter 5.

4.0 Chapter 4 Contribution of the F200Y isotype I β-tubulin mutation to the BZ resistance phenotype of the *MTci5* isolate and the potential role of other resistance mechanisms

4.1 Introduction

4.1.1 Survival of P200$^{Phe/Tyr}$ and P200$^{Phe/Phe}$ genotypes from *in vivo* BZ selection

The experiments presented in Chapter 3 demonstrated that the principal effect of BZ selection upon the *MTci5* isolate was to increase the frequency of the F200Y isotype I β-tubulin mutation. The significance of this increase was supported by the lack of any evidence of selection at five independent, neutral loci or of changes to the overall genetic structure of the population. These results strongly support the view of the F200Y isotype I β-tubulin mutation being an important genetic determinant of BZ resistance in the *MTci5* isolate. However, the extent to which this mutation contributes to the BZ resistance phenotype is still unclear as it could be the sole determinant of resistance or one of several different mechanisms. The survival of P200$^{Phe/Tyr}$ and P200$^{Phe/Phe}$ genotypes following BZ selection differs from previously published findings which suggest that BZ resistance is a recessive trait controlled by a single gene (Elard *et al.,* 1999; Kwa *et al.,* 1994, respectively). The body of evidence so far appears to suggest a functional role for this mutation in resistance (Kwa *et al.,* 1994, 1995; Elard *et al.,* 1996, Elard & Humbert, 1999, Njue *et al.,* 2004), and it is positively correlated with the BZ resistance phenotype as shown by the bioassays in Chapter 3. However, the role of this mutation must be defined more clearly, since:

1. The survival of P200$^{Phe/Tyr}$ genotypes post-BZ selection implies that either this trait is incompletely recessive and/or that other resistance mechanism(s) may be involved;
2. However, the survival of P200$^{Phe/Phe}$ genotypes occurred either as a result of some pharmacokinetic effect *in vivo* or as a result of some contributory resistance mechanism(s).

The work outlined in this Chapter attempts to investigate which of these hypotheses is true. *In vitro* experiments were performed to determine if $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes survive BZ exposure. These *in vitro* studies should not be affected by variability in pharmacokinetics and drug bioavailability that compromise the interpretation of the *in vivo* studies presented in Chapter 3. Following this, a number of other potential resistance mechanisms that might account for survival of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes (in terms of the F200Y isotype I β-tubulin mutation) are investigated for their possible contribution to the *MTci5* BZ resistance phenotype.

4.1.2 Expression of BZ resistance over time

The first issue to be addressed in this Chapter is an investigation of suggestions in the literature that BZ resistance does not remain fixed throughout the course of an infection. For instance, Borgsteede & Couwenberg (1987) reported that the expression of BZ resistance (as measured by the egg hatch assay) in both a susceptible and a resistant *H. contortus* isolate fluctuated by almost one order of magnitude over the course of 22 to 77 days post-infection. Peak resistance was observed at 40-60 days (weeks 6 to 9), and thereafter resistance began to decline to a level equal to that observed at weeks 3-4. Other possible contributory variables were excluded (as they were in the present study), i.e. the tests were conducted by one individual using one stock TBZ solution and the hosts were closely matched in terms of parasite history and age. Kerboeuf & Hubert (1987) reported similar findings from a susceptible *H. contortus* isolate, and studied the effects of dose administration and host immunity. In accordance with the results of Borgsteede & Couwenberg (1987), they observed peak ED_{50} values between days 50 and 65 post-infection in a population collected from a sheep which had received a single large dose (10,000 larvae). However, the authors also studied the change in BZ resistance over time in a population which had been administered via trickle infection; these populations showed much higher peaks of resistance. Since trickle infections were more similar to a field situation, this has implications for treatment regimes. Furthermore, populations derived from previously challenged, and thus, immune sheep showed much lower levels of resistance. This latter finding indicated that host immunity reduced parasite fitness. Finally, it was observed that a susceptible population showed a peak ED_{50} value, which was as high as a BZ resistant population at one stage in the course of the

infection. These finding are of great importance to this study, since the time at which a population is sampled for resistance assessment (whether in genotype or phenotype) may be unrepresentative. Furthermore, changes in immune status within and between hosts are highly likely and thus, these are important considerations for the egg hatch assay. Despite the known inaccuracies of the egg hatch assay, this phenomenon appears consistent amongst resistant and susceptible isolates of *H. contortus* and thus it should be investigated in *T. circumcincta* populations too. It is of further interest here, since the relationship between the F200Y isotype I β-tubulin mutation and BZ resistance phenotype over the course of an infection may aid our understanding of the role of this mutation.

4.1.3 Other isotype I β-tubulin mutations

It is difficult to accept that a single point mutation, F200Y, of a single gene (isotype I β-tubulin), is the universal cause of BZ resistance amongst nematodes, fungi and protozoans. Perhaps unsurprisingly, there have been reports of additional mechanisms. Silvestre & Cabaret (2002) observed a second mutation (F167Y) in the β-tubulin isotype I gene amongst two resistant populations of *H. contortus* and one of *T. circumcincta* from farms in SW France. They investigated the following possibilities, that:

1. A homozygous resistant genotype for one or both isotype I β-tubulin (F167Y and F200Y) mutations can confer resistance.
2. Double homozygous resistant genotypes (i.e. $P167^{Tyr/Tyr}$ / $P200^{Tyr/Tyr}$) are more resistant than single homozygous resistant genotypes.
3. Double heterozygous genotypes (i.e. $P167^{Phe/Tyr}$ / $P200^{Phe/Tyr}$) are more resistant than single heterozygous genotypes.

In these French *T. circumcincta* populations, it was apparent that a $P167^{Phe/Tyr}$ or $P167^{Tyr/Tyr}$ genotype conferred resistance in the absence of the F200Y isotype I β-tubulin mutation. Furthermore, the $P167^{Phe/Tyr}$ worms survived equally well as the $P200^{Tyr/Tyr}$ worms. These findings suggest that not only is the F167Y isotype I β-tubulin mutation capable of conferring BZ resistance in the absence of the F200Y mutation, but this locus is different from F200Y in that resistance is not recessive. In contrast, the absence of a $P200^{Tyr/Tyr}$ genotype conferred susceptibility upon the two *H. contortus* populations regardless of the F167Y isotype I β-

tubulin genotype. However, it is possible that this result was been influenced by small sample size since Prichard (2001) reported resistant *H. contortus* worms with $P167^{Phe/Tyr}$ or $P167^{Tyr/Tyr}$ genotypes, in the absence of the F200Y isotype I β-tubulin mutation. Moreover, recombinant β-tubulin incorporating the F167Y isotype I β-tubulin mutation was shown to suppress the binding of BZ, in the absence of the F200Y isotype I β-tubulin (Prichard *et al.,* AAVP abstract, 2000). Benomyl resistant *Neurospora crassa* also show the F167Y isotype I β-tubulin mutation (Ohrbach *et al.,* 1986). These findings indicate that if isotype I β-tubulin mutations are central to the development of BZ resistance, especially in other parasitic nematodes, it is equally important to determine the frequency of the F167Y isotype I β-tubulin mutation as it is to determine that of the F200Y isotype I β-tubulin mutation when undertaking BZ resistance diagnosis. It should be noted, however, that the F167Y isotype I β-tubulin mutation is not reported with the same frequency amongst Trichostrongylid nematode populations as F200Y, but appears to be a rarer mechanism of BZ resistance. For instance, Silvestre & Cabaret (2002) only found it in three out of 29 populations (including *T. circumcincta, H. contortus* and *T. colubriformis* species) and this amounted to 12 worms out of 171. This may be due to a fitness cost or that this mutation arises far less frequently than the F200Y isotype I β-tubulin mutation, but this remains to be investigated.

Recently another isotype I β-tubulin mutation (E198A) has been found in association with BZ resistance in *H. contortus*, occurring in the absence of F167Y and F200Y (Ghisi *et al.,* 2007). These populations were isolated from S. Africa and Australia, suggesting that the E198A isotype I β-tubulin mutation had arisen independently in these areas. The E198A isotype I β-tubulin mutation has been reported from BZ resistant strains of fungi, including *Monilinia fructicola* (Ma *et al.,* 2003). The proposed mechanism for resistance in this case follows that alanine precludes the formation of a hydrogen bond with BZ, which normally occurs in the negatively charged glutamate molecule (Robinson *et al.,* 2004). Several other resistance-permitting amino acids have been described at this locus in fungi, e.g. aspartate, lysine and glycine, however, alanine requires fewer base substitutions (Koenraadt *et al.,* 1992; Ma *et al.,* 2003).

4.1.4 Involvement of isotype II β-tubulin locus in BZ resistance

Although there has been no definitive proof that BZ resistance is caused by anything other than isotype I β-tubulin mutations, it has long been suggested that BZ resistance is a multigenic trait in *H. contortus* (Le Jambre *et al.,* 1979; Herlich *et al.* 1981; Le Jambre, 1993; Kwa *et al.,* 1993; Roos *et al.,* 1995 Sangster *et al.,* 1998) and in *T. colubriformis* (Martin *et al.,* 1988). However, there is good evidence for the involvement of a second β-tubulin isotype in several BZ resistant *H. contortus* isolates. In early experimental work, the distinction between the different isotypes of β-tubulin had not been made and experiments utilised techniques such as RFLP to examine pooled reactive β-tubulin fragments. These experiments demonstrated that a loss of polymorphism was associated with resistant phenotypes of *H. contortus* (Roos *et al.,* 1990). Characterisation of full length cDNA sequences from *H. contortus* allowed the discrimination of two isotypes of β-tubulin (Geary *et al.,* 1992; Lubega *et al.,* 1993), allowing the effects of BZ selection at each locus to be more accurately determined. Subsequently, a loss of diversity was described in both isotype I and isotype II genes following BZ selection (Kwa *et al.,* 1993; Beech *et al.,* 1994), and in highly resistant populations, a deletion event of the isotype II locus occurred (Kwa *et al.,* 1993). Gene deletion has also been reported for the *ben-1* β-tubulin locus in *C. elegans* as a mechanism of benzimidazole resistance (Driscoll *et al.,* 1989). Furthermore, it is known that isotype II β-tubulin is involved in BZ binding (Kwa *et al.,* 1993; Lubega *et al.,* 1994; Beech *et al.,* 1994); and that it appears to bind BZ with a greater affinity than isotype I β-tubulin (Oxberry *et al.,* 2001). It is thought that this is due to the formation of an additional binding site in the α/β-tubulin isotype II microtubule during polymerisation (Lubega & Prichard, 1991a,b,c; Oxberry *et al.,* 2001). Hence, a deletion event at this locus would provide an adaptive benefit to parasites by reducing the number of potential binding sites for the drug. This study will investigate the presence of this locus in the *MTci5* isolate, to assess the contribution of this mechanism towards BZ resistance.

4.1.5 Non-specific resistance mechanisms

4.1.5.1 P-glycoproteins

P-glycoproteins (P-gp) are ubiquitous, highly conserved transmembrane molecules which mediate the ATP-dependent transport of lipophilic peptides across the cell membrane

(Gottesman *et al.,* 1995; Higgins, 1997). A role for P-gp in the active efflux of xenobiotics from mammalian cell lines (van der Bliek & Borst, 1989), *C. elegans* (Broeks *et al.,* 1995), microbes (van Veen & Konings, 1997) and protozoan parasites (Ekong *et al.,* 1993) has led researchers to investigate their potential contribution to anthelmintic resistance in parasites of veterinary and medical importance. Associations between these genes and anthelmintic resistance have been documented and some anthelmintics are known substrates of these transporters. For instance, Schinkel *et al.* (1994) demonstrated that mice which were deficient in the *mdr1a* P-gp gene showed symptoms of neurotoxicity after exposure to IVM. The ligand potential of IVM for P-gp was later confirmed (Didier & Loor, 1996; Pouliot *et al.,* 1997). Similarly, binding of an analogue of FBZ by P-gp in MDR mammalian cell lines was demonstrated (Nare *et al.,* 1994). However, the most convincing evidence for the role of P-gp in MDR came with studies using Verapamil (VPL). VPL is a calcium channel blocker and its primary function is inhibition of P-gp (Shoji *et al.,* 1991). VPL competes for the substrate binding site of molecules which are lipophilic, with an amphipatic structure e.g. colchicine, taxol and vinblastine (Sangster, 1994; Blackmore *et al.,* 2001). These studies indicated that the drug target site was saturable and that the MDR phenotype could be reversed. In a study of three BZ resistant and two susceptible *H. contortus* populations, the egg hatch assay was used to show that VPL increased the potency of ABZ and TBZ anthelmintics in all five populations. Complete reversal of resistance, however, was not achieved in the resistant populations, suggesting that this is a secondary resistance mechanism (Beugnet *et al.,* 1997). In order to confirm the action of the inhibitors, however, Kerboeuf *et al.* (1999) utilised two P-gp substrate transport probes, Rhodamine 123 and Verapamil-Bodipy, in flow cytometry analysis of uptake by resistant vs. susceptible *H. contortus* eggs. The results showed that the resistant eggs had a far higher affinity for these compounds, showing similar activity to MDR cancer cells. Similar findings were made with resistant *T. circumcincta* eggs (unpublished data, Jackson & Kerboeuf, personal communication). Xu *et al.* (1998) analysed the effect of VPL and CL 347, 099 (a VPL homologue with a 70-fold less calcium channel blocking ability) administered in conjunction with either IVM or MOX upon a MOX-resistant isolate of *H. contortus in vivo* using Jirds (*Meriones unguiculatus*). Both inhibitors greatly enhanced the potency of these anthelmintics. The action of these inhibitors has also been assessed *in vitro* using a larval migration assay to assess the effect of the anthelmintic activity upon motility. The results supported those of the *in vivo* experiments, with the addition of VPL increasing the activity of IVM and MOX by 53 and 59%, respectively (Molento & Prichard, 2001). The

evidence suggests that VPL is an important inhibitor of P-gp in parasitic nematodes and that P-gp may be involved in resistance mechanisms. In this Chapter, the effect of VPL upon the resistance phenotype of *MTci5,* as well as a susceptible *T. circumcincta* isolate (*MTci2*), is investigated.

4.1.5.2 Cytochromes P450

Cytochromes P450 (CYP) are metabolic enzymes which specialise in the oxidation of endogenous compounds and xenobiotics (Guengerich, 1991). They are haem proteins, so called due to the presence of an iron atom at the centre of an organic ring structure. These enzymes have been described from the major phyla (Estabrook, 1984; Nelson *et al.,* 1993), however, their presence has only relatively recently been confirmed in nematodes (Kotze, 1999). To date, more than 80 CYP genes have been characterised in *C. elegans* (Menzel *et al.,* 2005). Furthermore, by blasting the *H. contortus* genome with known *C. elegans* CYP sequences, results have shown that at least 33 CYP loci are present (J. Gilleard, personal communication, unpublished data). The first indication of CYP activity in nematodes came from *in vivo* studies of *Heligmosomoides polygyrus* whereby the metabolism of aminopyrine (an antipyretic and analgesic drug) was demonstrated by sensitive gas chromatography (Kerboeuf *et al.,* 1995). A subsequent study proposed CYP activity in *H. contortus* and interestingly, this activity seemed to be stage-specific: whilst readily detectable amongst the egg and larval stages, it was almost undetectable in adults. The authors suggested that the anaerobic nature of the host environment may thwart the oxygen-dependent activity of these enzymes (Kotze, 1997). This is consistent with findings of a change in expression levels of metabolic components during the transition from anaerobic to aerobic lifestyle in the roundworm, *Ascaris suum* (Komuniecki & Vanover, 1987; Komuniecki & Komuniecki, 1995). Moreover, it is well known that GI parasites possess alternative anti-oxidant enzymes, which are usually employed in the parasite's defences against the host immune response, and in removal of endogenous toxins. For instance, the activity of peroxidases in adult *H. contortus* is very high in the soluble fraction compared with L_3 (Kotze, 1999); this is consistent with the theory that localisation of detoxifying enzymes in the cytosol indicates a first defence role in protection against the host response (Callahan *et al.,* 1988).

From the findings of Kotze (1997), it could be assumed that the expression of CYP in *T. circumcincta* would be confined to the free-living stages, making this resistance mechanism

important only under *in vitro* circumstances. However, latter studies (which finally confirmed the identity of these enzymes) showed that CYP could operate under anaerobic conditions *in vitro* (Kotze, 1999). That is, instead of acting as an NADPH-dependent monooxygenase, it can also act as a peroxygenase catalyst of hydroperoxide-dependent oxidation reactions. The role of a peroxide-supported pathway in GI nematodes in an *in vivo* situation has remained unclear. However, Kotze (1999) examined the potential of CYP in *H. contortus* L_3 to act as a peroxygenase (utilising cumene-hydroperoxide instead of molecular oxygen) *in vitro* and found that this anaerobic process was possible. This suggests that adult nematodes could still utilise such enzymes in the oxygen poor environment of the host GI tract; or perhaps for blood feeding nematodes like *H. contortus*, these parasites are able to utilise oxygen from host erythrocytes (Kotze *et al.,* 2006b). Moreover, Alvinerie *et al.* (2001) implied that these enzymes are active in adult *H. contortus* when they showed that the *in vitro* metabolism of MOX was occurring in the homogenate of a susceptible *H. contortus* isolate and that this process could be inhibited by treatment with carbon monoxide. However, whilst these results suggest that the anaerobic conditions prevented the metabolism of MOX, there is no proof that CYP was the metabolic agent. Although, the evidence for a role of CYP in detoxification of anthelmintics (including IVM and BZ) has also been reported in mammalian models (Gottschall *et al.,* 1990; Chui & Lu, 1989; Baliharova *et al.,* 2003; Velik *et al.,* 2004; Savlik *et al.,* 2006) and cancer cells (Kumaraguruparan *et al.,* 2006).

One way of assessing the potential contribution of CYP activity to anthelmintic resistance in nematodes, is by co-administration with an inhibitor, such as piperonyl butoxide (Jones, 1998). Kotze (1999) showed that both aerobic and anaerobic pathways were inhibited by piperonyl butoxide (PB) *in vitro*. In a more recent study, the use of PB revealed that CYP contributed to the detoxification of the anthelmintic, rotenone. The activity of rotenone was enhanced four-fold in both adult and larval stages of *H. contortus* and in the larval stage of *T. colubriformis*, when PB was added (Kotze *et al.,* 2006b). Indeed, a rotenone/ PB combination is already in use for culling of commercial fish populations when disease outbreaks occur (Ling, 2002). Although the metabolism of BZ by CYP has not been demonstrated in parasites, the characteristics required for CYP biotransformation of these anthelmintics are quite non-specific. That is, a planar structure (or ability to become planar); a sulphur component and the presence of substitution molecules, which would reduce the polarity of the overall structure, are all prerequisites. The exceptions to the last rule are 5-position hydrophilic groups and

nitrogen-substitution, which prevent induction of CYP (Velik *et al.,* 2004). Thus, most BZ anthelmintics are suitable for oxidative metabolism by CYP and there seems to be no obvious reason why this would not occur in parasitic nematodes (Kotze *et al.,* 2006b).

The wide substrate specificity of these enzymes raises concerns that this is yet another locus with the potential to propagate resistance-causing mutations in nematodes, as has already been witnessed in dipterans (Daborn *et al.,* 2002). The increased activity of CYP amongst insect species has long been suggested as a major cause of drug resistance (Oppennoorth, 1985). Over 90 individual CYP genes have been described in the fruit fly, *Drosophila melanogaster* (Tijet *et al.,* 2001) and numerous studies have implicated a role for these genes in insecticide resistance (Berge *et al.,* 1998; Feyereisen, 1999; Sabourault *et al.,* 2001). However, in a recent study of 20 (geographically disparate) resistant populations, the increased transcription of a single CYP gene, *Cyp6g1,* could confer DDT resistance and this was the only resistance allele found amongst all populations (Daborn *et al.,* 2002). The increased transcription of *Cyp6g1* in the order of 10 to 100 times was caused by the insertion of a single Accord transposon. Therefore, the potential enhancement of CYP activity amongst MDR nematodes is worth investigating.

TBZ, which is employed in the egg hatch assay, is known to induce CYP1A1 class enzymes (Galtier *et al.,* 1997). Thus, the potential contribution of CYP to BZ resistance in the *MTci5* isolate will be examined in this Chapter. PB will be used in conjunction with TBZ to investigate the hypothesised enhancement in toxicity of BZ in *MTci5* and a comparative susceptible *T. circumcincta* isolate (*MTci2*). Such a comparison, between resistant and susceptible isolates has only been conducted once before whereby ML resistant and susceptible *H. contortus* populations were studied (Kotze, 2000). The analysis revealed no increased CYP activity in the resistant population, suggesting that this mechanism had not been modified in any way to enhance the development of resistance in that population. However, these biochemical assays can be insensitive. Moreover, since there are at least 33 CYP genes in the *H. contortus* genome (J. Gilleard, personal communication, unpublished data), there is no guarantee that these assays will detect a difference in the specific activity of one relevant enzyme, if it was up-regulated, for example.

4.1.6 Aims and rationale

In order to examine the role of the F200Y isotype I β-tubulin mutation more closely, it was necessary to expose the *MTci5* parasites to BZ *in vitro* and then analyse their F200Y status following selection. The results of the *in vivo* characterization of the F200Y genotype suggest that some 'susceptible' worms, i.e. $P200^{Phe/Tyr}$ (rs) or $P200^{Phe/Phe}$ (ss), were able to survive the action of BZ when it was administered at the recommended dose rate. If parasites are selected *in vitro*, essentially there is no escape from the action of the drug, no pharmacokinetic host effects can be ascribed, therefore, this work will provide substantive evidence as to whether the F200Y isotype I β-tubulin mutation is wholly accountable for BZ resistance. Other potential mechanisms of BZ resistance are also investigated. The possible role of mutations in the isotype II β-tubulin gene and two non-specific mechanisms, the P-gp efflux pump and CYP metabolism will also be investigated. However, if these mechanisms do have a role in resistance, they are unlikely to be the only mechanisms involved and thus the results of this study will not provide a definitive list of all contributory resistance mechanisms in *T. circumcincta*. It should be noted that the egg hatch assays in Sections 4.2 & 4.3 of this Chapter were performed before the dissolution of TBZ was apparent, and thus the analysis of these data is limited to the trends observed as explained in Chapter 3. However, the assays described in Section 4.6 were conducted with known concentrations of TBZ and thus (see Section 2.2.1) the egg development values are likely to be a true representation of the BZ resistance level of the *MTci5* isolate, but for obvious reasons, cannot be compared directly to previous assay data. Comparative data was also obtained from a susceptible isolate (*MTci2*).

The overall aim of this Chapter was to determine the relative importance of the F200Y isotype I β-tubulin mutation to the BZ resistance phenotype of the *MTci5* isolate by assessing the potential contributions of other previously implicated mechanisms.

4.2 Pattern of BZ resistance over time: genotype vs. phenotype

4.2.1 *In vitro* pattern of BZ resistance over time: change in phenotype

It has been observed previously that the apparent resistance of a parasite population to BZ, as determined by the *in vitro* egg hatch assay, changes over the course of an experimental infection (Borgsteede & Couwenberg, 1987; Kerboeuf & Hubert, 1987). This was investigated in the *MTci5* isolate with a comparable susceptible isolate (*MTci1*) included as a control. Two lambs were infected with 15,000 L_3 from either the *MTci5* or the *MTci1* isolate. The egg hatch assay was performed weekly on eggs harvested from these lambs from three to ten weeks post-infection. Two replicates were performed for each TBZ concentration, these data were pooled and the ED values were calculated in Minitab V14 using Probit reliability/survival analysis. Week three was chosen as a starting point for this experiment as patency appears around days 19-21 and a sufficient egg count was attained at this stage. Due to the relatively short longevity of these parasites and the declining egg count, week ten was selected as a suitable end-point of the study. Unfortunately, the donor animal infected with the *MTci1* isolate mounted an immune response and expelled the worms. By this stage, infecting another donor for *MTci1* passage would not have provided a valid comparison, since the *MTci5* infection was well underway and other variables may have been introduced. For instance, after four weeks, there may have been seasonal differences which could have influenced the host-parasite relationship. Consequently, data were only obtained from the *MTci1* isolate for weeks three and four.

The level of BZ resistance changed over time in the *MTci5* isolate; peaking at week eight with a slight depression at week six (see Figure 4.1). Resistance factors were calculated (see Table 4.1) using week three as the reference point and the mean of these (ED_{50}, $_{96}$ and $_{99}$) showed that resistance had almost doubled (1.6) by week four, and quadrupled by week five (3.7) before returning to twice the level of resistance (2.5) by week ten. Peak resistance was observed at week eight (Rf of 4.1). The *MTci1* data also showed an increase in resistance (by a factor of 1.6 in week four when compared with week three). Thus, the *MTci1* isolate appeared to be following the same trend as the *MTci5* isolate (albeit at a much lower resistance level), however, it cannot be said whether this trend would have continued. A one-way ANOVA was performed using individual ED_{50} values calculated for each replicate (two per

week) and for each week from weeks three to ten post-infection. The results show that there was a highly significant change in ED_{50} values over time ($F_{7, 15} = 30.52$, $P < 0.0001$) and the level of variance between the ED_{50} values at each time point is displayed in the boxplot in Figure 4.2. The greatest variance was found within the week five data, however, there appears to be good consistency between replicates overall.

4.2.2 *In vitro* pattern of BZ resistance over time: change in the F200Y isotype I β-tubulin genotype

To determine whether the change observed in resistance phenotype was reflected at the F200Y isotype I β-tubulin locus, a number of L_3 parasites were collected via coproculture on a weekly basis for DNA extraction and genotyping by allele-specific PCR. To minimize disparity between the egg hatch assay samples and the L_3, those selected for coproculture and genotyping were collected on the same day as the eggs. Whilst it would have been better to directly genotype the L_1 from the egg hatch assays after enumeration, this was not possible due to the difficulty in attaining sufficient sensitivity when using such small amounts of genomic DNA template. This had been achieved by the time the next experiment was conducted, (see Section 4.3). However, there is no reason to believe that the L_3 and L_1 samples are likely to differ at each time point. Weeks five and ten were omitted from the analysis as the allele-specific PCR was a labour-intensive technique and it was believed that enough information could be sought from the other six time points. Figure 4.3 shows the genotype frequencies at the F200Y isotype I β-tubulin locus over the nine weeks post-infection. The sample sizes varied somewhat between weeks and this was due to the inefficiency of the allele-specific PCR technique. However, it is not thought that this biased the genotype proportions.

An 'estimation of the differences between genotype proportions' test with 95% confidence intervals was used to compare all genotype ratios across all time points. As Figure 4.3 indicates, there is no significant change ($p>0.05$) in F200Y isotype I β-tubulin genotype frequency over time; therefore, these parasites appear to display an increased level of BZ resistance independent of changes at the F200Y isotype I β-tubulin locus. The mean genotype ratios observed over the course of the infection were 42.7% $P200^{Tyr/Tyr}$, 46.6% $P200^{Phe/Tyr}$ and 10.7% $P200^{Phe/Phe}$. These results are in agreement (not significantly different at 99% CI using

the 'estimation of the differences between genotype proportions' test, see Table 4.2) with those ratios observed in Section 3.3 for adult and L_3 genotypes, which suggests that the genotypes are fixed and there is no change in the frequency of the F200Y isotype I β-tubulin mutation over time.

4.3 F200Y isotype I β-tubulin genotypes of MTci5 larvae surviving BZ selection from the in vitro egg hatch assay

In order to determine the survival of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes following BZ selection *in vitro*, L_1 were selected from various TBZ concentrations from the egg hatch assay for F200Y isotype I β-tubulin genotyping. The allele-specific PCR method was used for this Section, and as implied previously (Section 3.3), these data are subject to an error rate of between 5 and 10%. The results are displayed in Figure 4.4. In this egg hatch assay the mean ED_{50}, ED_{96} and ED_{99} values (Probit reliability/survival analysis calculated in Minitab V14) were 0.91, 2.06 and 2.44 µg/ml, respectively. The L_1 were selected from a wide range of TBZ concentrations with these egg development values in mind. For example, the concentrations from which the L_1 were sampled were: 0, 0.19, 0.5, 1 and 2µg/ml TBZ and these equate to the following egg development values of the population: ED_0, ED_6, ED_{20}, ED_{60} and ED_{96}. The egg development values have been estimated from Probit analysis of the egg hatch assay data (Minitab v14). Eight replicates were carried out for each TBZ concentration to provide sufficient numbers of L_1 survivors for genotyping. Even though the true TBZ concentrations are not known (see Section 2.2.1), the results of this assay followed the expected pattern (i.e. the range of TBZ concentrations used showed a 0 to 96% hatch range); therefore, L_1 collected from wells representing the ED_{96} value still represent the most resistant individuals in the population. Hence, one can still have confidence in this data-set. The maximum TBZ concentration used was 2µg/ml (i.e. the ED_{96} value) as it was necessary to permit survival of a small proportion of the population to provide sufficient L_1 for analysis.

From the F200Y isotype I β-tubulin frequency data, increasing numbers of $P200^{Tyr/Tyr}$ individuals as the concentration of TBZ increases (i.e. from 52% in the control to 78% at 2µg/ml) were observed. A concomitant decrease was also observed in the number of

$P200^{Phe/Tyr}$ (from 35% to 16%) and $P200^{Phe/Phe}$ worms (13% to 5%). The 'estimation of the differences between genotype proportions' test was used to compare the genotype ratios at each TBZ concentration to the control group (i.e. 0μg/ml TBZ) and the following significant differences were observed (see Table 4.3): significant increases in the number of $P200^{Tyr/Tyr}$ individuals at 0.5 and 2μg/ml TBZ (at the 99% CI); significant decreases in the number of $P200^{Phe/Tyr}$ worms at 0.5 and 2μg/ml TBZ (at the 95% CI) and significant decreases in the number of $P200^{Phe/Phe}$ individuals at 0.19 and 1μg/ml TBZ (at the 95% CI). The general trend showed a positive correlation between TBZ concentration and the proportion of $P200^{Tyr/Tyr}$ survivors. However, these data clearly indicate that there were L_1 survivors at high TBZ concentrations (at the ED_{60} & ED_{96} values) which had $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes. This is further evidence that the F200Y isotype I β-tubulin mutation does not fully explain BZ resistance.

4.4 Role of other isotype I mutations implicated in BZ resistance

Due to the observation that individuals with $P200^{Phe/Tyr}$ (rs) and $P200^{Phe/Phe}$ (ss) genotypes survived *in vivo* and *in vitro* BZ exposure, the investigation of additional potential resistance mechanisms was initiated. Firstly, the other β-tubulin isotype I mutations, which have been implicated previously in BZ resistant nematodes, were investigated. Here, the Pyrosequencer was employed to determine the presence of F167Y and E198A isotype I β-tubulin mutations in the *MTci5* isolate. Two additional BZ resistant isolates (*MTci3* and *MTci4*) were also included in this analysis to give an indication of whether these mutations may be important amongst other UK BZ resistant *T. circumcincta* populations. *MTci3* is a BZ resistant population which was isolated from a sheep farm in Central Scotland in 1983 and has been maintained as an unselected laboratory isolate since. *MTci4* is resistant to both BZ and IVM and was isolated in 1992 from a goat farm in the Scottish Borders (Jackson & Coop, 1992a,b,c). It too, has been maintained without selection at MRI since its isolation.

4.4.1 The E198A isotype I β-tubulin mutation

As discussed previously, some studies have described an amino acid substitution at position 198 of the isotype I gene amongst several fungal species (Koenraadt *et al.,* 1992; Ma *et al.,* 2003). A mutation at this site was described for the first time in nematodes in *H. contortus*; whereby alanine (A) was found to replace glutamic acid (E) in BZ-selected worms (Ghisi *et al.,* 2007). Due the close proximity of codon 198 to codon 200, this part of the sequence was included in the existing F200Y isotype I β-tubulin SNP assay (see Section 3.3). Therefore, a new SNP assay for position 198 was not required as any mutation in the sequence would have affected the F200Y isotype I β-tubulin SNP assay results. The expected sequence including any possible substitutions/ insertions must be determined in each SNP assay; if the Pyrosequencer encounters an unexpected mutation, the inbuilt quality control fails the sample. Any failed samples were analysed to determine the cause of failure (and repeated until successfully genotyped), thus, any mutation in codon 198 in any of the *MTci5* adults examined (from Trials One and Two) would have been identified. None was found, therefore, a mutation in codon 198 was ruled out in this population. Similarly, there was no evidence for this mutation in the *MTci3* and *MTci4* isolates, when 40 L3 were examined via Pyrosequencing for their F200Y isotype I β-tubulin genotype ratios (see Section 5.3.1)

4.4.2 The F167Y isotype I β-tubulin mutation

The F167Y isotype I β-tubulin mutation has been reported in a number of nematode and fungal species (Prichard *et al.*, 2000; Prichard, 2001; Silvestre & Cabaret, 2002; Ohrbach *et al.,* 1986) and a new Pyrosequencing SNP assay was designed to examine whether this mutation was present in the *MTci5* isolate (see Section 2.4.4.4). This allele was not found in any of the 72 *MTci5* adult worms (collected from Trials One and Two), that is, all individuals were $P167^{Phe/Phe}$. Similarly, two more BZ resistant isolates, *MTci3* and *MTci4*, were surveyed using this assay. Forty L_3 from each isolate were examined and no evidence of this mutation was found. Thus, the F167Y isotype I β-tubulin mutation was not contributing to BZ resistance in the *MTci5* isolate and is unlikely to be a common mechanism of resistance amongst Scottish resistant *T. circumcincta* isolates.

4.4.3 Other mutations

There have been reports of mutations at other residues in the β-tubulin isotype I gene in relation to BZ resistance in nematodes. For instance residue 165 is of interest (encoding alanine in isotype I and serine in isotype II) since it has been implicated previously in BZ binding (Geary *et al.,* 1992) and in TBZ resistance of *Aspergillus nidulans* (Jung & Oakley, 1990). In order to ascertain whether this mutation, or indeed any others, were contributing to the resistance status of the *MTci5* worms, part of the β-tubulin isotype I gene (residues 71 to 249) was sequenced from the survivors of *in vivo* (anthelmintic selection experiments) and *in vitro* (egg hatch assay) BZ exposure that had a $P200^{Phe/Phe}$ (ss) genotype. From each worm lysate, three separate PCR reactions were carried out using primers: Forward (P1) 5'- GGA ACA ATC GAC TCT GTT CG-3' and Reverse (P4) 5'- GAT CAG CAT TCA GCT GTC CA-3'. PCR products were subsequently cloned into pGEM-T Easy vector, which were transformed into JM109 competent cells. The cloned inserts were amplified by PCR with the original primers to ensure they were genuine and one clone from each original PCR was sequenced. That is, a total of three clones, each derived from separate PCR amplifications, were sequenced per worm and these sequences were aligned to give a consensus. This process was conducted to ensure that *Taq*-induced and sequencing errors would be excluded from the consensus sequence. The alignment of these sequences is displayed in Figure 4.5 alongside a published cDNA for *T. circumcincta* isotype I β-tubulin (Genbank accession number: Z96258). There were some differences between the sequences, however, these were synonymous mutations as indicated in Figure 4.6. This suggests that if there are additional resistance mechanisms in the β-tubulin isotype I gene, they do not occur at positions which have been implicated previously (i.e. positions 165, 167, 198 & 200). It is possible that additional mutations may be present out with codons 71 to 249, however, this is considered unlikely as mutations in such positions have not been implicated previously.

4.5 Determining the presence or absence of the isotype II locus in the MTci5 isolate

The isotype II β- tubulin gene has not previously been reported from *T. circumcincta*. It is not yet known what role, if any, this gene plays in BZ resistance. Previous studies have shown that in highly resistant *H. contortus* isolates, this locus is deleted (Kwa *et al.*, 1993). Others have demonstrated the same F200Y isotype I β-tubulin mutation occurring in isotype II (Beech *et al.*, 1994), however, it is not yet known what functional importance, if any, this mutation has. In order to ascertain what contribution mutations at the isotype II locus might have to the BZ resistance phenotype of *MTci5*, it was first of all necessary to isolate this gene from *T. circumcincta*.

4.5.1 Isolation of isotype II β-tubulin from *T. circumcincta*

A pool of male and female adult *T. circumcincta* worms from the *MTci2* (susceptible) isolate (approximately 100μg) was used to make cDNA, to provide a template for PCR amplification of the isotype II β-tubulin gene. A susceptible worm population was used as there was a possibility that the isotype II β-tubulin gene might be deleted from a resistant worm population (e.g. *MTci5*).

4.5.1.1 Initial unsuccessful attempts to isolate isotype II β-tubulin

Initial experiments involved designing primer pairs based on the consensus β-tubulin isotype II sequences from *Haemonchus contortus*, *Cooperia oncophora*, and some cyathostome species. These were forward, A: 5'- CGT GAG AT(CT)GTC CAC GTT CA -3'/ reverse, A: 5'- TAG GCT TGA GCT CCC TTT GCT GA -3' and forward, B: 5'- CCT GGC CAC GGA CGC TAT CTG -3'/ reverse, B: 5'- TCC TCG GGG TAC GGC TCT TCT G -3'. Some PCR products of the expected size were generated from each pair, however, after cloning and sequencing, these were found to be isotype I β-tubulin fragments. PCR conditions using greater stringency were attempted without further success.

Subsequently, primers based on those described by Clark *et al.* (2005) for isolation of this gene from cyathostomes were used [forward 5'- CGT GA(AG) AT(CT) GT(CT) CA(CT) GT (AGT) CA (AG) GC -3' and reverse: 5'- CCA TTT C(AG)T CCA TAC C(CT)T (GC) (AGT)C -3'], however, again, only isotype I fragments were generated.

4.5.1.2 Successful amplification of isotype II β-tubulin fragments

The isotype I and II β-tubulin polypeptide sequences from a number of closely related nematode species were aligned in order to identify amino acids which were conserved in all of the isotype I sequences, but were substituted in all the isotype II sequences (Figure 4.7). There are 23 amino acid positions which fulfil these criteria and a number of degenerate primers were designed such that their 3' ends matched the sequence encoding some of these amino acids. Hence, PCR amplification with these primer pairs was anticipated to amplify the isotype II β-tubulin gene but not the isotype I gene, providing these observed differences between the isotypes were conserved in *T. circumcincta* (Figure 4.7). All combinations of a total of three forward and three reverse primers (see Table 4.4) were used to amplify isotype II fragments from the *MTci2* cDNA template. Very faint PCR products were amplified for primer combinations: A for ⇔ 2 rev; B for ⇔ 2 rev and C for ⇔ 2 rev (data not shown). Two PCR products from each of these primer combinations were cloned. Successful clones (identified from blue-white selection of the transformed JM109 cells) were re-amplified using the same primers in order to confirm identity. The results of these reactions are shown in Figure 4.8. Most of the clones appeared genuine and some were selected for sequencing (in both directions). These were three A for ⇔ 2 rev (A2) clones, three C for ⇔ 2 rev (C2) clones and six B for ⇔ 2 rev (B2) clones. The consensus of the two sequences obtained for each clone was generated using Seqman (Lasergene v7; DNASTAR, 2006). None of the C2 clones produced reliable sequence data and were thus discarded. All consensus sequences for each clone were aligned (in GeneDoc, v 2.6; Nicholas & Nicholas, 1997) with a published isotype I β-tubulin cDNA sequence (Genbank accession number: Z96258) for comparison (Figure 4.9). The polypeptide sequence alignment of these clones is also displayed (Figure 4.10). The cloned fragments clearly encode a β-tubulin gene and there are five residues which differ from the published *T. circumcincta* isotype I β-tubulin gene sequence. These residues, at positions 90, 115, 153, 165 and 284, correspond to the conserved substitutions between isotype I and isotype II β-tubulin polypeptides (Figure 4.7) demonstrating that the *T. circumcincta* isotype II β-tubulin gene has been successfully isolated. The sequence obtained

for isotype II β-tubulin extends from residues 89 to 284. There were a few other nucleotide differences in sequence amongst the clones (Figure 4.9), some of which were translated into amino acid substitutions (Figure 4.10), i.e. positions 97, 100, 106, 159, 187, 244, 259 & 262. However, none of these were found in more than one clone and the polypeptide sequence alignments are highly conserved amongst species, apart from 23 consistent non-synonymous substitutions. Hence, it is thought that these differences could be due to either *in vitro* mutations associated with the imperfect fidelity of the *Taq* polymerase* or, more likely, sequencing errors. Alternatively, since the *MTci2* cDNA was generated from a pool of individuals, it is also possible that these differences represent different alleles.

4.5.2 PCR assay to investigate the possible deletion of the isotype II β-tubulin locus in the unselected *MTci5* isolate

Having successfully isolated isotype II β-tubulin fragments from the cDNA of a susceptible *T. circumcincta* worm population, it was necessary to determine whether this gene was deleted in the BZ resistant *MTci5* isolate. If such a deletion event had occurred in some or all individuals, this would indicate a further potential resistance mechanism. Using the sequence data obtained from the cDNA clones of isotype II β-tubulin, new primers were designed based on some of the original primer sites (see Figure 4.11). Primers 'B forward' and '4 reverse' were degenerate due to the sequence variability observed between the clones (see Table 4.5). All four combinations of these primers were used in attempts to amplify isotype II β-tubulin from six unselected *MTci5* adults (PT01 to PT06), using *MTci2* cDNA as a positive control. A negative PCR control (whereby water was used as template) was also included. The results of the PCR are shown in Figure 4.12. Single bands were generated for all individuals and all reactions. Worm PT04 (in lane 12) from the C ⇔ 4 reaction was weaker than the others, however, still apparent. The product sizes were consistent and appropriate amongst all individuals in each reaction. Note that the cDNA products appeared smaller than the *MTci5* products due to the lack of introns. The strongest bands were produced from the B ⇔ 1 primer pair, which was also the largest fragment, thus these were cloned in preparation for sequencing. One clone from each individual (after successful re-amplification using the same primers) was sequenced twice to confirm the identity of the products. The consensus sequences for each *MTci5* clone were aligned with the published *T. circumcincta* cDNA

* A high fidelity *Taq* polymerase had been selected for this purpose (Invitrogen Platinum *Taq* DNA polymerase).

sequence (Genbank accession number: Z96258) as well as the *MTci5* isotype I β-tubulin consensus sequence (which is based on 134 sequences) using GeneDoc (v 2.6; Nicholas & Nicholas, 1997, Figure 4.13). An alignment of the equivalent polypeptide sequences is shown in Figure 4.14. The amino acids at positions 90, 115, 153 and 165 which reveal the identity of these clones as isotype II β-tubulin gene fragments are shown by this alignment. However, the P167, P198 and P200 positions, which may be involved in BZ resistance in this gene, are not covered in the sequence. The *MTci5* polypeptide sequences were identical to each other and to the isotype II sequences of other nematode species, indicating that in this region, there were no additional mutations, which could be associated with resistance. None of the mutations found in the *MTci2* clones (see Section 4.5.1.2) were found in the *MTci5* sequences, further suggesting that these substitutions resulted from errors in sequencing.

Following confirmation of the single worm PCR products being isotype II β-tubulin, the primer pair B ⇔ 1 was then used to amplify this locus from 72 unselected and 72 BZ-selected *MTci5* adults (from Trials One and Two). IVM and LEV-selected *MTci5* worms were not assessed as the relevance of the deletion of the isotype II locus from previous studies has been confined to the effects of BZ selection. Isotype I β-tubulin plasmid template (extending over the same region) was included as a negative control and no products were generated using these primers, confirming the specificity of the isotype II primers. As Figure 4.15a, b shows, all individuals from both the unselected and BZ-selected *MTci5* populations produced a strong band, indicating that this locus was not deleted in this isolate. As Figure 4.15b shows, one PCR failed (worm PB16), however, this locus was amplified successfully on the second attempt. This failed amplification was an isolated event and likely to be due to a procedural error. Thus, the deletion of the isotype II β-tubulin locus can be ruled out as a contributory mechanism to BZ resistance in the *MTci5* isolate.

4.6 An in vitro investigation of the contribution of non-specific resistance mechanisms to BZ resistance of MTci5

From the work presented thus far, it is apparent that the previously implicated mechanisms of BZ resistance, which are confined to the β-tubulin genes (other than the F200Y isotype I β-tubulin mutation) are not prevalent or important in the *MTci5* isolate. However, the survival of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ individuals from the *in vitro* and *in vivo* BZ selection of this population still warrants further investigation into possible contributory mechanisms for resistance. The search for such mechanisms now extends to examination of the non-specific xenobiotic removal processes, which have been described in relation to MDR from other organisms. The p-glycoprotein (P-gp) drug efflux mechanism and the cytochromes P450 (CYP) -dependent drug metabolism pathway were investigated for their potential role in BZ resistance. The putative involvement of such mechanisms can be investigated using specific inhibitors of P-gp and CYP. Verapamil hydrochloride and piperonyl butoxide are inhibitors of P-gp and CYP, respectively, and were used in combination with TBZ in the egg hatch assay to analyse the effects of inhibition of these processes upon the BZ resistance phenotype. The *MTci5* isolate was examined and the susceptible *MTci2* isolate was also included in this study for comparison. L_1 survivors of the egg hatch assay were analysed for their F200Y isotype I β-tubulin genotype, in order to determine whether the role of this mutation was altered when the non-specific mechanisms were inhibited. Initial experiments involved testing the general toxicity of the inhibitors to *T. circumcincta* eggs (administered both separately and in combination) to determine the concentration ranges that could be used in the egg hatch assay. Once this had been established, subsequent experiments aimed to answer the following questions.

1. *Does inhibition of P-gp and CYP activity alter the BZ resistance phenotype of MTci5 (and MTci2)?*
2. *Is there a synergistic effect of using both inhibitors in combination?*
3. *How important is the F200Y isotype I β-tubulin mutation when these drug metabolism and efflux mechanisms are suppressed?*

It should be noted that the results of the egg hatch assays in this Section are the most accurate of those presented for the *MTci5* isolate in this thesis. This is due to the fact that the dissolution of TBZ in DMSO was discovered and rectified just prior to these experiments (see Chapter 2, Section 2.2.1).

4.6.1 Toxicity testing of inhibitors

The toxicity tests were set up using the same method as the egg hatch assays (see Section 2.2.1.2), except that thiabendazole (TBZ) was replaced with an inhibitor. The optimal concentrations of inhibitor were determined initially by using a dilution series ranging from 1.57μM to 50μM and then analysing the average reduction in the rate of egg hatch at each dilution. The concentrations at which no significant effect (using a one-way ANOVA, Minitab v 14) of an inhibitor was observed on the development and hatching of eggs were then selected for use in the test assays outlined below. The results of these toxicity tests are shown in Figure 4.16. There was a slight toxic effect of verapamil hydrochloride (VPL) at higher concentrations (>50μM); therefore, an optimum final concentration of 25μM was adopted for the test assays[1]. Piperonyl butoxide (PB) appeared to be far more toxic, exhibiting a significant effect on egg development at concentrations of 25μM and above. Therefore, the optimum concentration of 12.5μM was used for this drug. The inhibitors were dissolved and diluted in DMSO and it is thus possible that the carrier solution, dimethyl sulphoxide (DMSO) became toxic to the parasites when their xenobiotic removal mechanisms were interrupted. This must be taken into consideration when analysing the effects of inhibitors in combination with TBZ (i.e. VPL & PB & TBZ) due to potential synergistic effects.

4.6.2 Test assays with inhibitors: experimental design

The test assays were designed as follows. The TBZ was used alone, in combination with PB, in combination with VPL and in combination with both PB and VPL at each of the following concentrations: 0.013, 0.03, 0.05, 0.1, 0.2 and 0.4μg/ml. Controls included: 0.5% DMSO; water; 25μM VPL; 12.5μM PB and 25μM VPL & 12.5μM PB. Assays were carried out in duplicate for each test or control and the eggs and L_1 were counted from each well to allow estimation of ED_{50}, ED_{96} & ED_{99} values. L_1 (survivors) were selected for DNA extraction as

[1] A concentration of 10 or 20μM VPL was adopted in previous studies (Beugnet *et al.*, 1997)

described in Section 2.3.1. To provide sufficient larvae from each test and control well for genotyping, a [TBZ] of 0.2µg/ml showed an effect of the inhibitors without allowing the majority of eggs to hatch. The results of the test assays were corrected relative to the respective controls, i.e. the control samples containing VPL + PB were corrected to 100% hatch rate and the test samples (in this case TBZ + VPL + PB) were adjusted by the same margin to exclude individuals which had suffered poorer egg development as a result of the DMSO toxicity or some other factor. The control sample results did not show hatch rates of less than 80%, indicating that the effect of inhibitors and DMSO in isolation was not particularly toxic. This experiment was conducted on two separate occasions and the means of these data points are displayed in all tables and Figures except in Tables 4.7 & 4.8, where the statistical analysis required two data points per treatment. There were no significant differences (using a one way ANOVA, Minitab V14) between the results of either batch of tests, giving confidence in the accuracy and reproducibility of the improved egg hatch assay technique. Furthermore, these experiments were conducted at times where the fluctuation of resistance was predicted to be minimal as observed previously (Section 4.2). In the case of the *MTci5* population, the experiments were conducted in weeks 5 and 7 (missing out week 6 where a depression in resistance had been observed). The *MTci2* isolate was examined during weeks 10 and 11; although information regarding the pattern of resistance had not been obtained for this isolate, these estimates were later shown to be satisfactory given the consistency in ED values between assays.

4.6.3 Effects of inhibitors upon BZ resistance phenotype

4.6.3.1 MTci5 (Multiple resistant) isolate

The ED_{50} value for the *MTci5* isolate in the absence of inhibitors was 0.2µg/ml TBZ as shown in Figures 4.17 & 4.18. The addition of the VPL and PB inhibitors to the assay had the effect of reducing the mean ED_{50} values to 0.11µg/ml TBZ and 0.06µg/ml TBZ, respectively. Use of the inhibitors in combination rendered this isolate completely susceptible (i.e. ED_{50} = 0.001µg/ml TBZ). The pattern observed for the percentage of eggs developing successfully versus the increasing TBZ concentration gave the expected negative dose-response curve, which was shifted to the left in the presence of the inhibitors. PB appeared to be more effective in reducing the level of BZ resistance in this isolate than VPL, and in combination

these inhibitors rendered the *MTci5* population extremely sensitive to the drug. Susceptibility factors were calculated to compare the effects of each inhibitor (see Table 4.6). These were obtained by dividing the $ED_{50, 96 \& 99}$ values of the test groups by that of the TBZ treatment alone. Using the ED_{50} value as the most informative measure (of the effect of each drug upon the majority of parasites), VPL induced just less than a two-fold increase in susceptibility whereas PB had a greater effect causing a three- fold increase. In combination, these inhibitors rendered the *MTci5* population completely susceptible (i.e. 173 times less resistant). Interestingly, the ED_{96} and ED_{99} data do not reflect such a dramatic reduction. These represent the super-resistant minority proportion of the population and only show a two-fold increase in susceptibility when both inhibitors were introduced. The discrepancy is due to the non-linearity of the ED curve and the fact that the susceptibility factor is a relatively crude calculation. A one-way ANOVA was also performed using two data points (obtained from separate experiments) obtained for each ED_{50}, ED_{96} & ED_{99} value from each drug combination (as shown in Table 4.7). The test sample results (i.e. TBZ & VPL) were compared with the TBZ alone sample results in order to determine the effect of the inhibitors. In every case, the effect of the inhibitors in reducing the level of BZ resistance was statistically significant (see Table 4.7).

4.6.3.2 MTci2 (BZ susceptible) isolate

The *MTci2* isolate showed a similar pattern to the *MTci5* isolate. The ED_{50} value in the TBZ alone sample was 0.04µg/ml TBZ (Figures 4.19 & 4.20). The addition of the VPL and PB inhibitors to the assay had the effect of reducing the ED_{50} value to 0.03µg/ml TBZ and 0.02µg/ml TBZ, respectively. This translates into a 1.5- and 2- fold increase in susceptibility for VPL and PB inhibitors, respectively. When the inhibitors were used in combination, this isolate displayed an ED_{50} value of 0.01µg/ml TBZ, which is a four-fold increase in susceptibility. Despite the apparent reduction in ED values, the results of the one-way ANOVA (conducted in Minitab V14; see Table 4.8) showed the inhibitors to be far less effective in increasing BZ susceptibility in this population, compared with the *MTci5* population. A significant increase in susceptibility was found in the ED_{99} value of the TBZ + PB treated eggs and also in the $ED_{50, 96 \& 99}$ values of the TBZ + VPL + PB treated eggs. This suggests that the PB inhibitor is more potent amongst the susceptible population and there is possibly some synergy between the inhibitors.

4.6.4 Effects of inhibitors upon BZ resistance genotype

Since it was apparent from the egg hatch assays that the inhibitors did reduce the fitness of the parasites in terms of their ability to deal with BZ *in vitro*, it was decided to look at the F200Y isotype I β-tubulin ratios of the survivors from each treatment group. If there were changes in genotype with respect to the control group, this would give a clearer indication of the role of the F200Y isotype I β-tubulin mutation. All four groups were analysed at the 0.2μg/ml TBZ concentration. This was chosen as it was closest to the ED_{50} value of the isolate, meaning it was high enough to exert an effect upon the population, but low enough to gather enough L_1 survivors for genotyping. The Pyrosequencing assay for the F200Y isotype I β-tubulin mutation was utilized here and is considered to be 100% accurate. These data are summarized in Figure 4.21 and show substantial effects of the inhibitors upon F200Y isotype I β-tubulin genotypes. The ratios observed in the 0.2μg/ml TBZ alone group were 67% $P200^{Tyr/Tyr}$, 27% $P200^{Phe/Tyr}$ and 3% $P200^{Phe/Phe}$. The statistical significance of adding inhibitors was assessed by comparing the genotype ratios of all groups with the TBZ alone group using the 'estimation of the differences between genotype proportions' test (see Table 4.9). The results indicate that VPL had the effect of decreasing the number of $P200^{Tyr/Tyr}$ genotypes, whilst increasing the proportion of $P200^{Phe/Tyr}$ genotypes (both highly significant, $p < 0.01$), whilst the $P200^{Phe/Phe}$ genotype proportion remained unchanged. The addition of PB exerted the same influence, however, when the inhibitors were introduced in combination, the results were unexpected. The only significant deviation from the control genotypes was a decline in the number of $P200^{Tyr/Tyr}$ genotypes, and this was not highly significant ($p < 0.05$). This latter result seems inconsistent with the effect of adding the inhibitors separately.

Chapter 4 Tables & Figures

Figure 4.1: Pattern of BZ resistance as determined by the egg hatch assay over time in the *MTci5* isolate (limited data are displayed for the *MTci1* isolate due to the host animal mounting an unexpected immune response against the parasites).

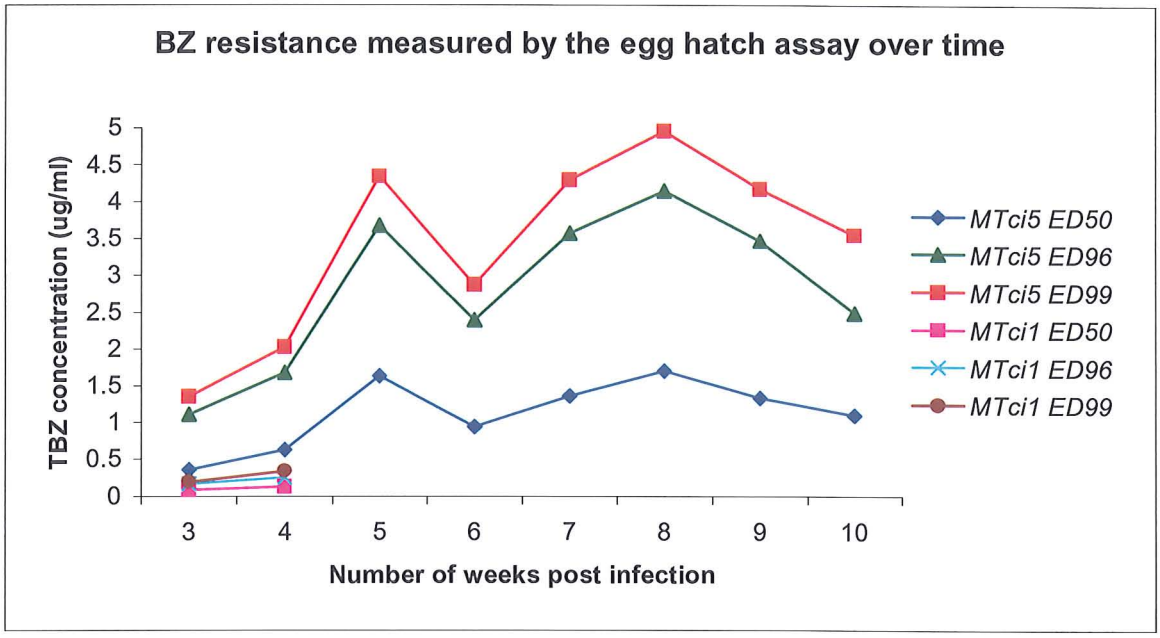


Table 4.1: Resistance factors calculated for the *MTci5* isolate. These were calculated by dividing each ED value by that of week 3 (as a reference point) to determine the increase or decrease in resistance over time. The means of all Rfs were calculated to give as accurate a value as possible.

Resistance factors	Number of weeks post-infection							
	3	4	5	6	7	8	9	10
ED_{50}	1.0	1.8	4.6	2.6	3.8	4.8	3.8	3.1
ED_{96}	1.0	1.5	3.3	2.2	3.2	3.8	3.1	2.2
ED_{99}	1.0	1.5	3.3	2.2	3.2	3.8	3.1	2.2
Mean	**1.0**	**1.6**	**3.7**	**2.3**	**3.4**	**4.1**	**3.3**	**2.5**

Figure 4.2: Boxplot showing the variance in ED_{50} values estimated from week's three to ten post-infection in the *MTci5* isolate (created in Minitab v14).

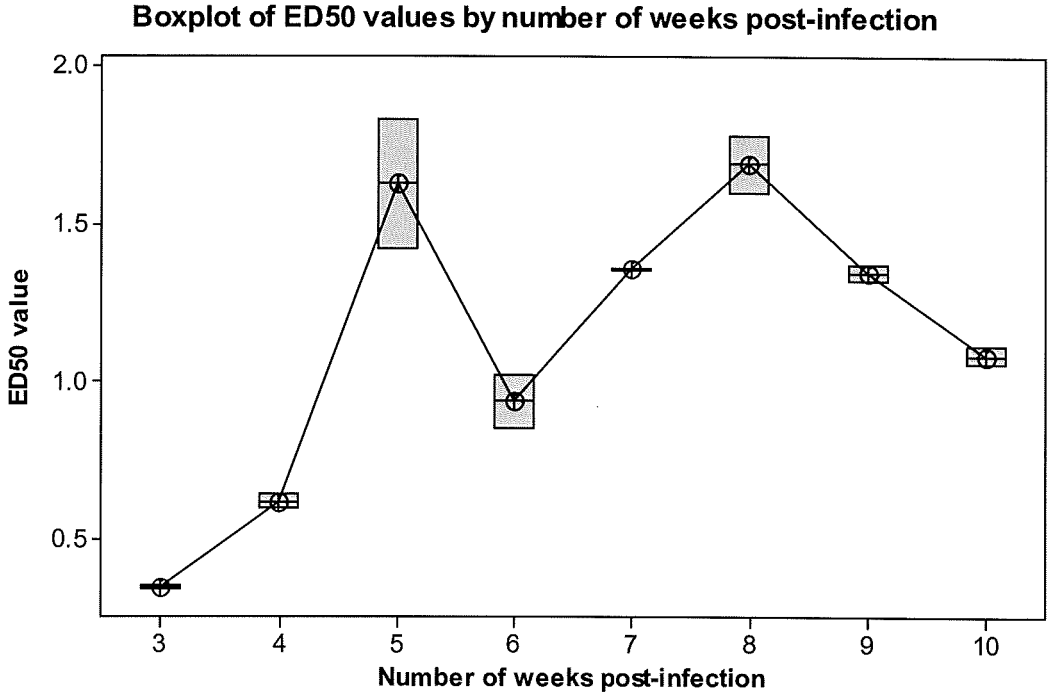


Figure 4.3: Graph displaying the genotype ratios of L_3 cultured from various time points throughout infection. The total numbers of L_3 genotyped (n) are also displayed.

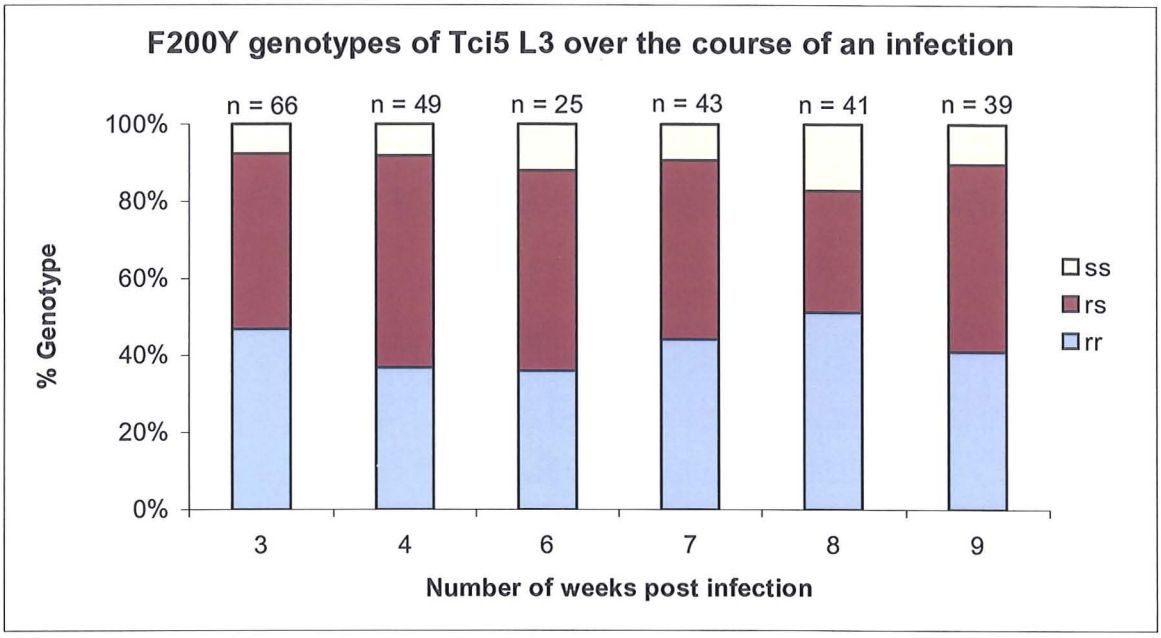


Table 4.2: The observed genotype counts per week and the 'estimation of the differences between genotype proportions' test results (where each value is compared to the week three counts) at the 95% confidence interval (CI estimates shown in brackets). No significant values were found.

Number of weeks PI	Number of $P200^{Tyr/Tyr}$ (CI) p-value	Number of $P200^{Phe/Tyr}$ (CI) p-value	Number of $P200^{Phe/Phe}$ (CI) p-value
3	31 -	30 -	5 -
4	18 (0.283, -0.079)	27 (0.087, -0.280)	4 (0.094, -0.106)
6	9 (0.333, -0.114)	13 (0.164, -0.295)	3 (0.098, -0.187)
7	19 (0.219, -0.163)	20 (0.181, -0.202)	4 (0.090, -0.125)
8	21 (0.152, -0.237)	13 (0.324, -0.049)	7 (0.037, -0.227)
9	16 (0.255, -0.136)	19 (0.165, -0.230)	4 (0.088, -0.141)

Figure 4.4: F200Y isotype I β-tubulin genotype ratios of L_1 survivors from the egg hatch assay. N refers to the total sample size of L_1 collected from each TBZ concentration. The TBZ concentrations shown (0, 0.19, 0.5, 1 and 2μg/ml TBZ) equate to the following egg development values of the population: ED_0, ED_6, ED_{20}, ED_{60} and ED_{96}.

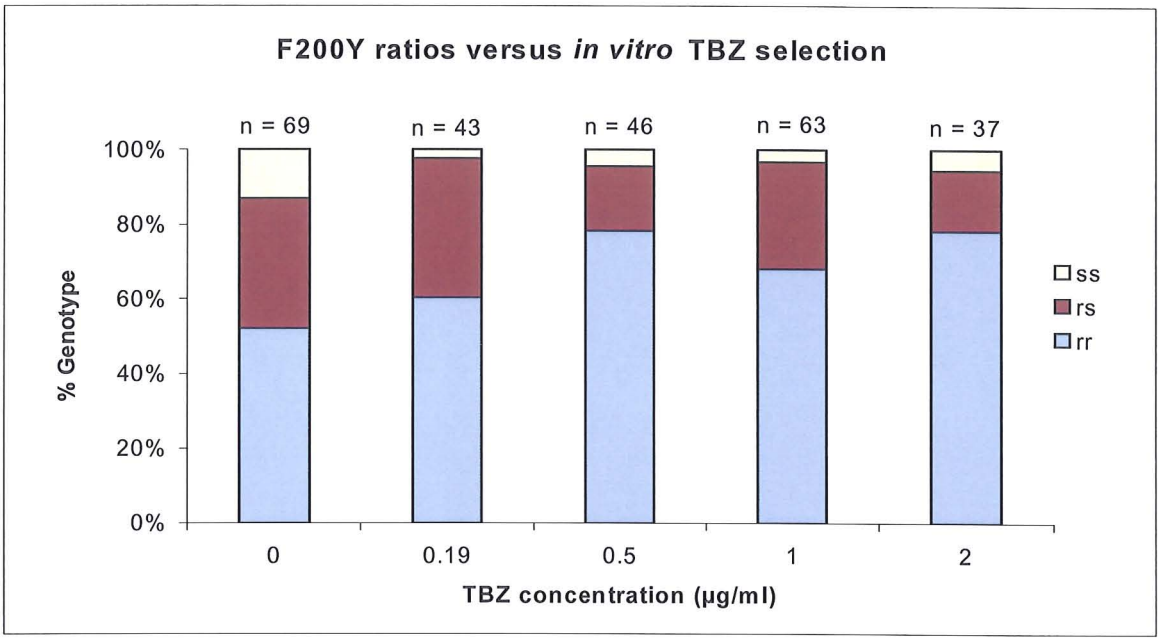


Table 4.3: The observed genotype counts per TBZ concentration and the 'estimation of the differences between genotype proportions' test results (comparing each value to the control group i.e. 0µg/ml TBZ) at the 95% and 99% confidence intervals (CI estimates are shown in brackets). All significant values are shown in bold.

[TBZ] (µg/ml)	Number of $P200^{Tyr/Tyr}$ (CI) p-value	Number of $P200^{Phe/Tyr}$ (CI) p-value	Number of $P200^{Phe/Phe}$ (CI) p-value
0	36 -	24 -	9 -
0.19	26 (0.104, -0.271) p>0.05	16 (0.159, -0.207) p>0.05	**1** **(0.199, 0.016) p<0.05**
0.5	**36** **(-0.041, -0.481) p<0.01**	**8** **(0.331, 0.017) p<0.05**	2 (0.186, -0.012) p>0.05
1.0	43 (0.003, -0.325) p>0.05	18 (0.220, -0.096) p>0.05	**2** **(0.189, 0.008) p<0.05**
2.0	**29** **(-0.029, -0.495) p<0.01**	**6** **(0.349, 0.022) p<0.05**	2 (0.184, -0.031) p>0.05

Figure 4.5: Sequence alignment of five individual P200$^{Phe/Phe}$ survivors from the *in vitro* egg hatch assay (at ED_{96} value) and the sole P200$^{Phe/Phe}$ survivor from the *in vivo* anthelmintic selection experiment. Introns have been removed and a published *T. circumcincta* beta-tubulin isotype I sequence (TcTub isol) (GenBank accession number Z96258), is displayed alongside the other sequences. PB33_T1 refers to a single post-BZ selected adult worm (number 33 from Trial 1). L_1 means first stage larva, the letters A- E represent their identity and 05/05 refers to the date (May 2005) that these worms were selected for genotyping and sequencing. The consensus is shown on the top line and the discrepancies in sequence are displayed. The sequence is 538 bases long and the primer regions are highlighted in pink.

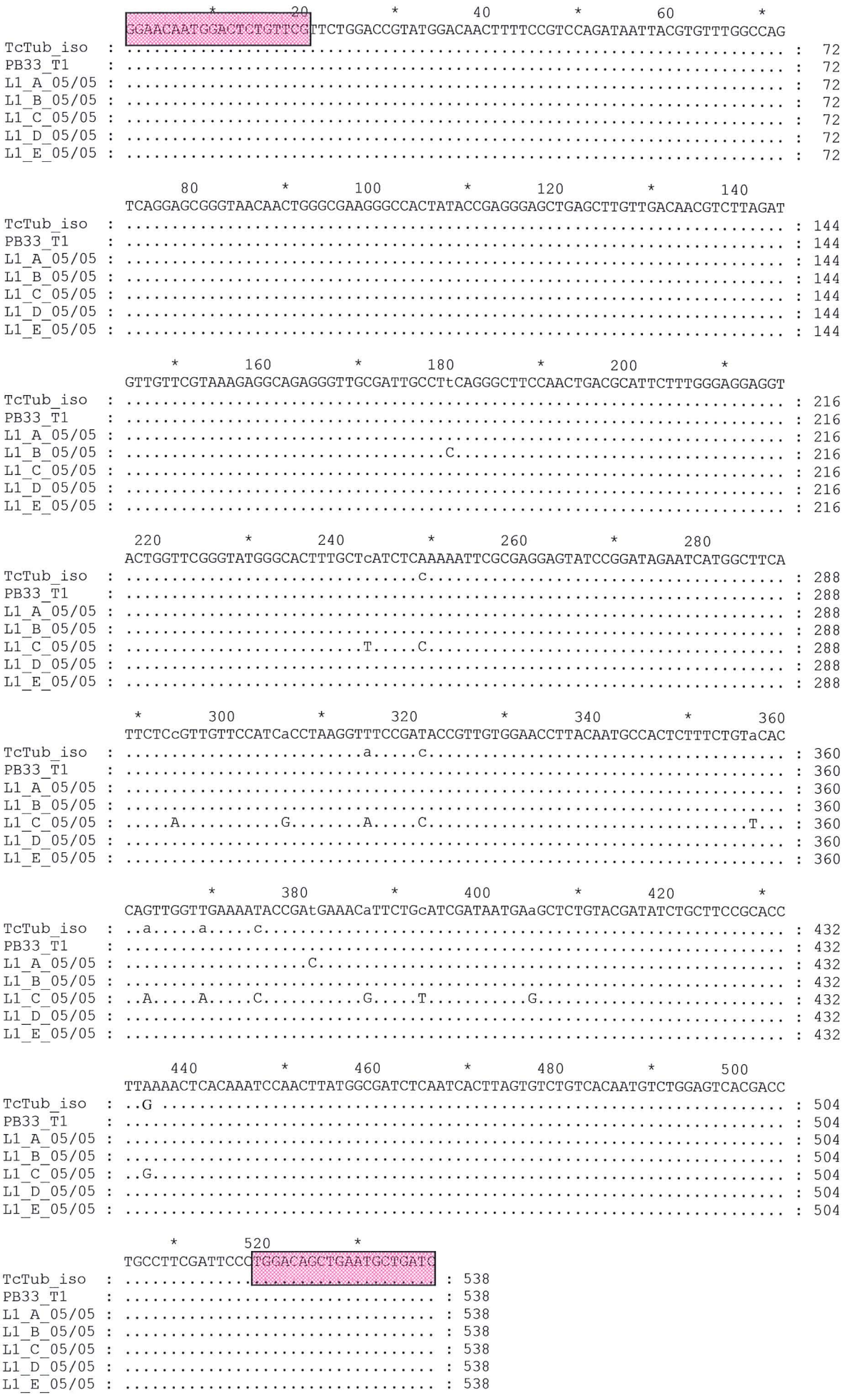

GGAACAATGGACTCTGTTCGTTCTGGACCGTATGGACAACTTTTCCGTCCAGATAATTACGTGTTTGGCCAG
TCAGGAGCGGGTAACAACTGGGCGAAGGGCCACTATACCGAGGGAGCTGAGCTTGTTGACAACGTCTTAGAT
GTTGTTCGTAAAGAGGCAGAGGGTTGCGATTGCCTtCAGGGCTTCCAACTGACGCATTCTTTGGGAGGAGGT
ACTGGTTCGGGTATGGGCACTTTGCTcATCTCAAAAATTCGCGAGGAGTATCCGGATAGAATCATGGCTTCA
TTCTCcGTTGTTCCATCaCCTAAGGTTTCCGATACCGTTGTGGAACCTTACAATGCCACTCTTTCTGTaCAC
CAGTTGGTTGAAAATACCGAtGAAACaTTCTGcATCGATAATGAaGCTCTGTACGATATCTGCTTCCGCACC
TTAAAACTCACAAATCCAACTTATGGCGATCTCAATCACTTAGTGTCTGTCACAATGTCTGGAGTCACGACC
TGCCTTCGATTCCCTGGACAGCTGAATGCTGATC
TcTub_iso
PB33_T1
L1_A_05/05
L1_B_05/05
L1_C_05/05
L1_D_05/05
L1_E_05/05

Figure 4.6: Polypeptide sequence alignment of the P200$^{Phe/Phe}$ survivor sequences from the previous Figure (4.5). The sequence extends from residue 71 to 249 and the primer regions are highlighted in pink. The mutations shown in the previous Figure (4.5) did not alter the protein alignment. Note that there are no mutations at positions 76 (the forward primer would not have annealed if there was a mutation here), 167, 198 and 200 which have been shown to contribute to BZ resistance in a number of nematodes and other species. There were no novel mutations.

```
                    80          *         100         *         120
                GTMDSVRSGPYGQLFRPDNYVFGQSGAGNNWAKGHYTEGAELVDNVLDVVRKEAEGCD
TcTub_isoI  : ..........................................................  :  58
PB33_T1     : ..........................................................  :  58
L1_A_05/05  : ..........................................................  :  58
L1_B_05/05  : ..........................................................  :  58
L1_C_05/05  : ..........................................................  :  58
L1_D_05/05  : ..........................................................  :  58
L1_E_05/05  : ..........................................................  :  58

                 *        140         *        160         *        180
               CLQGFQLTHSLGGGTGSGMGTLLISKIREEYPDRIMASFSVVPSPKVSDTVVEPYNAT
TcTub_isoI  : ..........................................................  : 116
PB33_T1     : ..........................................................  : 116
L1_A_05/05  : ..........................................................  : 116
L1_B_05/05  : ..........................................................  : 116
L1_C_05/05  : ..........................................................  : 116
L1_D_05/05  : ..........................................................  : 116
L1_E_05/05  : ..........................................................  : 116

                    *        200         *        220         *        240
               LSVHQLVENTDETFCIDNEALYDICFRTLKLTNPTYGDLNHLVSVTMSGVTTCLRFPG
TcTub_isoI  : ..........................................................  : 174
PB33_T1     : ..........................................................  : 174
L1_A_05/05  : ..........................................................  : 174
L1_B_05/05  : ..........................................................  : 174
L1_C_05/05  : ..........................................................  : 174
L1_D_05/05  : ..........................................................  : 174
L1_E_05/05  : ..........................................................  : 174

               QLNAD
TcTub_isoI  : .....  : 179
PB33_T1     : .....  : 179
L1_A_05/05  : .....  : 179
L1_B_05/05  : .....  : 179
L1_C_05/05  : .....  : 179
L1_D_05/05  : .....  : 179
L1_E_05/05  : .....  : 179
```

Figure 4.7: Polypeptide sequence alignment of full-length β-tubulin isotype I and II genes (e.g. *iso I or iso II*) of various nematode species, created using Megalign (Lasergene v7; DNASTAR, 2006). The abbreviations *T. circ*, *H. con*, *C. onc*, *C. cat* and *T. col* refer to *Teladorsagia circumcincta* (isotype I β-tubulin GenBank accession number: Z96258); *Haemonchus contortus* (isotype I and II β-tubulin GenBank accession numbers: M76493, M76491); *Cooperia oncophora* (isotype I and II β-tubulin GenBank accession numbers: AY259994, AY259995); *Cyathostomum catinatum* (isotype I and II β-tubulin GenBank accession numbers: AY666162, AY666161) and *Trichostrongylus colubriformis* (isotype II β-tubulin GenBank accession number: L23506), respectively. Iso I and II refer to isotype I and II β-tubulin genes. The degenerate primer sites are highlighted in pink. Three forward (A, B and C) and three reverse primers (1, 2 and 3) were designed initially (see Table 4.4), however a fourth reverse primer (4) was later used (see Table 4.5) after genomic sequence data were obtained (see Figure 4.13).

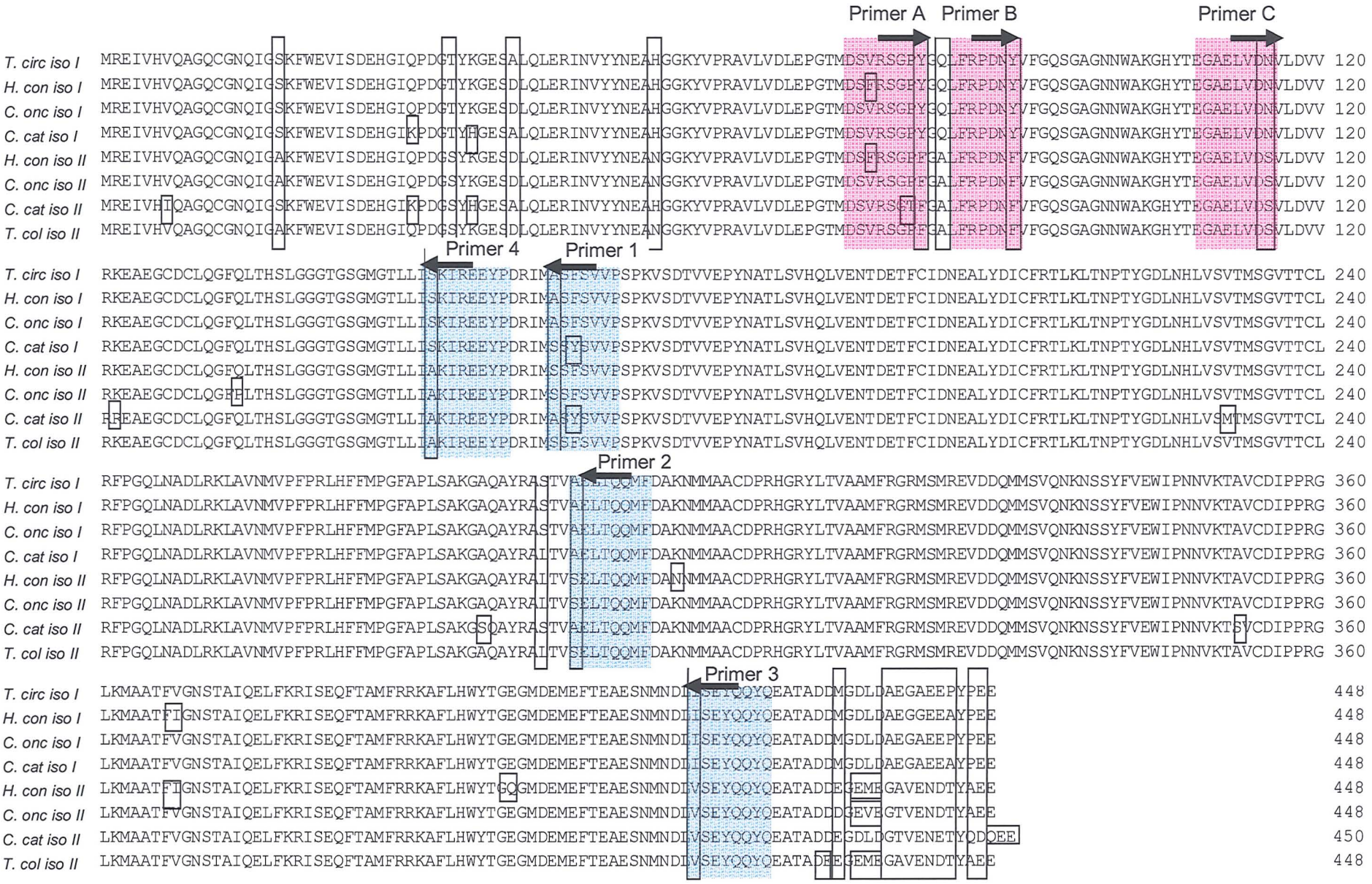
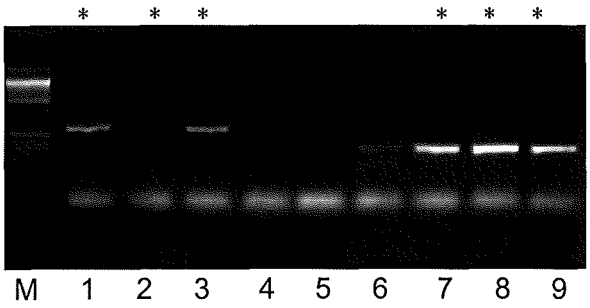

Primer A
Primer B
Primer C
Primer 4
Primer 1
Primer 2
Primer 3
T. circ iso I MREIVHVQAGQCGNQIGSKFWEVISDEHGIQPDGTYKGESALQLERINVYYNEAHGGKYVPRAVLVDLEPGTMDSVRSGPYGQLFRPDNYVFGQSGAGNNWAKGHYTEGAELVDNVLDVV 120
H. con iso I MREIVHVQAGQCGNQIGSKFWEVISDEHGIQPDGTYKGESDLQLERINVYYNEAHGGKYVPRAVLVDLEPGTMDSFRSGPYGQLFRPDNYVFGQSGAGNNWAKGHYTEGAELVDNVLDVV 120
C. onc iso I MREIVHVQAGQCGNQIGSKFWEVISDEHGIQPDGTYKGESDLQLERINVYYNEAHGGKYVPRAVLVDLEPGTMDSVRSGPYGQLFRPDNYVFGQSGAGNNWAKGHYTEGAELVDNVLDVV 120
C. cat iso I MREIVHVQAGQCGNQIGSKFWEVISDEHGIKPDGTYHGESALQLERINVYYNEAHGGKYVPRAVLVDLEPGTMDSVRSGPYGQLFRPDNYVFGQSGAGNNWAKGHYTEGAELVDNVLDVV 120
H. con iso II MREIVHVQAGQCGNQIGAKFWEVISDEHGIQPDGSYKGESDLQLERINVYYNEANGGKYVPRAVLVDLEPGTMDSFRSGPFGALFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVV 120
C. onc iso II MREIVHVQAGQCGNQIGAKFWEVISDEHGIQPDGSYKGESDLQLERINVYYNEANGGKYVPRAVLVDLEPGTMDSVRSGPFGALFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVV 120
C. cat iso II MREIVHIQAGQCGNQIGSKFWEVISDEHGIKPDGSYHGESALQLERINVYYNEAHGGKYVPRAVLVDLEPGTMDSVRSGTFGALFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVV 120
T. col iso II MREIVHVQAGQCGNQIGAKFWEVISDEHGIQPDGSYKGESDLQLERINVYYNEANGGKYVPRAVLVDLEPGTMDSVRSGPFGALFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVV 120
T. circ iso I RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYPDRIMASFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLTNPTYGDLNHLVSVTMSGVTTCL 240
H. con iso I RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYPDRIMASFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLTNPTYGDLNHLVSVTMSGVTTCL 240
C. onc iso I RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYPDRIMASFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLTNPTYGDLNHLVSVTMSGVTTCL 240
C. cat iso I RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYPDRIMSSYSVVPSPKVSDTVVEPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLTNPTYGDLNHLVSVTMSGVTTCL 240
H. con iso II RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLIAKIREEYPDRIMSSFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLTNPTYGDLNHLVSVTMSGVTTCL 240
C. onc iso II RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLIAKIREEYPDRIMSSFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLTNPTYGDLNHLVSVTMSGVTTCL 240
C. cat iso II RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLIAKIREEYPDRIMASYSVVPSPKVSDTVVEPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLTNPTYGDLNHLVSMTMSGVTTCL 240
T. col iso II RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLIAKIREEYPDRIMSSFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLTNPTYGDLNHLVSVTMSGVTTCL 240
T. circ iso I RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLSAKGAQAYRASTVAELTQQMFDAKNMMAACDPRHGRYLTVAAMFRGRMSMREVDDQMMSVQNKNSSYFVEWIPNNVKTAVCDIPPRG 360
H. con iso I RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLSAKGAQAYRASTVAELTQQMFDAKNMMAACDPRHGRYLTVAAMFRGRMSMREVDDQMMSVQNKNSSYFVEWIPNNVKTAVCDIPPRG 360
C. onc iso I RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLSAKGAQAYRASTVAELTQQMFDAKNMMAACDPRHGRYLTVAAMFRGRMSMREVDDQMMSVQNKNSSYFVEWIPNNVKTAVCDIPPRG 360
C. cat iso I RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLSAKGAQAYRAITVAELTQQMFDAKNMMAACDPRHGRYLTVAAMFRGRMSMREVDDQMMSVQNKNSSYFVEWIPNNVKTAVCDIPPRG 360
H. con iso II RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLSAKGAQAYRAITVSELTQQMFDANNMMAACDPRHGRYLTVAAMFRGRMSMREVDDQMMSVQNKNSSYFVEWIPNNVKTAVCDIPPRG 360
C. onc iso II RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLSAKGAQAYRAITVSELTQQMFDAKNMMAACDPRHGRYLTVAAMFRGRMSMREVDDQMMSVQNKNSSYFVEWIPNNVKTAVCDIPPRG 360
C. cat iso II RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLSAKGSQAYRASTVAELTQQMFDAKNMMAACDPRHGRYLTVAAMFRGRMSMREVDDQMMSVQNKNSSYFVEWIPNNVKTSVCDIPPRG 360
T. col iso II RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLSAKGAQAYRAITVSELTQQMFDAKNMMAACDPRHGRYLTVAAMFRGRMSMREVDDQMMSVQNKNSSYFVEWIPNNVKTAVCDIPPRG 360
T. circ iso I LKMAATFVGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADDMGDLDAEGAEEPYPEE 448
H. con iso I LKMAATFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLISEYQQYQEATADDMGDLDAEGGEEAYPEE 448
C. onc iso I LKMAATFVGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLISEYQQYQEATADDMGDLDAEGAEEPYPEE 448
C. cat iso I LKMAATFVGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLISEYQQYQEATADDMGDLDAEGAEEAYPEE 448
H. con iso II LKMAATFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGQGMDEMEFTEAESNMNDLVSEYQQYQEATADDEGEMEGAVENDTYAEE 448
C. onc iso II LKMAATFVGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADDDGEVEGTVENDTYAEE 448
C. cat iso II LKMAATFVGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADDEGDLDGTVENETYQDQEE 450
T. col iso II LKMAATFVGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVSEYQQYQEATADDEGEMEGAVENDTYAEE 448

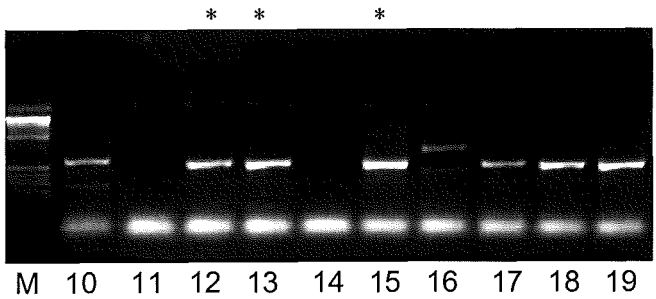
Table 4.4: Degenerate primers used in the amplification of isotype II-specific β-tubulin products. The underlined amino acid is the one upon which the sequence was designed to discriminate for isotype II β-tubulin. The primer names are arbitrary, except 'for' means forward (direction) and 'rev' means reverse. Tann refers to the annealing temperature.

Name	Sequence	To match protein (sense):	Tann
A for	5′- gay wmn gtn mgn wsn ggn ccn tt - 3′	DSVRSGPF	63.3 °C
B for	5′- ytn tty mgn ccn gay aay tt - 3′	LFRPDNF	53.2 °C
C for	5′- gar ggn gcn gar ytn gtn gay ws - 3′	EGAELVDS	64.2 °C
1 rev	5′- ggn can can swr aan swn sw - 3′	SSFSVVP	57.3 °C
2 rev	5′- aac aty tgy tgn gtn ary tcn sw - 3′	SELTQQMF	58.0 °C
3 rev	5′- tgr tay tgy tgr tay tcn swn ac - 3′	VSEYQQYQ	58.0 °C

Figure 4.8: The clones derived from amplification of isotype II β-tubulin fragments from *MTci2* cDNA were re-amplified using the same primers (A forward, B forward, C forward and 2 reverse) in order to determine which clones were genuine for sequencing. The results of these reactions are shown below. This table describes the products and reactions in each well. On the gel photographs, M denotes the molecular marker (1Kb ladder, Roche) and an asterisk (*) highlights the clones, which were selected for sequencing. More B2 clones were sequenced as these products showed the greatest cloning success.

Lanes	Original PCR from *MTci2* cDNA	Cloned products from original PCR	PCR check upon clones	Clones selected for sequencing*
1 – 3	A for ⇔ 2 rev	A2 clones: 1, 2 & 3	A for ⇔ 2 rev	A2 clones: 1, 2 & 3
4 – 5	-	Negative controls	A for ⇔ 2 rev	-
6 – 10	C for ⇔ 2 rev	C2 clones: 1, 2, 3 & 4	C for ⇔ 2 rev	C2 clones: 2, 3 & 4
11	-	Negative control	C for ⇔ 2 rev	-
12 – 26	B for ⇔ 2 rev	B2 clones: 1 to 15	B for ⇔ 2 rev	B2 clones:1, 2, 4, 9, 10 & 11
27	-	Negative control	B for ⇔ 2 rev	-





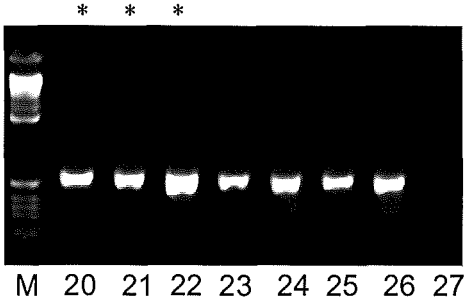
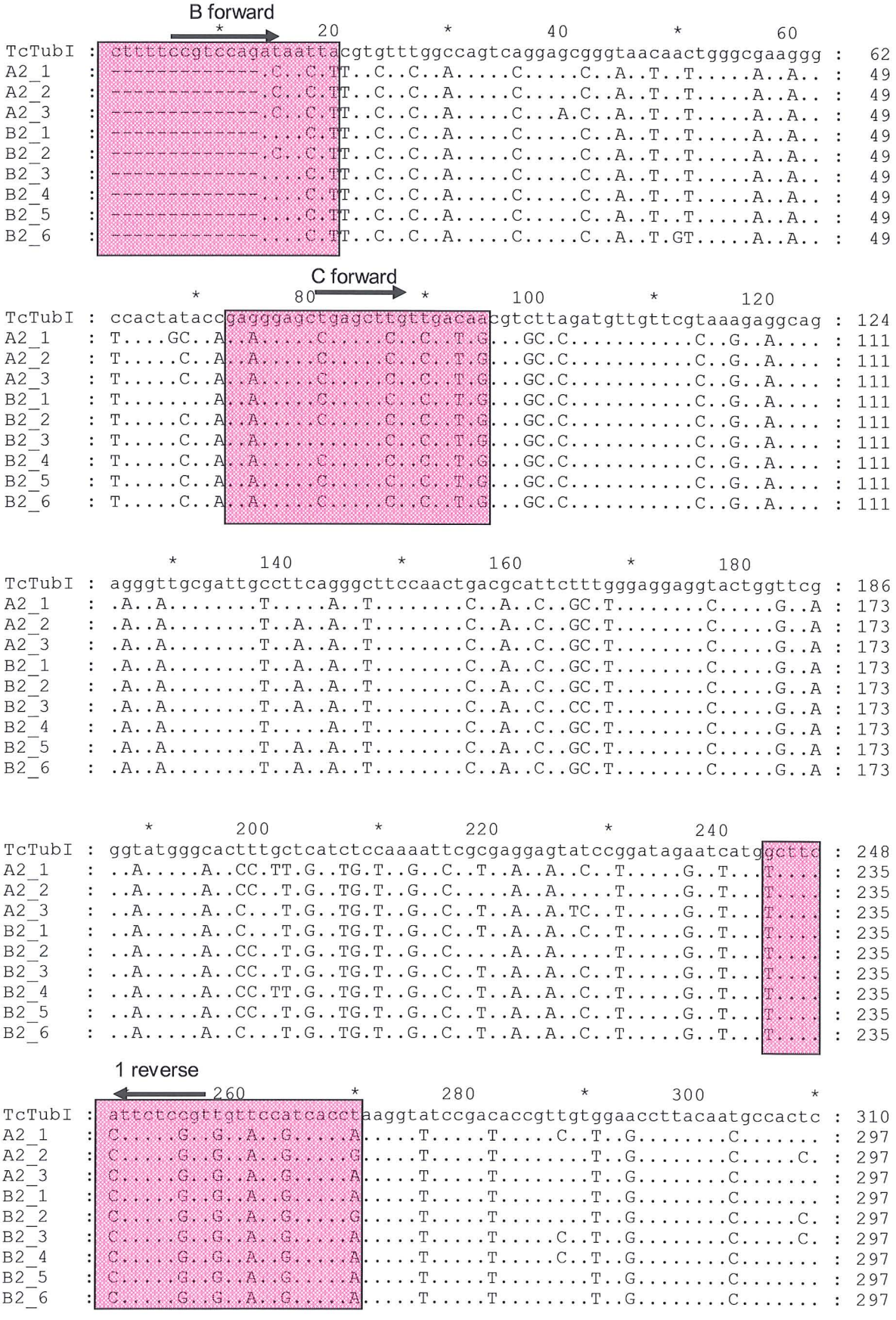


Figure 4.9: Sequence data obtained from consensus clones of suspected isotype II β-tubulin PCR products (592bp). A published *T. circumcincta* isotype I β-tubulin sequence (GenBank accession number Z96258) is included in the alignment (TcTubI) for comparison. Only three of the primers are shown here (in pink) as the sequence data did not extend to the sites of A forward and 2 reverse. The clone names refer to the primers used in their amplification.



```
                320         *       340         *       360         *
TcTubI : tttctgtacaccaattggtagaaaacaccgatgaaacattctgcatcgataatgaagctctg : 372
A2_1   : ....G..T......C....T..G.....G..C.............T..C..C.....G..C : 359
A2_2   : .C..G..T......C.T..T..G.....T..C.............T..C..C.....G..C : 359
A2_3   : C...G..T......C....T..G.....G..C.............T..C..C.....G..C : 359
B2_1   : .C..G..T......C....T..G.....G..C.............T..C..C.....G..C : 359
B2_2   : .C..G..T......C.T..T..G.....T..C.............T..C..C.....G..C : 359
B2_3   : .C..G..T......C....T..G.....G..C.............T..C..C.....G..T : 359
B2_4   : ....G..T......C....T..G.....G..C.............T..C..C.....G..C : 359
B2_5   : ....G..T......C....T..G.....G..C.............T..C..C.....G..C : 359
B2_6   : ....G..T......C....T..G.....G..C.............T..C..C.....G..C : 359

              380         *       400         *       420         *
TcTubI : tacgatatctgcttccgcaccttgaaactcacaaatccaacttatggcgatctcaatcactt : 434
A2_1   : .....C..T........A..GC.C..G.....T..C..T........A..C..T..C..TC. : 421
A2_2   : .....C..T........A..GC.C..G..T..T..C..T........A..C..T..C..TC. : 421
A2_3   : .....C..T........A..GC.C..G..T..T..C..T........A..C..T..C..TC. : 421
B2_1   : .....C..T........A..GC.C..G..T..T..C..T........A..C..T..C..TC. : 421
B2_2   : .....C..T........A..GC.C..G..T..T..C..T........A..C..T..C..TC. : 421
B2_3   : .....C..T........A..GC.C..G.....T..C..T........A..C..T..C..TC. : 421
B2_4   : .....C..T........A..GC.C..G.....T..C..T........A..C..T..C..TC. : 421
B2_5   : .....C..T........A..GC.C..G..T..T..C..T........A..C..T..C..TC. : 421
B2_6   : .....C..T........A..GC.C..G..T..T..C..T........A..C..T..C..TC. : 421

             440         *       460         *       480         *
TcTubI : agtgtctgtcacaatgtctggagtcacgacctgccttcgattccctggacagctgaatgctg : 496
A2_1   : T..A..C..A..............A..A..G.....G........C.AC..A..A....... : 483
A2_2   : T..A..C..A..............A..A..G.....G........C..C.....A....... : 483
A2_3   : T..A..C..A..............A..A..G.....G........C..C..A..A....... : 483
B2_1   : T..A..C..A..............A..A..G.....G........C..C..A..A....... : 483
B2_2   : T..A..C..A..............A..A..G.....G........C..C.....A....... : 483
B2_3   : T..A..C..A..............A..A..G.....G........C..C..A..A....... : 483
B2_4   : T..A..C..A..............A..A..G.....G........C..C..A..A....... : 483
B2_5   : T..A..C..A..............A..A..G.....G........C..C..A..A....... : 483
B2_6   : T..A..C..A..............A..A..G.....G........C..C..A..A....... : 483

           500         *       520         *       540         *       5
TcTubI : atcttcgcaagttagccgtgaacatggttccattccctcgtcttcacttcttcatgccaggt : 558
A2_1   : .......T..AC.G.....C........A..C........S..G..T...........T..A : 545
A2_2   : .......T..AC.G.....C........A..C........C..G..T...........T..A : 545
A2_3   : .......T..AC.G.....C........A..C........C..G..T...........T..A : 545
B2_1   : .......T..AC.G.....C........A..C........C..G..T...........T..A : 545
B2_2   : .......T..AC.G.....C........AT.C........C..G..T...........T..A : 545
B2_3   : .......T..AC.G.....C........A..C.....C..CT.G..T...........T..A : 545
B2_4   : .......T..AC.G.....C........A..C........C..G..T...........T..A : 545
B2_5   : .......T..AC.G.....C........A..C........C..G..T...........T..A : 545
B2_6   : .......T..AC.G.....C........A..C........C..G..T...........T..A : 545

          60         *       580         *       600
TcTubI : ttcgccccactgtctgctaagggtgctcaagcgtatcgCGCTTCAAC : 605
A2_1   : ..T..G........A..G.....A........A..C..T..ACTT.. : 592
A2_2   : ..T..G........A..G.....A........A..C..T..ACTT.. : 592
A2_3   : ..T..G........A..G.....A........A..C..T..ACTT.. : 592
B2_1   : ..T..G........A..G.....A........A..C..T..ACTT.. : 592
B2_2   : ..T..G........A..G.....A........A..C..T..ACTT.. : 592
B2_3   : ..T..G.....A..A..G.....A........A..C..T..ACTT.. : 592
B2_4   : ..T..G........A..G.....A........A..C..T..ACTT.. : 592
B2_5   : ..T..G........A..G.....A........A..C..T..ACTT.. : 592
B2_6   : ..T..G........A..G.....A........A..C..T..ACTT.. : 592
```

Figure 4.10: Polypeptide sequence alignment of suspected β-tubulin isotype II clones (each is a consensus of three sequences) with published *T. circumcincta* β-tubulin isotype I sequence (TcTubI). The clone names refer to the primers used in their amplification. The clone sequences start at residue 89 and end at 284. The gaps at the start represent unreliable sequence data. Note the consistent differences between the isotype II β-tubulin clones and isotype I β-tubulin sequence at residues 90, 115, 153, 165 and 284. Only three of the primers are shown here (in pink) as the sequence data did not extend to the sites of A forward and 2 reverse.

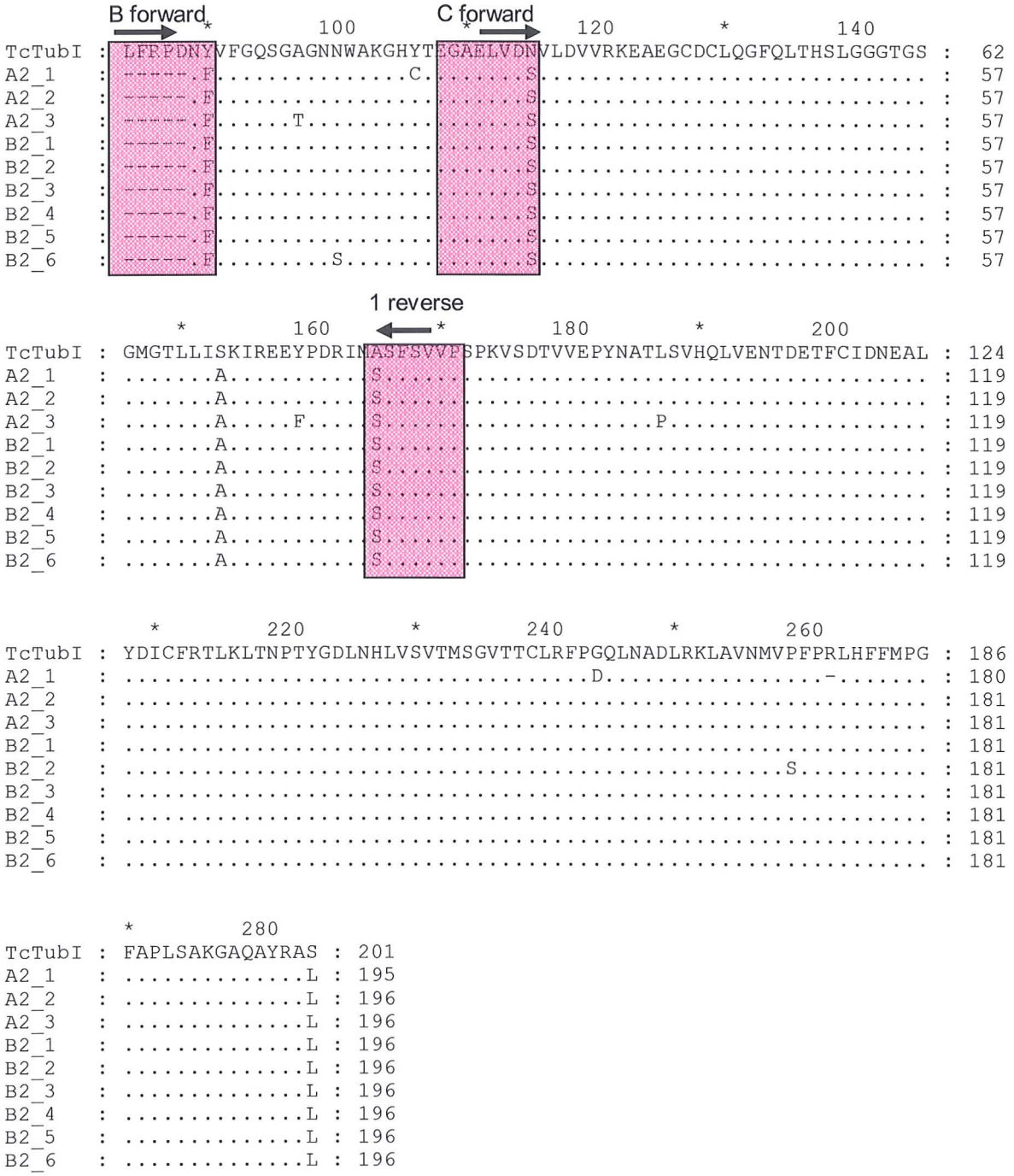


Figure 4.11: Schematic diagram showing the primer sites used for the amplification of isotype II β-tubulin products from individual *MTci5* adults. Primers B, C and 1 target sites used previously (see Figure 4.10), but primer 4 is new. All primers are 3' specific for isotype II β-tubulin as shown by the underlined residue below. The information in brackets e.g. Y90F indicates the amino acid position and difference between isotype I and II β-tubulin genes, upon which the primers are designed to discriminate.

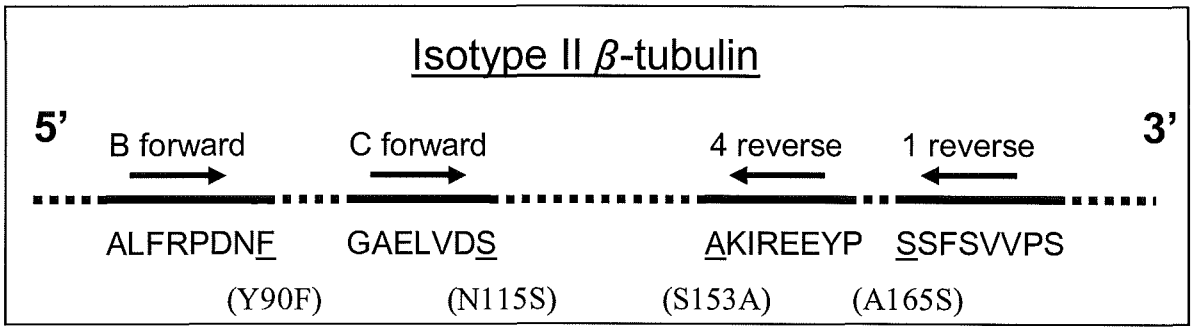
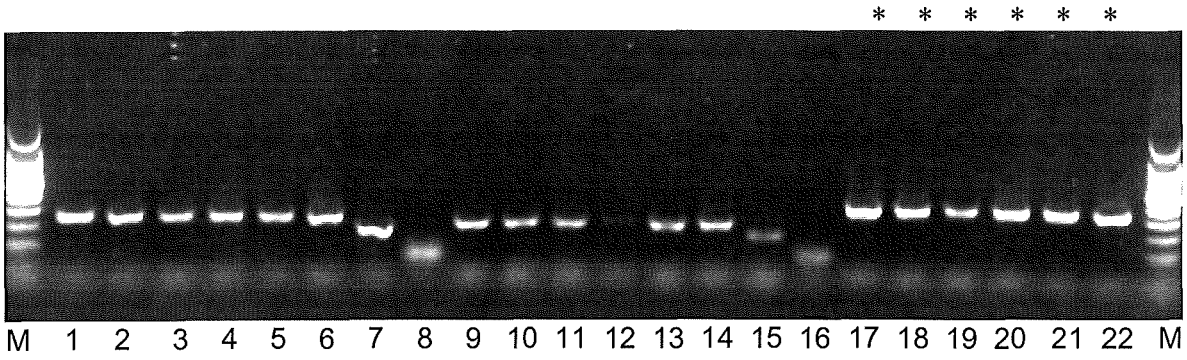


Table 4.5: Information about the isotype II (β-tubulin) specific primers used to determine the presence of this gene in *MTci5* adults. These were based upon the sequence information obtained from *MTci2* cDNA (see Figure 4.9). The underlined amino acid is the one upon which the sequence is designed to discriminate for isotype II products. The primer names refer to a more accurate version of those displayed in Table 4.4, except 'for' means forward and 'rev' means reverse. Tann refers to the annealing temperature.

Name	Sequence	To match protein (sense):	Tann
B for	5' - gcw ctr tty cgt cca gac aac ttt - 3'	ALFRPDNF	68.0 °C
C for	5' - agg agc cga gct cgt cga tag - 3'	GAELVDS	63.7 °C
1 rev	5' - ggt gac ggt acc acc gag aag gaa ga - 3'	SSFSVVPS	68.0 °C
4 rev	5' - tca ggr tat tct tcr cgg atc tta gc - 3'	AKIREEYP	63.2 °C

Figure 4.12: Gel images showing the results of the β-tubulin isotype II-specific PCR using four primer combinations. Six unselected *MTci5* adults (PT01 to PT06) were used as template and the *MTci2* cDNA was included as a positive control. On the gel photographs, M denotes the molecular marker (1Kb ladder, Roche) and those lanes marked with an asterisk* represent products which were sequenced to confirm identity (i.e. all B⇔1 products).

Lanes	Reaction	Template
1 – 6	C ⇔ 1	*MTci5* adults PT01 to PT06
7		*MTci2* cDNA (positive control)
8		Water (negative control)
9 – 14	C ⇔ 4	*MTci5* adults PT01 to PT06
15		*MTci2* cDNA (positive control)
16		Water (negative control)
17 – 22	B ⇔ 1	*MTci5* adults PT01 to PT06
23		*MTci2* cDNA (positive control)
24		Water (negative control)
25 – 30	B ⇔ 4	*MTci5* adults PT01 to PT06
31		*MTci2* cDNA (positive control)
32		Water (negative control)



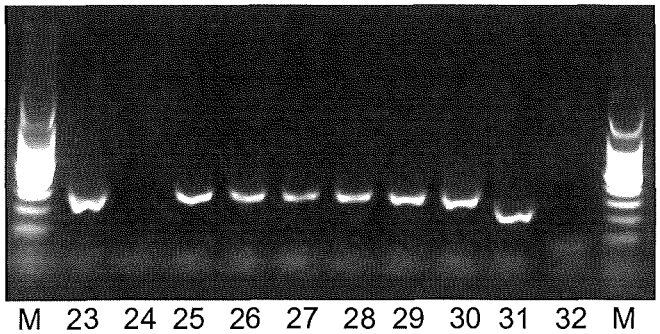


Figure 4.13: Sequence alignment of *MTci5* adult isotype II *β*-tubulin sequences alongside published cDNA sequence for *T. circumcincta* isotype I (TcTubI) and *MTci2* cDNA clones (A2, B2 etc). The consensus *MTci5* isotype I (*MTci5* isoI) sequence (based on 134 sequences) is also included for comparison. Bases that distinguish isotype II from isotype I are highlighted. The introns of the genomic sequence have been removed from this alignment. The length of the genomic sequences is shorter than the cDNA clones, at around 258bp.

```
                  *        20         *        40         *        6
TcTubI     : cttttccgtccagataattacgtgtttggccagtcaggagcgggtaacaactgggcgaa :  59
Tci5_isoI  : ............................................................ :  59
A2_1       : ----------.C..C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
A2_2       : ----------.C..C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
A2_3       : ----------.C..C.TT..C..C..A.....C...A.C..A..T..T.....A.. :  46
B2_1       : ----------....C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
B2_2       : ----------.C..C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
B2_3       : ----------....C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
B2_4       : ----------....C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
B2_5       : ----------....C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
B2_6       : ----------....C.TT..C..C..A.....C.....C..A..T.GT.....A.. :  46
PT01_II    : ----------.C..c.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
PT02_II    : ----------.C..C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
PT03_II    : ----------.C..C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
PT04_II    : ----------.C..c.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
PT05_II    : ----------.C..C.TT..C..C..A.....C.....C..A..T..T.....A.. :  46
PT06_II    : ----------.C..c.TT..C..C..A.....C.....C..A..T..T.....A.. :  46

            0         *        80         *       100         *       1
TcTubI     : gggccactataccgagggagctgagcttgttgacaacgtcttagatgttgttcgtaaag : 118
Tci5_isoI  : ............................................................ : 118
A2_1       : A..T....GC..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
A2_2       : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
A2_3       : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
B2_1       : A..T........A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
B2_2       : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
B2_3       : A..T.....C..A..A..........C..C..T.G...GC.C..........C..G. : 105
B2_4       : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
B2_5       : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
B2_6       : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
PT01_II    : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
PT02_II    : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
PT03_II    : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
PT04_II    : A..T.....C..A..A.....c.....C..C..T.G...GC.C..........C..G. : 105
PT05_II    : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105
PT06_II    : A..T.....C..A..A.....C.....C..C..T.G...GC.C..........C..G. : 105

            20        *       140        *       160        *
TcTubI     : aggcagagggttgcgattgccttcagggcttccaactgacgcattctttgggaggaggt : 177
Tci5_isoI  : ............................................................ : 177
A2_1       : .A.....A..A........T.....A..T........C..A..C..GC.T........C : 164
A2_2       : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
A2_3       : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
B2_1       : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
B2_2       : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
B2_3       : .A.....A..A........T..A..A..T........C..A..C..CC.T........C : 164
B2_4       : .A.....A..A........T.....A..T........C..A..C..GC.T........C : 164
B2_5       : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
B2_6       : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
PT01_II    : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
PT02_II    : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
PT03_II    : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
PT04_II    : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
PT05_II    : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
PT06_II    : .A.....A..A........T..A..A..T........C..A..C..GC.T........C : 164
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```
            180         *       200         *       220         *
TcTubI     : actggttcgggtatgggcactttgctcatctccaaaattcgcgaggagtatccggatag : 236
Tci5_isoI  : ........................T.............................. : 236
A2_1       : .....G..A..A.....A..CC.TT.G..TG.T..G..C..T..A..A..C..T..... : 223
A2_2       : .....G..A..A.....A..CC..T.G..TG.T..G..C.....A..A.....T..... : 223
A2_3       : .....G..A..A.....A..C...T.G..TG.T..G..C..T..A..A.TC..T..... : 223
B2_1       : .....G..A..A.....A..C...T.G..TG.T..G..C..T..A..A..C..T..... : 223
B2_2       : .....G..A..A.....A..CC..T.G..TG.T..G..C.....A..A.....T..... : 223
B2_3       : .....G..A..A.....A..CC..T.G..TG.T..G..C..T..A..A..C..T..... : 223
B2_4       : .....G..A..A.....A..CC.TT.G..TG.T..G..C..T..A..A..C..T..... : 223
B2_5       : .....G..A..A.....A..CC..T.G..TG.T..G..C..T..A..A..C..T..... : 223
B2_6       : .....G..A..A.....A..C...T.G..TG.T..G..C..T..A..A..C..T..... : 223
PT01_II    : .....G..A..A.....A..C...T.G..TG.T..G..C..T..A..A..C..T..... : 223
PT02_II    : .....G..A..A.....A..C...T.G..TG.T..G..C..T..A..A..C..T..... : 223
PT03_II    : .....G..A..A.....A..CC.TT.G..TG.T..G..C..T..A..A..C..T..... : 223
PT04_II    : .....G..A..A.....A..CC..T.G..TG.T..G..C..T..A..A..C..T..... : 223
PT05_II    : .....G..A..A.....A..CC..T.G..TG.T..G..C..T..A..A..C..T..... : 223
PT06_II    : .....G..A..A.....A..CC..T.G..TG.T..G..C..T..A..A..C..T..... : 223

             240         *       260         *       280         *
TcTubI     : aatcatggcttcattctccgttgttccatcacctaaggtatccgacaccgttgtggaac : 295
Tci5_isoI  : ............................R..W...........Y............. : 295
A2_1       : G..T...T....C.....G..G..A..G.....A.....T.....T.....C..T..G. : 282
A2_2       : G..T...T....C.....G..G..A..G.....G.....T.....T........T..G. : 282
A2_3       : G..T...T....C.....G..G..A..G.....A.....T.....T........T..G. : 282
B2_1       : G..T...T....C.....G..G..A..G.....A.....T.....T........T..G. : 282
B2_2       : G..T...T....C.....G..G..A..G.....G.....T.....T........T..G. : 282
B2_3       : G..T...T....C.....G..G..A..G.....A.....T.....T.....C..T..G. : 282
B2_4       : G..T...T....C.....G..G..A..G.....A.....T.....T.....C..T..G. : 282
B2_5       : G..T...T....C.....G..G..A..G.....A.....T.....T........T..G. : 282
B2_6       : G..T...T....C.....G..G..A..G.....A.....T.....T........T..G. : 282
PT01_II    : G..T...T....C.....G..G..A..G.....A.----------------------- : 258
PT02_II    : G..T...T....C.....G..G..A..G.....A.----------------------- : 258
PT03_II    : G..T...T....C.....G..G..A..G.....A.----------------------- : 258
PT04_II    : G..T...T....C.....G..G..A..G.....A.----------------------- : 258
PT05_II    : G..T...T....C.....G..G..A..G.....A.----------------------- : 258
PT06_II    : G..T...T....C.....G..G..A..G.....A.----------------------- : 258

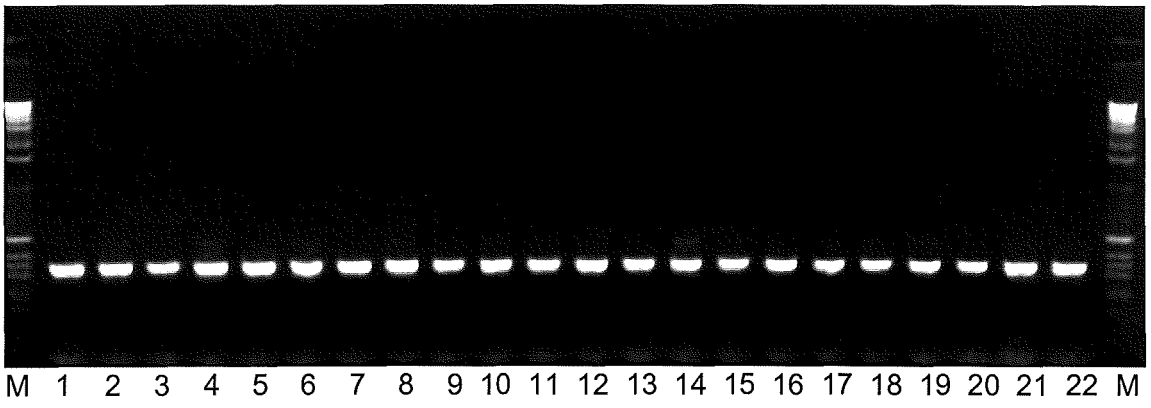
             300
TcTubI     : cttacaatgc : 305
Tci5_isoI  : .......... : 305
A2_1       : .......C.. : 292
A2_2       : .......C.. : 292
A2_3       : .......C.. : 292
B2_1       : .......C.. : 292
B2_2       : .......C.. : 292
B2_3       : .......C.. : 292
B2_4       : .......C.. : 292
B2_5       : .......C.. : 292
B2_6       : .......C.. : 292
PT01_II    : ---------- :   -
PT02_II    : ---------- :   -
PT03_II    : ---------- :   -
PT04_II    : ---------- :   -
PT05_II    : ---------- :   -
PT06_II    : ---------- :   -
```

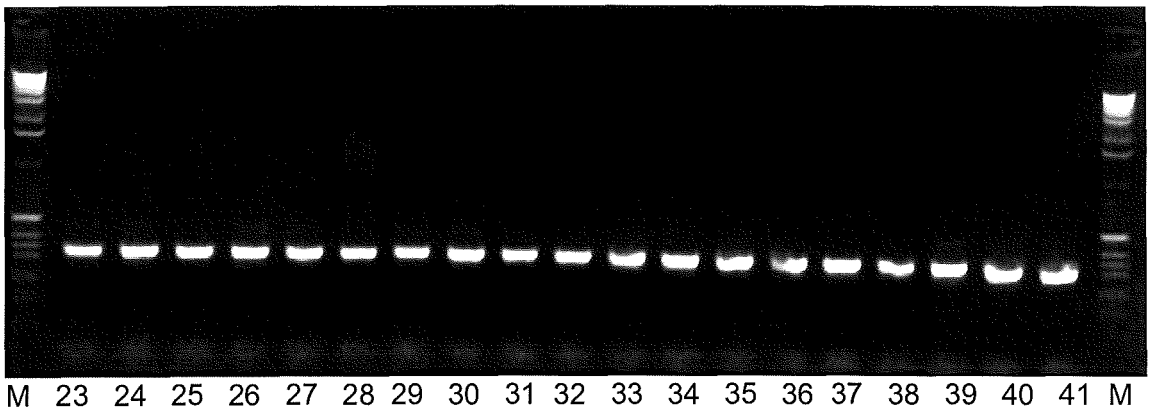
Figure 4.14: Polypeptide sequence alignment of adult *MTci5* isotype II β-tubulin sequences alongside published cDNA sequence for *T. circumcincta* isotype I ('TcTubI') and *MTci2* cDNA clones ('A2', 'B2' etc). The consensus *MTci5* isotype I β-tubulin sequence ('*MTci5* isoI', based on 134 sequences) is also included for comparison. Residues that distinguish isotype II from isotype I β-tubulin are highlighted. The length of the sequence is shorter than achieved for the cDNA clones, only showing residues 89 to 173. However, residues 90, 115, 153 and 165 can still be used to identify the genomic sequences as isotype II β-tubulin gene fragments.

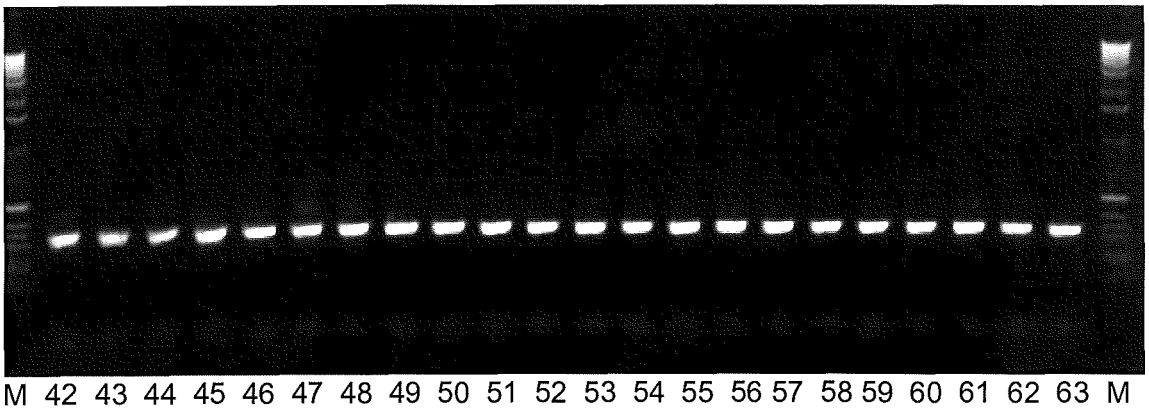
```
                 *        100        *        120        *        140
TcTubI     : LFRPDNYVFGQSGAGNNWAKGHYTEGAELVDNVLDVVRKEAEGCDCLQGFQLTHSLGGG :  59
Tci5_isoI  : ............................................................ :  59
A2_1       : -----.F..............C........S............................ :  54
A2_2       : -----.F.........................S............................ :  54
A2_3       : -----.F......T..................S............................ :  54
B2_1       : -----.F.........................S............................ :  54
B2_2       : -----.F.........................S............................ :  54
B2_3       : -----.F.........................S............................ :  54
B2_4       : -----.F.........................S............................ :  54
B2_5       : -----.F.........................S............................ :  54
B2_6       : -----.F.........S...............S............................ :  54
PT01_II    : -----.F.........................S............................ :  54
PT02_II    : -----.F.........................S............................ :  54
PT03_II    : -----.F.........................S............................ :  54
PT04_II    : -----.F.........................S............................ :  54
PT05_II    : -----.F.........................S............................ :  53
PT06_II    : -----.F.........................S............................ :  54

                  *        160        *        180
TcTubI     : TGSGMGTLLISKIREEYPDRIMASFSVVPSPKVSDTVVEPYN : 101
Tci5_isoI  : ........................................... : 101
A2_1       : ..........A...........S.................... :  96
A2_2       : ..........A...........S.................... :  96
A2_3       : ..........A.....F.....S.................... :  96
B2_1       : ..........A...........S.................... :  96
B2_2       : ..........A...........S.................... :  96
B2_3       : ..........A...........S.................... :  96
B2_4       : ..........A...........S.................... :  96
B2_5       : ..........A...........S.................... :  96
B2_6       : ..........A...........S.................... :  96
PT01_II    : ..........A...........S........----------- :  85
PT02_II    : ..........A...........S........----------- :  85
PT03_II    : ..........A...........S........----------- :  85
PT04_II    : ..........A...........S........----------- :  85
PT05_II    : ..........A...........S........----------- :  84
PT06_II    : ..........A...........S........----------- :  84
```

Figure 4.15a: Gel images showing the results of the diagnostic PCR used to amplify isotype II β-tubulin from 72 unselected adults from the *MTci5* isolate (lanes 1 to 72). *MTci2* cDNA isotype II β-tubulin was included as a positive control (lane 73), and *MTci1* isotype I β-tubulin cDNA was included as a negative control (lane 74), along with two water-only PCR negative controls (lanes 75 and 76). M denotes the molecular marker (1 Kb ladder, Roche).







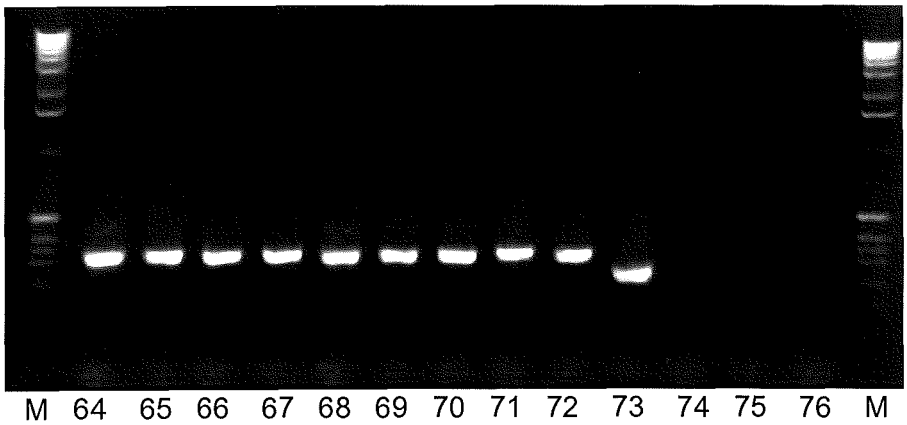
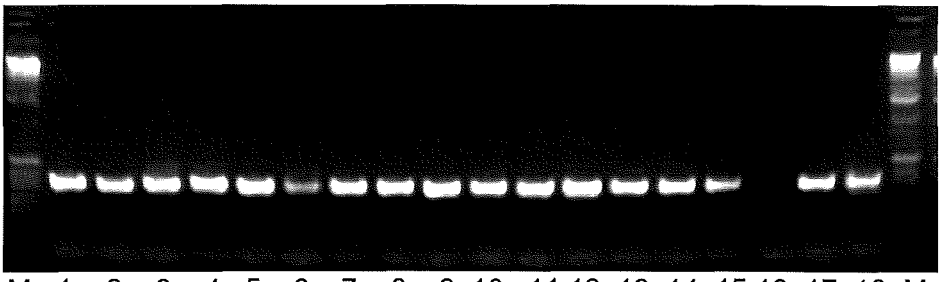
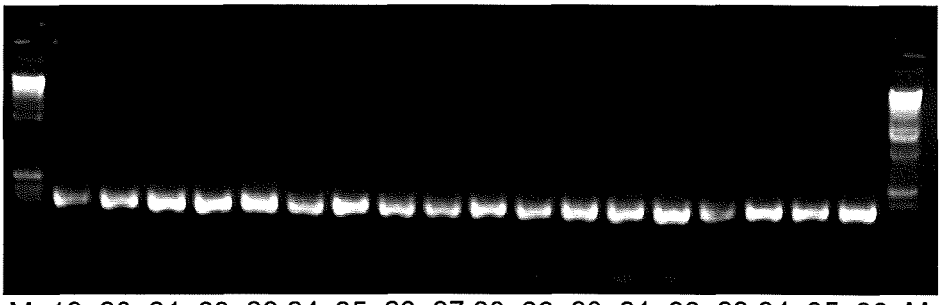


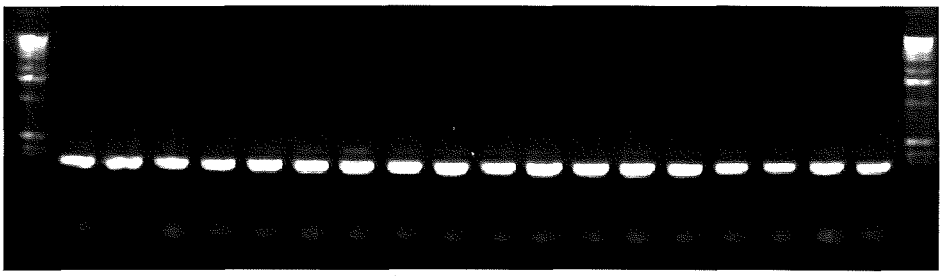
Figure 4.15b: Gel images showing the results of the diagnostic PCR used to amplify isotype II β-tubulin from 72 BZ selected adults from the *MTci5* isolate (lanes 1 to 72). Note that isotype II β-tubulin did not amplify successfully from worm PB16 (lane 16) on first attempt, but was repeated with success (lane 73). *MTci2* cDNA isotype II β-tubulin was included as a positive control (lane 74), and *MTci1* isotype I β-tubulin cDNA was included as a negative control (lane 75), along with two water-only PCR negative controls (lanes 76 & 77). M denotes the molecular marker (1 Kb ladder, Roche).



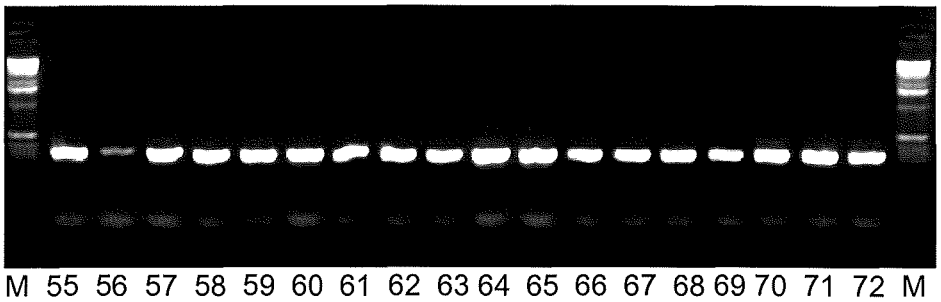
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M



M 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 M



M 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 M



M 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 M

M 73 74 75 76 77 M

Figure 4.16: Toxicity test results of verapamil hydrochloride (VPL) and piperonyl butoxide (PB) upon egg development of *MTci5* isolate. Egg hatch values have been corrected to the DMSO control to exclude the fraction of eggs that fail to hatch for other reasons.

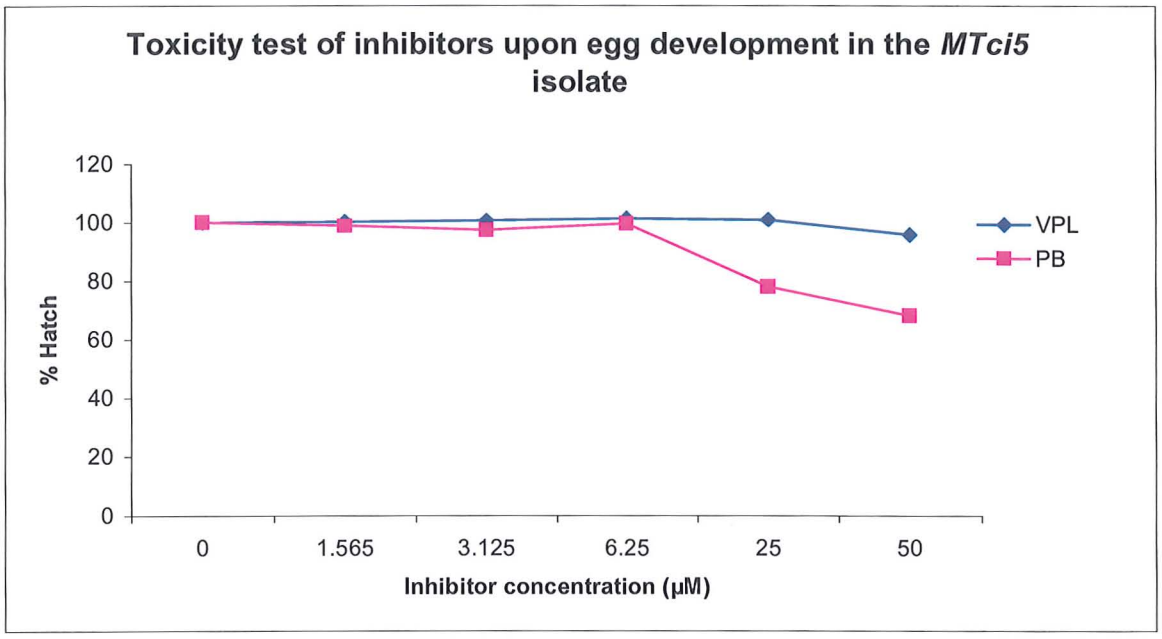


Figure 4.17: Graph showing the effect of verapamil hydrochloride and piperonyl butoxide inhibitors upon egg development in the *MTci5* isolate at different thiabendazole concentrations.

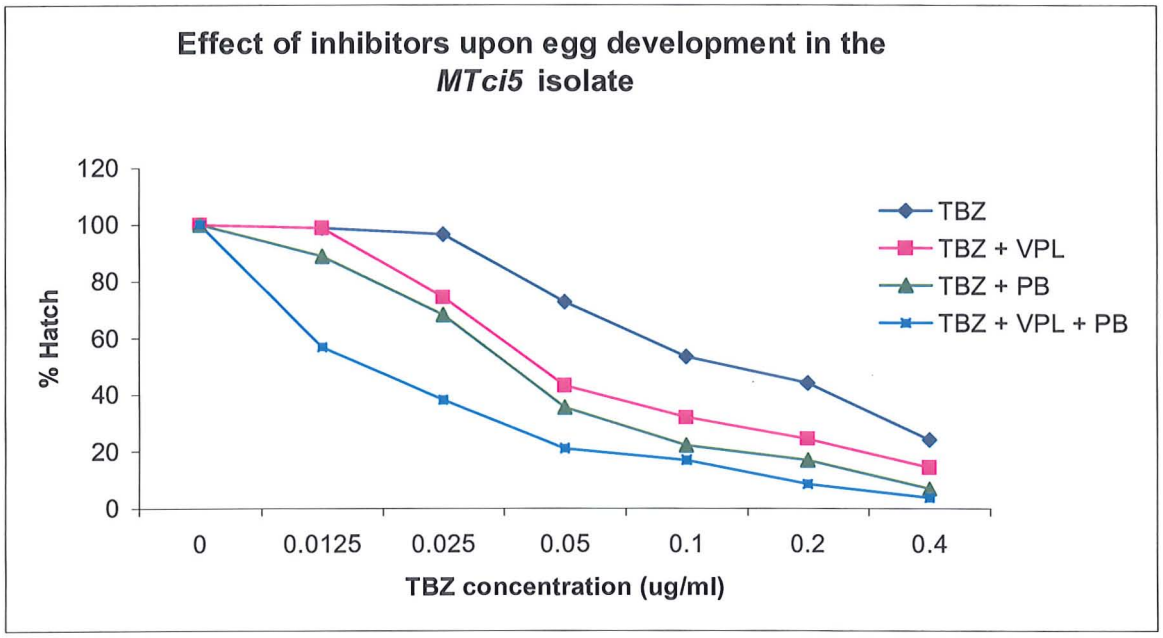


Figure 4.18: Graph showing the effect of verapamil hydrochloride and piperonyl butoxide inhibitors upon egg development values (ED50, 96 & 99) in the *MTci5* isolate. Actual ED values are labelled on the graph.

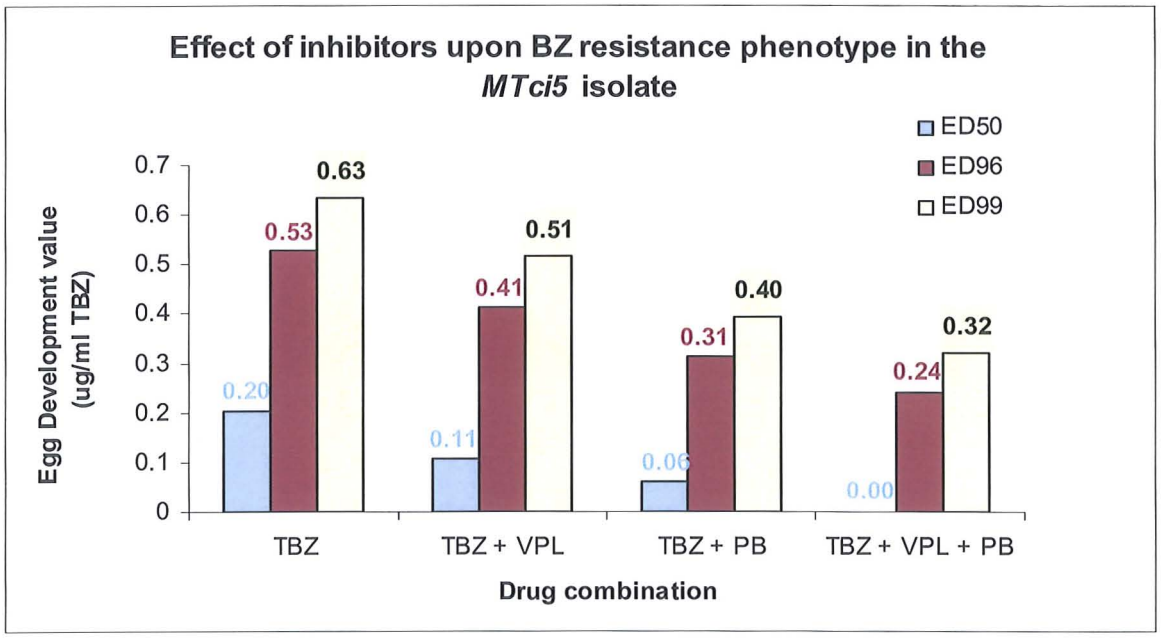


Figure 4.19: Graph showing the effect of verapamil hydrochloride and piperonyl butoxide inhibitors upon egg development in the *MTci2* isolate.

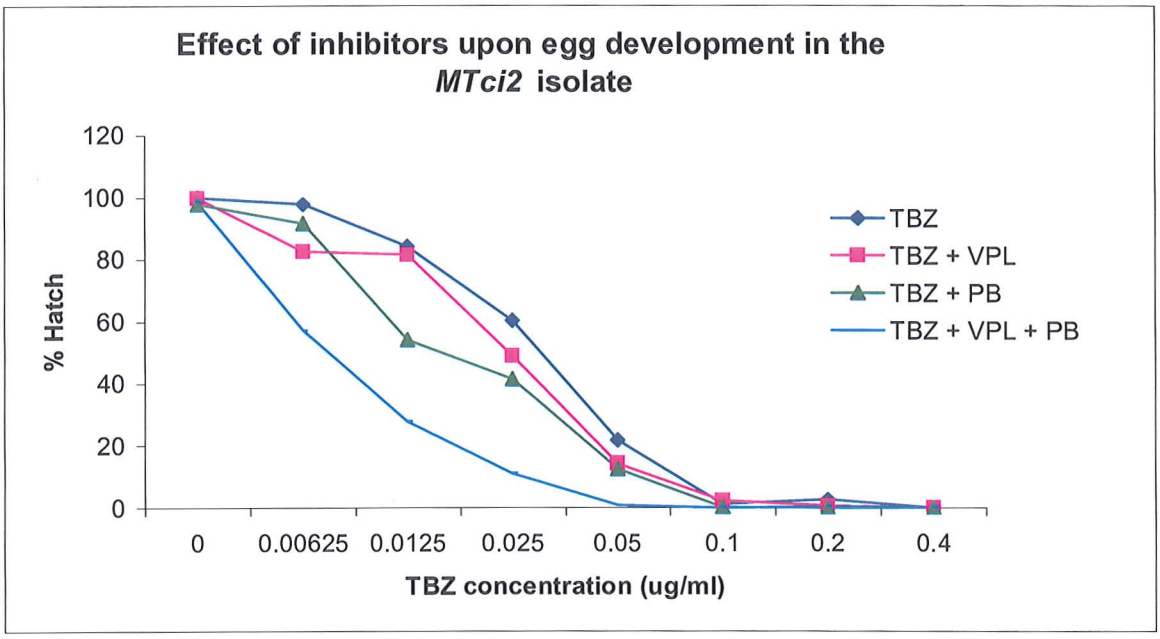


Figure 4.20: Graph showing the effect of verapamil hydrochloride and piperonyl butoxide inhibitors upon egg development values ($ED_{50, 96 \& 99}$) in the *MTci2* isolate. Actual ED values are labelled on the graph.

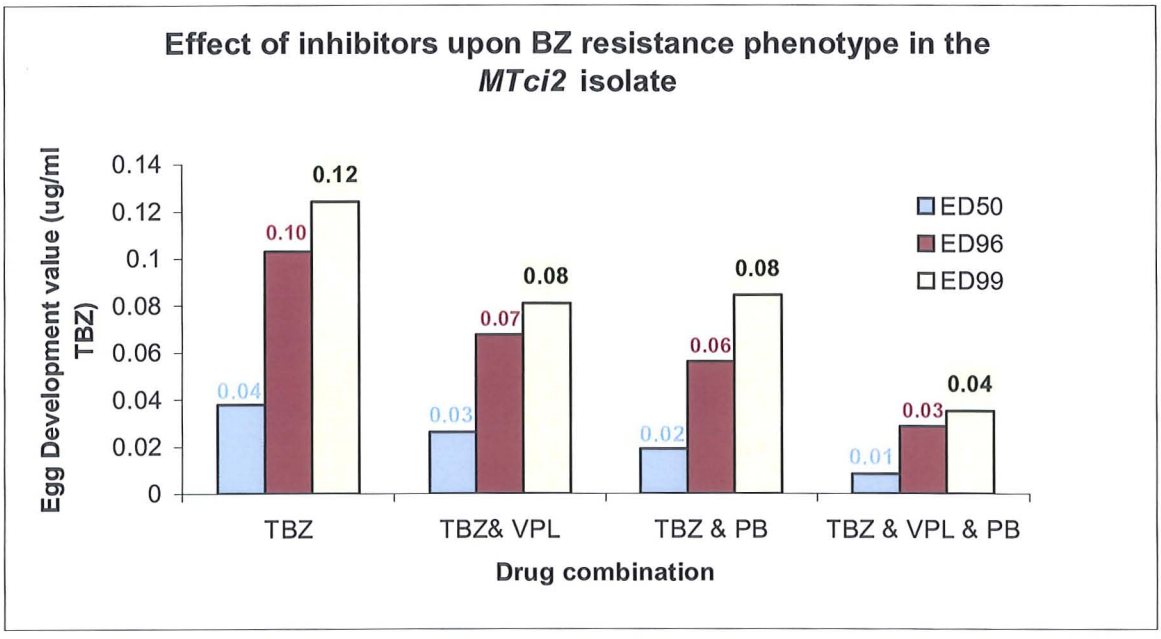


Table 4.6: Susceptibility factors induced by inhibitors in the *MTci5* and *MTci2* isolates. Susceptibility factors were obtained by dividing ED values of the test group by that of TBZ alone value.

		MTci5				*MTci2*			
		TBZ	**TBZ + VPL**	**TBZ + PB**	**TBZ + VPL + PB**	**TBZ**	**TBZ + VPL**	**TBZ + PB**	**TBZ + VPL + PB**
ED values (µg/ml)	**ED_{50}**	0.203	0.106	0.062	0.001	0.038	0.026	0.019	0.009
	ED_{96}	0.528	0.414	0.313	0.241	0.103	0.068	0.056	0.029
	ED_{99}	0.634	0.515	0.395	0.320	0.124	0.081	0.084	0.035
Susceptibility factors	**ED_{50}**		1.91	3.26	172.85		1.45	1.98	4.44
	ED_{96}		1.28	1.69	2.19		1.53	1.84	3.61
	ED_{99}		1.23	1.60	1.99		1.53	1.47	3.54

Table 4.7: Input and output of one-way ANOVA performed on the egg hatch assay data for the effect of inhibitors upon BZ resistance in the *MTci5* isolate, (Minitab v 13). F statistic and p value refer to the result of the statistical comparison between the TBZ alone sample and each of the other test samples. Significant p values (with 95% or 99% confidence) are shown in bold.

Treatment	Mean ED_{50} (μg/ml TBZ)		$F_{(1, 3)}$ statistic	P value	Mean ED_{96} (μg/ml TBZ)		$F_{(1, 3)}$ statistic	P value	Mean ED_{99} (μg/ml TBZ)		$F_{(1, 3)}$ statistic	P value
	Test 1	Test 2			Test 1	Test 2			Test 1	Test 2		
TBZ alone	0.203	0.203	-	-	0.521	0.540	-	-	0.625	0.651	-	-
TBZ & VPL	0.096	0.117	84.46	**0.012**	0.395	0.426	43.57	**0.022**	0.493	0.527	35.77	**0.027**
TBZ & PB	0.057	0.069	544.44	**0.002**	0.289	0.340	63.01	**0.016**	0.366	0.428	51.40	**0.019**
TBZ & VPL & PB	-0.015	0.024	103.62	**0.010**	0.216	0.268	108.62	**0.009**	0.292	0.348	106.11	**0.009**

Table 4.8: Input and output of one-way ANOVA performed on the egg hatch assay data for the effect of inhibitors upon BZ resistance in the *MTci2* isolate, (Minitab v 14). F statistic and p value refer to the result of the statistical comparison between the TBZ alone sample and each of the other test samples. Significant p values (with 95% or 99% confidence) are shown in bold.

Treatment	Mean ED_{50} (μg/ml TBZ)		$F_{(1, 3)}$ statistic	P value	Mean ED_{96} (μg/ml TBZ)		$F_{(1, 3)}$ statistic	P value	Mean ED_{99} (μg/ml TBZ)		$F_{(1, 3)}$ statistic	P value
	Test 1	Test 2			Test 1	Test 2			Test 1	Test 2		
TBZ alone	0.040	0.035	-	-	0.086	0.110	-	-	0.122	0.135	-	-
TBZ & VPL	0.033	0.024	3.06	0.223	0.093	0.060	1.11	0.402	0.112	0.087	4.24	0.176
TBZ & PB	0.026	0.016	8.71	0.098	0.062	0.052	9.95	0.088	0.074	0.079	55.75	**0.017**
TBZ & VPL & PB	0.011	0.008	92.24	**0.011**	0.027	0.028	34.46	**0.028**	0.032	0.043	114.22	**0.009**

Figure 4.21: Graph showing the effect of verapamil hydrochloride and piperonyl butoxide inhibitors upon F200Y isotype I β-tubulin genotype in the *MTci5* isolate. This graph displays percentage ratios.

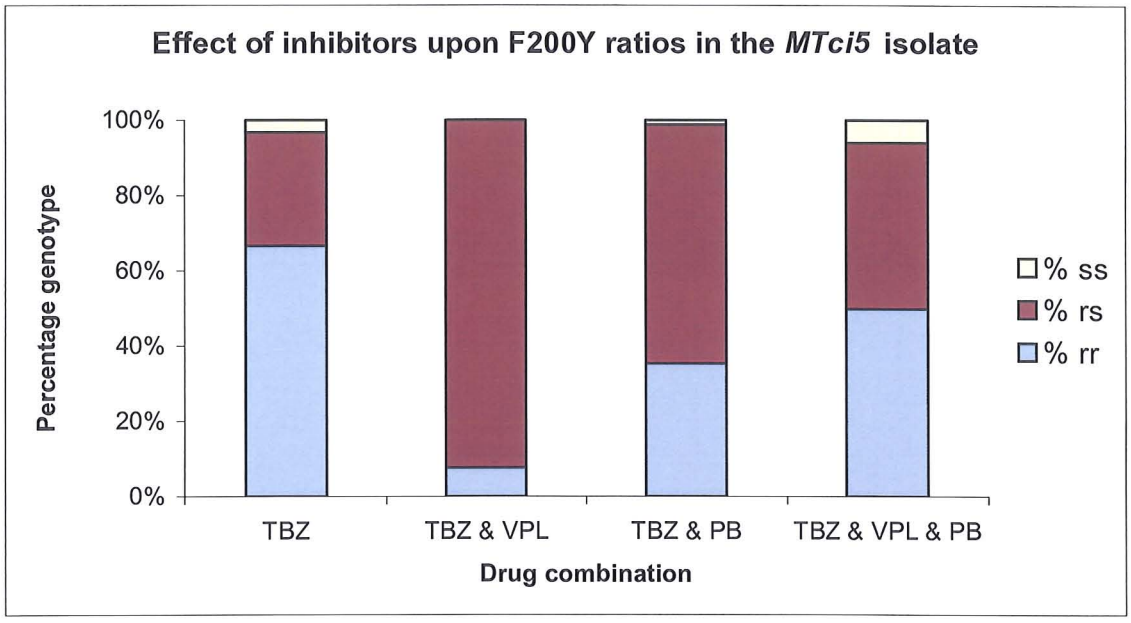


Table 4.9: Table showing the results of the 'estimation of the differences between genotype proportions' test (*MTci5* isolate). The number of $P200^{Tyr/Tyr}$, $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes found in each group have been compared to those of the control group (i.e. TBZ alone) at the 95% and 99% confidence intervals (CI estimates are shown in brackets). All significant values are shown in bold.

Treatment	$P200^{Tyr/Tyr}$ genotypes (CI) p-value	$P200^{Phe/Tyr}$ genotypes (CI) p-value	$P200^{Phe/Phe}$ genotypes (CI) p-value
TBZ & VPL	(0.743, 0.436) **p < 0.01**	(-0.472, -0.774) **p < 0.01**	(0.070, -0.004) p>0.05
TBZ & PB	(0.499, 0.129) **p < 0.01**	(-0.152, -0.519) **p < 0.01**	(0.065, -0.022) p>0.05
TBZ & VPL & PB	(0.322, 0.012) **p < 0.05**	(0.013, -0.292) p>0.05	(0.041, -0.096) p>0.05

4.8 Discussion

4.8.1 Evidence for the existence of multiple BZ resistance mechanisms in parasitic nematodes

The F200Y isotype I β-tubulin mutation is widely accepted as a key mechanism of BZ resistance in many parasite species (reviewed by Wolstenholme *et al.,* 2004). However, there is evidence that additional mechanisms might be involved (Kwa *et al.,* 1993; Beech *et al.,* 1994; Blackhall *et al.,* in press). This is an important issue if molecular markers are to be used for diagnosis and management of anthelmintic resistance.

The survival of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes following *in vivo* and *in vitro* BZ selection of the *MTci5* isolate prompted the work in this Chapter and aimed to investigate the potential role of other resistance mechanisms in the *MTci5* isolate. Given that only one worm survived *in vivo* BZ selection with a $P200^{Phe/Phe}$ genotype and that the egg hatch assay has been highlighted for its inaccuracies, we should first examine the significance of the survival of $P200^{Phe/Tyr}$ genotypes, for which there is far stronger evidence. There are two possibilities that could explain the survival of $P200^{Phe/Tyr}$ genotypes:

1. The BZ resistance trait is not recessive as previously determined (Elard *et al.,* 1998; Elard & Humbert, 1999) and/or
2. There are other BZ resistance mutations/mechanisms involved.

If the $P200^{Phe/Phe}$ survival is a *bona fide* observation, as this Chapter aims to determine, then it would appear that the second of these possibilities is true. However, the first possibility is much more difficult to determine since nematodes do not have isogenic backgrounds. Elard *et al.,* (1998; Elard & Humbert, 1999) observed that $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes always succumbed to BZ treatment at the recommended dose rate in an experimental infection. Hence, the authors concluded that the F200Y isotype I β-tubulin mutation was recessive. However, this finding has not been observed from field situations (in sheep and goats), whereby treatment of all (naturally-infected) members of a flock at the recommended dose rate does not produce 100% $P200^{Tyr/Tyr}$ survivors (Leignel *et al.,* 2002; Silvestre unpublished data, cited in Silvestre *et al.,* 2001). Whether this is due to a stage-specific BZ tolerance of

part of the infrapopulation is still unclear. However, the survival of 19% of $P200^{Phe/Tyr}$ genotypes following *in vivo* BZ selection of the *MTci5* isolate in an experimental infection (see Chapter 3, Section 3.3.2) clearly indicates that there is more than just a stage-specific tolerance to BZ to consider. Several studies have implied that BZ resistance is incompletely recessive. A study by Silvestre *et al.* (2001) revealed that under-dosing to the extent of one quarter of the recommended dose rate resulted in the removal of $P200^{Phe/Phe}$ genotypes, however, a significant proportion of $P200^{Phe/Tyr}$ genotypes were able to survive. In fact, $P200^{Phe/Tyr}$ heterozygotes had a five times greater fitness advantage over $P200^{Phe/Phe}$ homozygotes. This suggests that BZ resistance is incompletely recessive, or that this allele is genetically linked with another locus that is responsible for the resistant phenotype, perhaps one which has a quantitative influence, i.e. one resistance allele gives the effect of *X*, but two resistance alleles will give the effect of *2X*. Furthermore, Martin *et al.,* (1988) described BZ resistance as a co-dominant trait following backcross experiments between resistant and susceptible *T. colubriformis* parents. Other studies have also contested the recessiveness of BZ resistance in *H. contortus* (Le Jambre *et al.,* 1979; Herlich *et al.,* 1981; Sangster *et al.,* 1998). However, most of these studies have suggested that BZ resistance, at its fullest expression, is a multigenic trait, and thus, it would be very difficult to determine whether these observations were a result of incomplete recessiveness or of multigenic influences.

Much of the recent literature suggests that there are multiple steps or influences in the development of BZ resistance. For instance, one round of BZ selection of a susceptible *H. contortus* population (SE) resulted in the loss of alleles, presumably associated with $P200^{Phe/Phe}$ and $P200^{Phe/Tyr}$ genotypes (see Figure 4.8.1, reproduced from Roos *et al.,* 1990, with permission). A second round of selection reduced the variation to only one allele, presumably with a $P200^{Tyr/Tyr}$ genotype (Roos *et al.,* 1990). Successive selection events did not have any observable effect upon the number of isotype I β-tubulin-reactive fragments detectable by RFLP. However, if we compare this finding to the phenotypic expression of BZ resistance observed from those same populations (see Figure 4.8.2, reproduced from Otsen *et al.,* 2001, with permission), it appears that the dramatic increase in the ED_{50} values is not accounted for by the change in RFLP patterns. Furthermore, the ED_{50} values continued to rise beyond the point (RE2) where only one isotype I β-tubulin-reactive fragment remained. Despite the fact that RFLP is somewhat insensitive in terms of estimating the allelic diversity of a population, there is much evidence elsewhere for the involvement of other resistance

mechanisms. For example, with continued BZ selection, changes in the isotype II β-tubulin allelic constitution are also apparent (Beech *et al.,* 1994) and in some cases, a complete loss of the isotype II β-tubulin locus has been observed (Kwa *et al.,* 1993). Otsen *et al.,* (2001) used AFLP to examine the genetic diversity of the same *H. contortus* populations shown in Figures 4.8.1 and 4.8.2 at the individual worm level to assess the genome-wide effects of BZ selection. Analysis of more than 200 markers indicated that a change in the genetic constitution occurred after the first round of BZ selection, however, there was no evidence of a reduction in genetic diversity with further BZ selection (see Jaccard indices in Figure 4.8.2, which are more or less static after population RE1). Despite previous indications that other genes are involved in BZ resistance, Otsen *et al.* (2001) could find little evidence of BZ selection across random loci, which may indicate that the strongest effects of BZ selection are confined to the β-tubulin loci.

However, there may be more unknown changes within the β-tubulin loci that are important in BZ resistance, since recent studies have indicated that the frequency of the F200Y isotype I β-tubulin mutation is not well correlated with the BZ resistance phenotype. For example, in one highly BZ resistant (experimentally selected) cyathostomin population, the frequency of the $P200^{Tyr}$ allele was only 29.8% (Pape *et al.,* 2003). Moreover, a phenotypically BZ resistant field population of cyathostomins was shown to have an equally low level of $P200^{Tyr}$ alleles (33.3%), with the proportion of $P200^{Tyr/Tyr}$ genotypes at a mere 7.8% (von Samson-Himmelstjerna *et al.* 2003). Furthermore, a phenotypically BZ resistant *T. colubriformis* population from Australia has been found completely lacking the F200Y isotype I β-tubulin mutation (Le Jambre, personal communication). Ghisi *et al.,* (2007) have also reported a different isotype I β-tubulin mutation, E198A, in association with BZ resistance of a *H. contortus* isolate. Similarly $P167^{Phe/Tyr}$ and $P167^{Tyr/Tyr}$ genotypes (of the isotype I β-tubulin gene) have been reported in association with BZ resistant *H. contortus*, again, in the absence of the F200Y mutation (Prichard, 2001).

There are also non-tubulin resistance mechanisms to consider. In recent years, it has been shown that P-glycoprotein (P-gp) and cytochromes P450 (CYP) can, respectively, transport and metabolise some anthelmintics. Associations between such mechanisms and anthelmintic resistance have thus been made (Sangster, 1994; Kotze, 1999). To this end, several *in vitro* studies have shown that P-gp mechanisms play a crucial role in the detoxification of ML and BZ anthelmintics in parasitic nematodes (Beugnet *et al.,* 1997; Xu *et al.,* 1998; Kerboeuf *et*

al., 1999; Molento & Prichard, 2001) and CYP is known to be a key determinant of resistance in insects (Daborn *et al.,* 2002).

Taken together, this evidence provides strong motivation to investigate other potential mechanisms of BZ resistance. Specifically, this Chapter aimed to characterise BZ resistance in the *MTci5* isolate through examination of previously implicated mechanisms of resistance. However, this Chapter has also examined the *in vitro* pattern of BZ resistance over time in the *MTci5* isolate to allow a better understanding of the role of the F200Y isotype I β-tubulin mutation in the phenotypic expression of BZ resistance.

1. *Does the BZ resistance phenotype change over time in the MTci5 isolate, as observed previously by Borgsteede & Couwenberg (1987) and Kerbouef & Hubert, 1987? If so, is this change correlated with a change in genotype, that is, frequency of the F200Y isotype I β-tubulin mutation?*
2. *Are other previously implicated isotype I β-tubulin mutations (e.g. F167Y, E198A) present in the MTci5 isolate? If so, how do the frequencies of these mutations respond to selection with different broad-spectrum anthelmintics?*
3. *Is there evidence of a deletion of the isotype II β-tubulin locus in MTci5 and is there an effect of BZ selection upon the frequency of this event?*
4. *Is there any effect of the P-gp inhibitor, verapamil hydrochloride, upon the BZ resistance phenotype of the MTci5 and comparable MTci2 isolate, when co-administered with BZ?*
5. *Is there any effect of the CYP inhibitor, piperonyl butoxide, upon the BZ resistance phenotype of the MTci5 and comparable MTci2 isolate, when co-administered with BZ?*
6. *Is there any effect of addition of both inhibitors upon the BZ resistance phenotype of the MTci5 and comparable MTci2 isolate when administered in combination with BZ?*
7. *What relevance does the F200Y isotype I β-tubulin mutation have to survival of MTci5 parasites from in vitro BZ selection in the presence of inhibitors?*

4.8.2 Variation in the BZ resistance phenotype of *MTci5* over time in an experimental infection

There are a number of variables which could feasibly influence the expression of BZ resistance exhibited by a parasite population derived from an experimental infection including:

1. age, number and challenge method of parasites constituting the infection;
2. climatic and seasonal influences upon the host-parasite relationship;
3. effects of xenobiotics (e.g. agents of host immunity) upon the parasite population
4. the sensitivity and execution of the laboratory technique (see Section 4.8.3).

The age of the parent population has been shown to affect the level of resistance of the offspring in the *MTci5* isolate. BZ resistance peaked between weeks five and eight with a depression at week six. These results were remarkably similar to that observed by Borgsteede & Couwenberg (1987), whereby the egg hatch assay was used to compare the expression of BZ resistance over time between a susceptible and resistant population of *H. contortus*. Despite the difference in ED_{50} values between the populations, the authors found the pattern of resistance over the course of 11 weeks (post-infection) to be consistent between populations. There are a number of possible explanations for such a phenomenon. Borgsteede & Couwenberg (1987) suggested that the resistant population would likely comprise some homozygous susceptible females which may mature more quickly and live longer than their resistant counterparts explaining the baseline of the graph; whereas the resistant worms may mature more slowly, releasing their eggs over a shorter period, thus constituting the peak of the graph. However, this does not provide a feasible explanation for the same curve witnessed in the susceptible isolate, since it was unlikely to have contained sufficient homozygous resistant worms to constitute the peak. Furthermore, the F200Y isotype I β-tubulin genotyping of the *MTci5* progeny at various time points throughout the infection did not support a theory that resistant worms are developmentally stunted or otherwise. There is no proof that the F200Y isotype I β-tubulin mutation is the sole mechanism for BZ resistance in these parasites. In fact, this change in phenotype, which is not correlated with genotype, provides strong evidence that the F200Y isotype I β-tubulin mutation is not the sole determinant of BZ resistant in the *MTci5* isolate. Moreover, it seems unlikely that this phenomenon is entirely controlled by resistance genes, because this pattern was mirrored in the susceptible isolate, which ostensibly has no resistance genes (Borgsteede & Couwenberg, 1987). It was

unfortunate that the comparable susceptible (*MTci1*) isolate could not provide sufficient data to investigate this further in this study.

Another similarity between the data presented in this Chapter and that described by Borgsteede & Couwenberg (1987) is highlighted by Figure 4.8.3 (reproduced from Borgsteede & Couwenberg, 1987 with permission). The depression in fecundity of the adults and of the egg development (and thus, resistance of the offspring) observed between weeks 6 and 7 in both *H. contortus* isolates corresponds with the depression in egg development observed in the *MTci5* isolate. This may be coincidental or it may suggest that some process is occurring at this stage of the adult worm's life cycle, which temporarily compromises the expression of BZ resistance in their offspring. Furthermore, it is possible that some concomitant activity in host immune response (the time course of which is probably fairly consistent amongst hosts), affects the parasites' fitness. Host immune responses, particularly IgA activity, are known to affect worm length and fecundity in *T. circumcincta* (Stear *et al.*, 1995); therefore, it could feasibly cause a reduction in the fitness of both adults and offspring. Such a transient decline (i.e. week 6 to 7) may represent a quick recovery whereby the parasites employ counter-immune responses. Interestingly, studies by Kerboeuf & Hubert (1987) suggested that host immune responses may be involved as they observed different levels of resistance depending on the size and administration of the larval challenge. For instance, populations which had been administered via trickle infection showed much higher peaks of resistance than the single challenge dose infections. Furthermore, populations derived from previously challenged, and thus, immune sheep, showed much lower levels of resistance. This latter finding indicated that host immunity does have an effect upon parasite fitness, however, this effect was not transient as postulated above and the pattern of ED_{50} values over time did not show a depression around week six, although a decrease in hatching rates and fecundity were apparent at this time.

Another possible theory may be that parasites naturally exhibit a peak reproductive fitness which is preceded by immaturity and succeeded by senility. There is also the possibility that other resistance mechanisms develop with maturity, these may be inherent xenobiotic removal/ metabolic mechanisms present in both resistant and susceptible worms, which are simply up-regulated in resistant worms. Whatever the answer may be, the importance of this phenomenon is probably restricted to experiments conducted in the laboratory, since the

variation in worm maturity of a natural infection would probably have a stabilizing effect upon the overall expression of resistance at any given time. Indeed, Borgsteede & Couwenberg (1987) questioned the importance of this phenomenon, stating that the ovicidal activity of TBZ *in vitro* did not closely reflect the efficacy of FBZ upon adult worms *in vivo*. That is, by selecting a population, the adults became notably more resistant, whilst the ED_{50} value of the progeny remained constant. However, it is important to be aware of this phenomenon to allow consistency in laboratory experiments. For instance, a recent study has shown great disparity between the results of the egg hatch assay when the same populations of nematodes were analysed in different laboratories across Europe (von Samson-Himmelstjerna *et al.,* 2005, WAAVP abstract). This is of great importance since the results of these assays were so varied as to cast doubt over whether an isolate was susceptible or resistant. The expense and complex technology surrounding the more reliable genetic assays make genotyping for resistance less available for routine diagnosis and thus, the phenotypic assays must be calibrated across the board.

4.8.3 Reliability of the *in vitro* egg hatch assay

As described above, the potential for resistance to fluctuate over the course of an experimental infection is inherent and can be of significant magnitude. For instance, Kerbouef & Hubert, 1987 have shown that the peak ED_{50} values of a susceptible population were as high as a BZ resistant population at one stage in the course of the infection. These findings are of great importance, since the time at which a population is sampled for resistance assessment (whether in genotype or phenotype) may be inappropriate. Furthermore, changes in immune status within and between hosts are highly likely and thus, these are also important considerations for the egg hatch assay.

Personal observations and those of colleagues (A. Donnan & D. Bartley, personal communication), have also highlighted that the reliability of the egg hatch assay can be seasonal. For instance, the hatch success of eggs harvested in the winter months can be much lower than normal. It is possible that seasonal effects upon the host influence the parasites and the resultant viability of their offspring. Whilst a cessation in egg laying over the winter months would make more sense in evolutionary terms for these parasites, it is feasible that less

reproductive investment is made during periods when survival of the free living stages is far less likely.

In terms of the experiments described in this thesis, by far the most important issue was the dissolution of the TBZ stock solution. This phenomenon was only recognised latterly and thus, the actual egg development data generated from the *in vitro* egg hatch assays could not be compared between experiments. However, the results can still be useful in terms of interpreting the resistance factors in comparison with susceptible isolates, since the pattern of egg development from 0 to 100% hatch success was achieved for each assay within a given range of TBZ concentrations (whatever these concentrations actually were). Moreover, it is believed that a reliable estimate of BZ resistance phenotype was made in this Chapter, where an ED_{50} value of 0.2μg/ml was determined for the *MTci5* isolate, which was five times more resistant than the susceptible *MTci2* isolate (ED_{50} = 0.04μg/ml). This seems low in comparison with a number of published estimates for other highly BZ resistant nematode populations, however, it is likely that other laboratories have unknowingly encountered the same problem. The relevance of the dissolution of TBZ is of greater consequence in the experiments which have highlighted the survival of $P200^{Phe/Phe}$ individuals. This is discussed below.

4.8.4 Survival of susceptible genotypes ($P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$) in *MTci5*

From the results of Chapter 3, the *in vivo* BZ selection of the *MTci5* isolate permitted the survival of both $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes. Whilst the survival of the single $P200^{Phe/Phe}$ genotype could have been a chance event, it is unlikely that such a large proportion of surviving $P200^{Phe/Tyr}$ genotypes (19%) could have escaped the action of the drug. This Chapter examined the survival of all genotypes from BZ exposure *in vitro*, where extraneous factors (e.g. inter-host variation in immunity, resilience, metabolism and gut flow), which are synonymous with *in vivo* studies were removed, showing only the effect of the drug upon the parasites. The findings showed that both $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes were able to survive high levels of TBZ exposure. Perhaps this indicates that the F200Y isotype I β-tubulin mutation is not the sole mechanism of BZ resistance in the *MTci5* isolate. However, there are three possible explanations to consider:

1. A small proportion of $P200^{Phe/Phe}$ genotypes (and indeed all genotypes) survive by chance alone
2. Some biological phenomenon is responsible for the survival of $P200^{Phe/Phe}$ genotypes, i.e. thicker or less permeable egg shells/ membranes
3. There are other unknown BZ resistance mechanisms operating in $P200^{Phe/Phe}$ (and $P200^{Phe/Tyr}$) genotypes which aid survival.

The first possibility is less likely due to the correction that is applied to the egg hatch assay data. That is, in the control wells, there is a small proportion of eggs which fail to hatch and this proportion is applied to the test wells. Furthermore, we would not expect those eggs which failed to hatch to represent any one genotype as previous studies have shown that there is no fitness cost associated with any F200Y isotype I β-tubulin genotype when there is no selection pressure applied (Elard *et al.,* 1998). Moreover, the percentage of eggs which would not hatch due to chance should be equal amongst all F200Y isotype I β-tubulin genotypes and across all TBZ concentrations tested. Hence, if the lowest survival of $P200^{Phe/Phe}$ genotypes was observed at 2.3% in the 0.19µg/ml TBZ sample, then we could say that all the other samples could be expected to show around a 2% survival of $P200^{Phe/Phe}$ genotypes by chance alone. However, in the 2.0µg/ml TBZ test, double this proportion of $P200^{Phe/Phe}$ genotypes survived (5.4%), hence, this is unlikely to have happened by chance alone. The second point is worth considering, since it is known that there is an optimum time frame within which the egg hatch assay can be performed. Eggs have to be collected and either stored at 4°C under anaerobic conditions, or used in the assay within two hours of collection to prevent development occurring. In this early phase of collection, the egg shells are permeable and thus, vulnerable to drug exposure, after a certain period of time, however, this permeability is reduced and the eggs could hatch regardless of the drug concentration they are exposed to if they are exposed after this period (F. Jackson, personal communication). It is possible, indeed likely, that this window of permeability varies between individuals. Thus, it is conceivable that some eggs will hatch without actually having a resistance phenotype. However, the proportion of these individuals is, again, likely to be equal amongst all F200Y isotype I β-tubulin genotypes and the latter point about the survival of $P200^{Phe/Phe}$ genotypes not being constant amongst different drug concentrations would also reject this possibility. Furthermore, it would be unlikely that only $P200^{Phe/Phe}$ genotypes would survive due to reduced egg shell permeability unless this was an advantageous adaptation. For this to be the case, there must be a selection pressure and it is questionable how much direct anthelmintic

selection the egg stage of the worm receives in an *in vivo* situation, in comparison with say, L_4 and adult parasites. Moreover, if this was a selectable trait, we would expect to see co-selection with the F200Y isotype I β-tubulin mutation, which would then cause a steady increase in the proportion of $P200^{Phe/Phe}$ genotype survivors as the drug concentration increased, and this was not observed. Finally, there is much more evidence in the literature for the third possibility and indeed from the results of this Chapter, whereby a change in the phenotypic expression of BZ resistance over the course of an experimental infection was not accompanied by a change in the frequency of the F200Y isotype I β-tubulin mutation.

The conclusion that the F200Y isotype I β-tubulin mutation is not the sole determinant of BZ resistance could be criticised due to the unknown concentrations of TBZ at which these $P200^{Phe/Phe}$ genotypes were shown to survive (due to the TBZ dissolution). However, as Figure 4.8.4 demonstrates, L_1 survivors were genotyped at TBZ concentrations equivalent to the ED_0, ED_6, ED_{20}, ED_{60} and ED_{96} values of that assay (see Section 4.3) and $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ survivors were found at each level. Hence, regardless of the actual concentration of TBZ used, it can be said that at the ED_{96} value, this concentration was high enough to prevent egg development in 96% of the population, which in itself is a strong indicator of BZ resistance.

4.8.5 Contribution of other isotype I β-tubulin mutations to BZ resistance in *MTci5*

Whilst the F200Y isotype I β-tubulin mutation appears to be a good marker, the evidence for it being the sole determinant of BZ resistance is diminishing. Indeed, other mechanisms have been described for the same gene, which are capable of conferring a resistant phenotype. Two such mutations, F167Y and E198A were surveyed in *MTci5* and in other BZ resistant isolates in this study and neither polymorphism was found. However, occurrences of these mutations seem to be rare, with only three reports of F167Y in different nematode species (Prichard *et al.,* AAVP abstract, 2000; Silvestre & Cabaret, 2002; Clark *et al.,* 2005) and one report of E198A from *H. contortus* (Ghisi *et al.,* 2007). The F167Y mutation appears to be more heritable than the F200Y isotype I β-tubulin mutation according to Silvestre & Cabaret (2002). For instance, the $P167^{Phe/Tyr}$ genotypes survived equally well as the $P200^{Tyr/Tyr}$ genotypes

suggesting it is a dominant trait. Prichard *et al.,* (AAVP abstract, 2000) reported similar findings in *H. contortus*. This raises the question: why have F167Y genotypes not been reported more frequently? Perhaps there is a fitness cost associated with this mutation, although this seems unlikely as this mutation has reached such high frequency as to be detectable in relatively small samples from three different trichostrongylid species (Silvestre & Cabaret, 2002). Instead, it may be that the perceived rarity of this allele is due to a lack of surveys, or that it simply does not arise as often as the F200Y isotype I β-tubulin mutation.

Other mutations have been reported, including F76V and I368V isotype I β-tubulin mutations (Kwa *et al.,* 1994), however, there was not a strong correlation between these mutations and resistance, unlike the F200Y SNP in the same population. It is possible that these mutations are accumulated as resistance increases, or perhaps they confer resistance but have a fitness cost, and thus, do not persist. It should be noted that the full length of this gene was not examined from this isolate; therefore, it is possible that there are yet more mutations which may be contributing to BZ resistance. Only those mechanisms which have been previously implicated in BZ resistant nematodes have been examined in this study, and further work should take this into account.

4.8.6 Role of isotype II β-tubulin locus in BZ resistance of *MTci5*

This study also aimed to isolate isotype II β-tubulin from *T. circumcincta* in order to assess whether this locus had been deleted in *MTci5* during the process of developing BZ resistance. Partial cDNA sequence was successfully obtained from a pool of susceptible adult *T. circumcincta*. The sequence alignments between these and the published isotype I and isotype II cDNAs from closely related species identified this as an isotype II homologue. This was based on five isotype-defining conserved residues from the alignment of closely related nematode species. These were positions: Y90F, N115S, S153A, A165S and S284L (where the isotype I residue, e.g. Y and P90, is replaced by isotype II residue, F). The amplification of isotype II gene fragments was then achieved from all unselected and BZ-selected adults of the *MTci5* isolate. Thus, it is apparent that the deletion of the isotype II locus, a mechanism reported previously in highly resistant isolates of *H. contortus* (Kwa *et al.,* 1993), was not contributing to the BZ resistance status of the *MTci5* isolate. With continued BZ selection, it is possible that this deletion event may occur, however, the importance of such a mechanism

would be low, given that the *MTci5* isolate is already highly BZ resistant. There was some variation between the *MTci5* cDNA and the *MTci2* cDNA sequences, showing 92% homology. It is possible that the BZ selection of the *MTci5* isolate has led to this moderate differentiation. When these cDNAs were compared with the published accession sequence for *T. circumcincta* isotype I β-tubulin (Z96258), *MTci5* and *MTci2* shared 67 and 68% homology, respectively, with this gene. Given that the protein alignments are highly conserved between isotype I and II genes (with the exception of 19 residues), the homology estimates confirm that approximately two out of three bases per amino acid are conserved.
A deletion of the isotype II locus has only been reported in one study and this involved laboratory passage through multiple rounds of selection, which does not reflect the natural field situation. Kwa *et al.* (1993) used RFLP to monitor the effects of continued BZ selection in a number of BZ resistant and susceptible *H. contortus* populations. In all populations, it was apparent that the first few rounds of selection reduced the genetic variation of the isotype I alleles until one variant remained. However, in two of the populations, complete elimination of individuals carrying isotype II variants was achieved. These populations had different origins, one having been isolated from the field, suggesting that this mechanism was not an irregularity.

The RFLP technique has been criticized as the results were based on pooled genomic DNA samples hybridised with isotype I- and II-specific probes, which only confirms that many of the bands present in susceptible isolates are absent in resistant isolates. From these images (see Figure 4.8.1), it is difficult to determine whether a number of alleles have been lost or whether an entire locus has been deleted as a result of selection, since rare alleles may be obscured (Beech *et al.,* 1994). Furthermore, a study which analysed the consequences of BZ selection at the individual worm level failed to reproduce this deletion event. Beech *et al.* (1994), studied individuals of two resistant *H. contortus* populations and found the diversity of isotype I and II alleles to be significantly reduced as selection was applied, however, the isotype II locus remained. This study provided clear evidence that both loci were actively involved in BZ resistance. Moreover, the authors stated that deletion of isotype II was unlikely to arise in these two populations even with continued selection, as the level of diversity had been reduced to such a degree that gene flow would have been necessary to restore genetic variation. However, they conceded that there may be two separate mechanisms

for BZ resistance, either: selection for resistant alleles at both loci; or selection at both loci culminating in the deletion of the isotype II locus.

Whether or not this deletion event is possible in *T. circumcincta* remains to be seen, however, it appears from the latter experiments in this Chapter, that there are other non-specific resistance mechanisms, which have arisen in these parasites; perhaps selection is applied more strongly at these loci where multiple anthelmintics have been used in short succession. As yet, there are no published sequences for the isotype II gene in *T. circumcincta*; thus, the effects of BZ selection at this locus remain to be investigated in other isolates.

Whilst this Chapter employed a simple diagnostic PCR for the presence of isotype II, other studies have looked at expression of these genes. According to a recent study in cyathostomes (Clark *et al.,* 2005), the expression of this isotype seems to be stage-specific, since it could only be isolated from adult worms. There is no information regarding the developmental expression of isotype II in trichostrongylid nematodes as yet, but if it were expressed only in the adult stage, this would cast further doubt over a functional role of this gene in BZ resistance.

It is unfortunate that the characterisation of isotype II did not encompass a survey of the same mutations found in isotype I, as F200Y has been found in isotype II of other trichostrongylid nematode populations (Beech *et al.,* 1994). Pyrosequencing assays were designed for F167Y, E198A and F200Y mutations of the isotype II β-tubulin gene, however, time constraints prevented this work being undertaken. It would have been particularly useful to rule in/out these potential BZ resistance mechanisms from the *in vivo* and *in vitro* $P200^{Phe/Phe}$ survivors of BZ selection. This should be a priority for future studies. Beech *et al.* (1994) found the F200Y isotype II β-tubulin allele at a frequency of 12% in a resistant *H. contortus* isolate. This may indicate that a mutation at this residue is important in terms of altering the conformation of the protein or simply that this mutation arises frequently. It may also suggest a common gene ancestor of both isotypes and add weight to the theory that the F200Y SNP is a pre-adaptation, or it may alternatively suggest that this residue represents a 'mutational hotspot' in both genes. Characterisation of isotype II from BZ resistant cyathostomes did not show the presence of the F200Y mutation at this locus, despite the fact that it occurs at the isotype I locus of these parasites (Clark, personal communication). As Prichard (2001)

suggests, there appear to be a number of possible tubulin mutations which can confer BZ resistance amongst nematodes and a survey of the F200Y isotype I β-tubulin mutation no longer provides a satisfactory estimate of BZ resistance at the individual worm level. For instance, it seems highly possible that isotype II β-tubulin could have a role in BZ resistance, since the high homology between isotype I and II genes amongst different species suggests that divergence is constrained by function. Furthermore, if isotype II is an important target, it will be subject to a selection pressure for modification of this site to enhance resistance.

4.8.7 Contribution of P-gp and CYP to the BZ resistance phenotype of *MTci5* & *MTci2* isolates

In recent years, the emergence of multiple drug resistance has brought more attention towards the non-specific resistance mechanisms. There seems to be a trend that each successor to the last introduced anthelmintic becomes ineffective at a faster rate than its predecessors. For instance, IVM was introduced to the market in 1981 (Shoop, 1993) and after just 33 months, the first case of IVM resistance was reported in South Africa in an already BZ-resistant isolate (Carmichael *et al.,* 1987). Furthermore, the first case of IVM resistance reported in the UK (and indeed Europe) developed after just two seasons of use (Jackson *et al.,* 1992a,b,c, whereas BZ resistance in this goat flock had been diagnosed in 1988 after three years of use in the current locale (the extent of BZ treatments prior to the relocation of the goat herd is unknown). The idea that there may be a drug resistance mechanism which has little specificity is a worrying concept and the potential up-regulation of such a mechanism is not unlikely given the evidence for this phenomenon from other phyla. Since the full genetic basis of BZ resistance in the MDR *MTci5* isolate remains unexplained, it was pertinent to investigate the contribution of P-gp and CYP, two recently implicated mechanisms of drug resistance with little substrate specificity regarding anthelmintics.

This Chapter provided clear evidence of the role of P-gp and CYP in drug resistance in the *MTci5* isolate. Most of the decreases in $ED_{50,\ 96\ \&\ 99}$ values were highly significant (i.e. $p < 0.01$) and all were at least significant at the 95% CI. In contrast, the *MTci2* isolate showed little effect of inhibition, with only the combination of inhibitors producing any consistent significant decrease in ED values. These results appear to show that the *MTci5* isolate is comparatively more sensitive to the removal of its non-specific resistance mechanisms than

the *MTci2* isolate. This may indicate an up-regulation of these mechanisms in *MTci5* or it is possible that this assay is not sensitive enough to detect a reduction in susceptibility when the ED values are so low (i.e. in the *MTci2* isolate), thus, making the susceptibility factors appear greater in the resistant isolate. Further experimentation would be required to investigate this finding, ideally by examining the expression of candidate P-gp and CYP genes at the RNA or protein level, either constitutively or in response to drug selection. The susceptibility factors and statistics suggested that, of the two inhibitors, PB was more potent in both isolates. This suggests that the metabolic detoxification of BZ is a more important process than the P-gp drug efflux mechanism in these parasites. This could be due to the location of P-gp being restricted to the membrane of particular cells, whereas the CYP enzymes might be free to operate anywhere in the tissues.

Alternatively, these findings could represent a developmental effect, whereby one mechanism matures more quickly than the other, depending on the selective advantage of each. Eggs may be subject to different xenobiotic challenges than larvae or adults and so different mechanisms may be involved. For example, there is evidence that P-gp is developmentally regulated in the filarial nematode, *Onchocerca volvulus*, and an implication that this may be the case in a number of other filarial worm species (Huang & Prichard, 1999). For instance, the expression of *ovpgp-1* was around 40% higher in adults relative to the expression of the control gene (β-tubulin) compared with the larval stages, although the significance of this finding has yet to be established. The reason for the higher IVM tolerance exhibited by adult parasites of these filarial species is still unknown. Now it seems possible that the upregulation of these mechanisms in adults (for whatever biological reason) may explain the decreased sensitivity to IVM. It is possible that the P-gp mechanism is developmentally expressed in *T. circumcincta* also. Analysis of the effects of these inhibitors on the adult stages would be interesting, although the necessary *in vivo* conditions make such experiments problematic. Such studies have been carried out, however, using *H. contortus* in both sheep and jirds (Molento & Prichard, 1999; Xu *et al.,* 1998). The results showed that VPL and a VPL analogue (CL 347, 099) did dramatically increase the efficacy of MOX and IVM upon the ML resistant worms as demonstrated *in vitro*. However, there is a critical threshold of the VPL dose which is toxic in mammals regardless of the presence or absence of an anthelmintic (Molento & Prichard, 2001), therefore, such experiments should be planned carefully. Despite the question of whether P-gp genes are naturally over-expressed in the adult stages of these parasites, this

study has demonstrated that both mechanisms are undoubtedly important and when inhibited in combination, render this highly BZ resistant isolate completely susceptible (ED_{50} = 0.001μg/ml TBZ).

The F200Y isotype I β-tubulin genotyping data did not provide clear answers as to the importance of this mutation when the non-specific resistance mechanisms were interrupted. If the CYP - and P-gp - dependent mechanisms were responsible for the survival of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes following BZ treatment in previous experiments, one would expect fewer of these genotypes to survive BZ treatment once these mechanisms had been inhibited. Consequently, the proportion of $P200^{Tyr/Tyr}$ individuals would be expected to be greater in the larvae genotyped from the EHA in the presence of the inhibitors, than in their absence. However, this was not the case. By inhibiting the P-gp mechanism, the number of $P200^{Tyr/Tyr}$ individuals was vastly reduced, whilst the $P200^{Phe/Tyr}$ individuals survived better. Interestingly, there was no change in the proportion of $P200^{Phe/Phe}$ genotypes. Taken alone, this result may imply that the F200Y isotype I β-tubulin mutation is unimportant in BZ resistance when this crucial non-specific mechanism is inhibited. The effect of inhibiting CYP also discriminated against the survival of $P200^{Tyr/Tyr}$ genotypes, in favour of $P200^{Phe/Tyr}$ genotypes. Perhaps by interrupting the metabolism of BZ, the drug becomes so toxic that having a $P200^{Tyr/Tyr}$ genotype is insufficient to allow it to survive. For instance, if the drug acts on multiple targets (which may only be important at much higher does of BZ); when the metabolism of the drug is inhibited, these targets will become available and the worm will succumb to the effects of the drug. This would happen even if BZ could not bind to the isotype I β-tubulin target as a consequence of the $P200^{Tyr/Tyr}$ genotype. However, this would only explain why $P200^{Tyr/Tyr}$ worms have no advantage over $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ worms in the presence of the CYP inhibitor; it does not explain why $P200^{Phe/Tyr}$ worms have a survival advantage.

To complicate matters further, adding both inhibitors in combination had no effect upon genotype ratios, bar a moderately significant reduction in $P200^{Tyr/Tyr}$ genotypes. This appears to suggest that when both mechanisms are interrupted, survival is unaffected by the F200Y isotype I β-tubulin status. This would appear to suggest that there are multiple targets for the action of BZ. It should be noted that at this concentration (0.2μg/ml TBZ), only 8% of *MTci5*

parasites were able to survive. In contrast, 0% of *MTci2* parasites survived under the same conditions; therefore, clearly there is still some resistance advantage within *MTci5* worms when the P-gp and CYP mechanisms are removed. Given that the recessiveness of the F200Y isotype I β-tubulin mutation remains uncertain, it is possible that a $P200^{Phe/Tyr}$ genotype confers partial resistance (thus explaining the large proportion of $P200^{Phe/Tyr}$ survivors after P-gp and CYP inhibition). It follows that the F200Y isotype I β-tubulin mutation may have been the first resistance mechanism to evolve and this was subsequently overtaken by a more efficient system, e.g. P-gp or CYP. A recent study has shown selection of one allele of one gene (Pgp-A) in association with TBZ and cambendazole (CBZ) resistance in *H. contortus* (Blackhall *et al.,* in press). The authors suggest that P-gp efflux is a more crucial resistance mechanism to these parasites, in removing BZ before it reaches the target site, thus making the F200Y isotype I β-tubulin mutation superfluous. Studies such as this and those described by Beugnet *et al.* (1997) and Kerbeouf *et al.* (2003) do indicate a strong correlation between P-gp and BZ resistance, however, neither provides proof of a causal link. Genetic hitchhiking or epistasis remain possible causes of such associations. Similarly, the same P-gp gene has been implicated in the expression of ML resistance in the same species. Examination of Pgp-A in ML resistant and susceptible *H. contortus* revealed a reduction in the number of hybridisation patterns associated with IVM resistance (Xu *et al.,* 1998). Selection of a particular P-gp allele was again demonstrated amongst three IVM resistant populations when compared to the susceptible isolates from which they were derived (Blackhall *et al.,* 1998). Again, it remains unclear whether these changes were effected through linkage with the actual (unknown) resistance gene or whether they represent true resistance alleles. Sangster *et al.* (1999) also reported association of one P-gp allele with ML resistance in *H. contortus*, as did Le Jambre *et al.,* (1999), however, the latter study was able to prove that this allele was not the cause of ML resistance. The apparent lack of substrate-specificity makes P-gp a valid candidate as a MDR gene.

The effect of adding the inhibitors in isolation seemed to indicate that $P200^{Phe/Tyr}$ individuals have a better survival advantage and that a $P200^{Tyr/Tyr}$ genotype may even compromise fitness. Perhaps there are synergistic effects when the inhibitors are used in combination which restores the balance between the F200Y isotype I β-tubulin genotypes. For instance, it is possible that these chemicals may react and reduce the reciprocal effects upon the target mechanisms. Alvinerie *et al.* (2001) suggested that use of a synergist will increase

susceptibility in both resistant and susceptible isolates independently of other mechanisms. Such experiments need to be repeated and it is unfortunate that the *MTci2* isolate could not also have been examined in this way, due to the presence of too few F200Y isotype I β-tubulin alleles to highlight the effects of these inhibitors.

Kotze (1998, 2000) suggested that there was no evidence for a role of CYP in ML, BZ or imidazothiazole resistance, however, these results suggest otherwise. Similarly, CYP has been shown to be an important resistance mechanism in other organisms. For instance, increased transcription of *Cyp6g1* was observed in *Drosophila melanogaster*, where between 10 and 100 times more mRNA was observed in DDT resistant versus susceptible populations (Daborn *et al.,* 2002). It appears that the insertion of an Accord transposon is the cause of this enhanced transcription. DDT resistance persists within a population without continued selection implying that there is no fitness cost associated with this mutation. One resistance allele of one gene is responsible for the global expression of resistance in *D. melanogaster*, despite the massive number of CYP genes transcribed (over 90) in this species. Moreover, cross-resistance was observed with a number of other compounds including organochlorine, carbamate and organophosphorous. This indicates that a relatively simple mutation can lead to resistance against a number of chemically unrelated compounds, and the potential for such a mechanism in parasites must be considered, particularly since two anthelmintics have already been shown to be metabolised by CYP, namely rotenone (Fukami *et al.,* 1967) and MOX (Alvinerie *et al.,* 2001). Kotze *et al.* (2006b) studied the effects of rotenone upon *H. contortus* adults and larvae *in vitro*, and found the addition of PB to increase the effect of the anthelmintic by a magnitude of three to four. Moreover, the use of PB-anthelmintic combinations has been studied *in vivo,* in order to extend the period of drug bioavailability in the host. For instance, pre-treatment of goats with PB induced a three-fold increase in the bioavailability of FBZ (Benchaoui & McKellar, 1996). This treatment may also directly inhibit parasite detoxification mechanisms since the addition of PB increased efficacy of FBZ from 7.9% to 97.8% in the FBZ-resistant *MTci3* isolate (Benchaoui & McKellar, 1996). However, the results of a field study which used a FBZ-PB combination on the less-resistant parent field isolate of *MTci3* were not as striking, showing an increase in efficacy from 25.7% (FBZ alone) to 53.1% (FBZ-PB treatment) (M. Barrett, PhD Thesis, 1997). Similarly, the work presented here seems to suggest that both P-gp and CYP are more crucial resistance

mechanisms in the *MTci5* isolate than the F200Y isotype I β-tubulin mutation, or an increase in the proportion of $P200^{Tyr/Tyr}$ individuals would have been observed.

4.8.8 Summary

In summary, the work presented in this Chapter has indicated that there are multiple BZ resistance mechanisms at play in the MDR *MTci5* isolate. There are four findings which support this statement. The first observation was that the pattern of BZ resistance fluctuated over the course of an experimental infection, in a manner consistent with the findings of others (Borgsteede & Couwenberg, 1987; Kerboeuf & Hubert, 1987), however, this change was not supported by a change in the frequency of the isotype I β-tubulin mutation. Secondly, the *in vivo* and *in vitro* survival of $P200^{Phe/Tyr}$ genotypes may indicate that the F200Y isotype I β-tubulin mutation is partially dominant. Thirdly, the survival of $P200^{Phe/Phe}$ genotypes following BZ selection suggests that must be other mechanisms contributing to the BZ resistance phenotype of these worms. Finally, whilst none of the other previously implicated isotype I β-tubulin mutations could be found, nor was the proposed deletion of the isotype II β-tubulin locus apparent in *MTci5*; there was strong evidence of a role of P-gp and CYP resistance mechanisms upon the removal and detoxification of BZ *in vitro* in the *MTci5* isolate. Furthermore, the F200Y isotype I β-tubulin mutation was less important to survival when these mechanisms were interrupted, suggesting that BZ may have multiple targets.

Figure 4.8.1: Blots showing restriction fragment length polymorphism (RFLP) analysis of *H. contortus* populations sequentially selected with BZ (reproduced from Roos *et al.*, (1995) with permission). From Figure A, note that population SE is the susceptible *H. contortus* population and after one round of BZ selection (RE1) there is a reduction in the number of isotype I β-tubulin-reactive fragments from three to two, and then to one fragment following a second round of BZ selection (RE2). Figure B shows the number of isotype II β-tubulin-reactive fragments in the same populations. One fragment is lost after the first round of selection and another after the third round of selection. RE4 represents appears to show a complete loss of the isotype II β-tubulin locus.

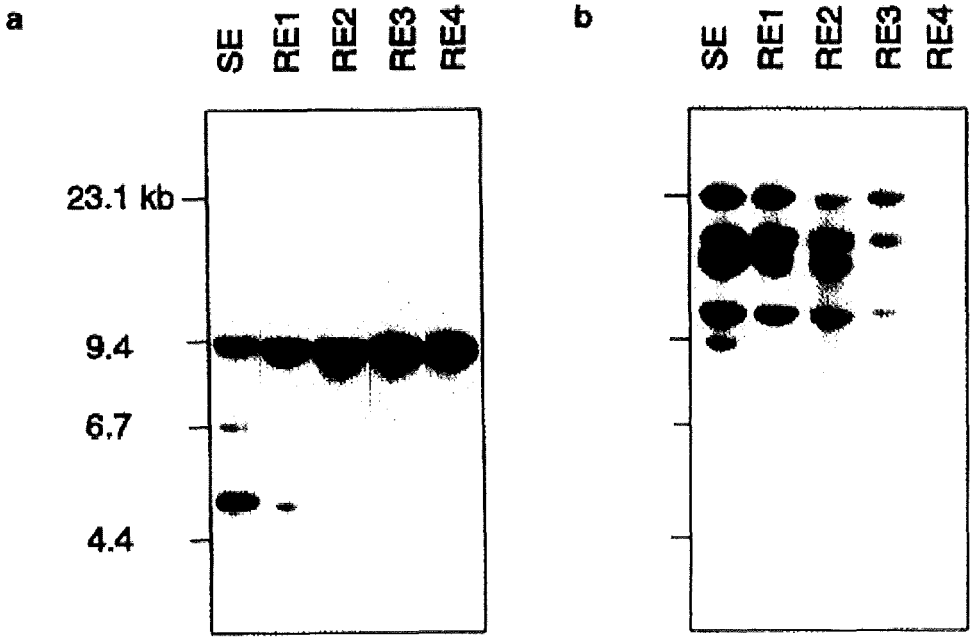


Fig. 1. Restriction fragment length polymorphism (RFLP) analysis of the *H. contortus* generations obtained by *in vitro* selection for BZ resistance[14]. Normally 2 μg genomic DNA was digested with XbaI. Identical blots were hybridized with: β-tubulin isotype 1 probe (a), and β-tubulin isotype 2 probe (b). Phage lambda DNA, digested with HindIII was used as a molecular weight marker. SE, susceptible population Edinburgh; RE1, population after first selection; RE2, population after second selection; RE3, population after third selection; RE4, after fourth selection[14,20]. (See also Table 1.)

Figure 4.8.2: Graph showing the amplified fragment length polymorphism (AFLP) analysis of *H. contortus* populations sequentially selected with BZ (reproduced from Otsen *et al.*, 2001 with permission). Notice the line graph representing the increase in ED50 values with each round of BZ selection. The populations in this Figure represent the same populations shown in Figure 4.8.1.

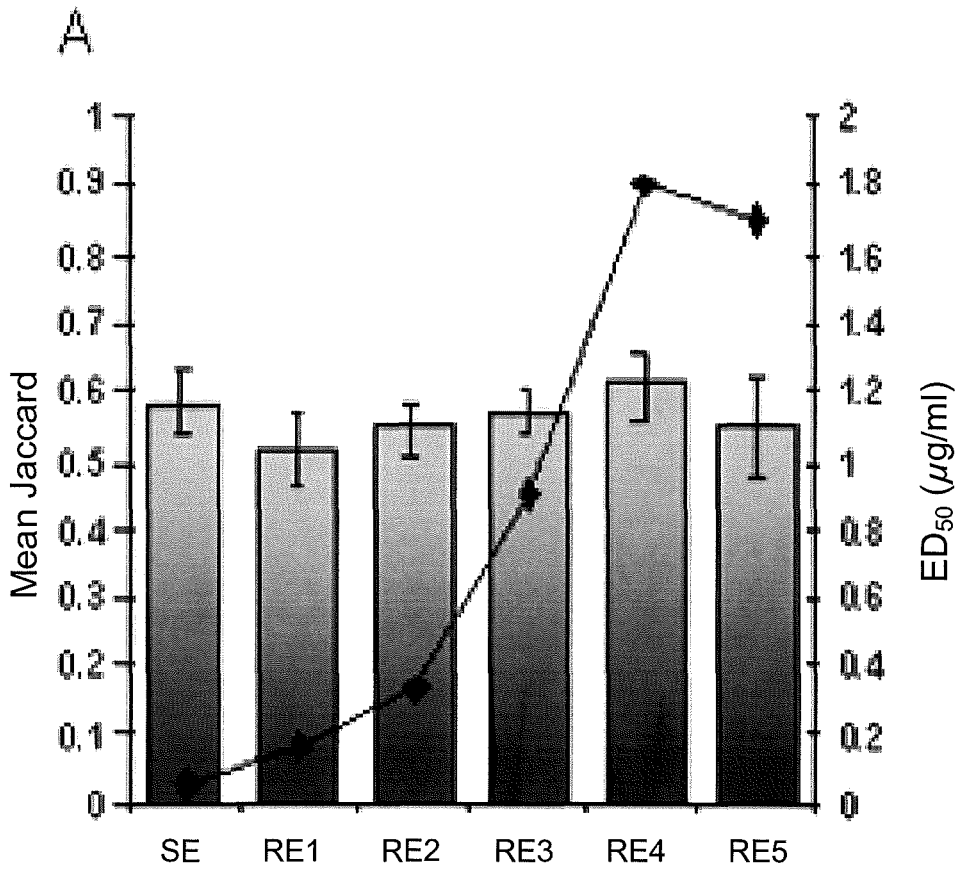
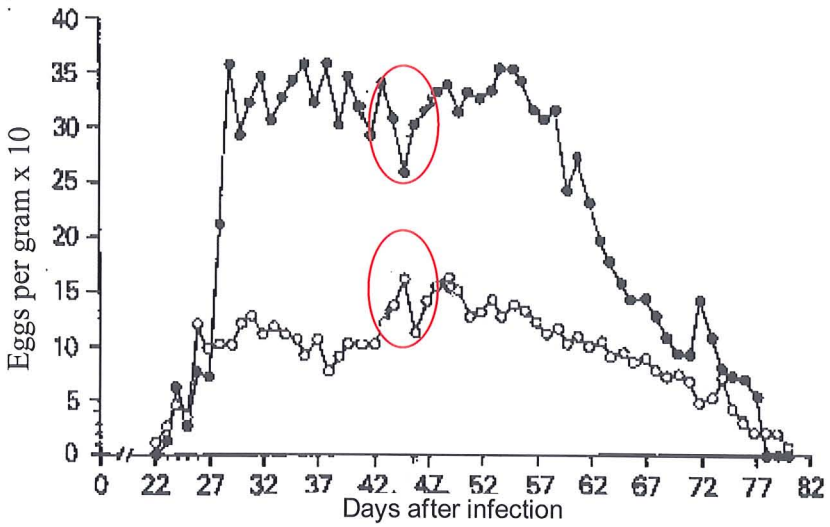


Fig. 2. AFLP analysis of populations with increasing resistance to benzimidazole, based on 224 polymorphic and monomorphic AFLP markers derived from the 12 individuals of each population. The SE population used had been propagated for five generations without benzimidazole selection, after the benzimidazole selection on part of the population had started. Bars with the scale on the left Y-axis. (A, B and C) indicate Jaccard indices with standard deviations, averaged over all pairwise comparisons. Standard deviations are indicated. (A) Pairwise comparison of individuals from the same population. Two-tailed P values of Student's t-test of J values of consecutive populations were 0.0015 or less. Matching ED50 values indicating the degree of resistance to benzimidazole are indicated as a line diagram with the scale on the right Y-axis.

Figure 4.8.3: Graphs showing the expression of BZ resistance over time in resistant and susceptible *H. contortus* isolates (A, B: reproduced from Borgsteede & Couwenberg, 1987 with permission). Figure A shows the number of eggs per gram released over time and note the depression in fecundity between days 42 and 47 (circled in red). In Figure B, a concomitant decline in egg development is also observed around the same time point (circled in red). Compare this with Figure C, where the *MTci5* isolate shows a depression in egg development at six weeks post-infection (or 42 days).

A



G 1: Eggs per gram pattern of the susceptible strain of *Haemonchus contortus* (○——○ sheep 1) and of the resistant rain (●——● sheep 2)

B

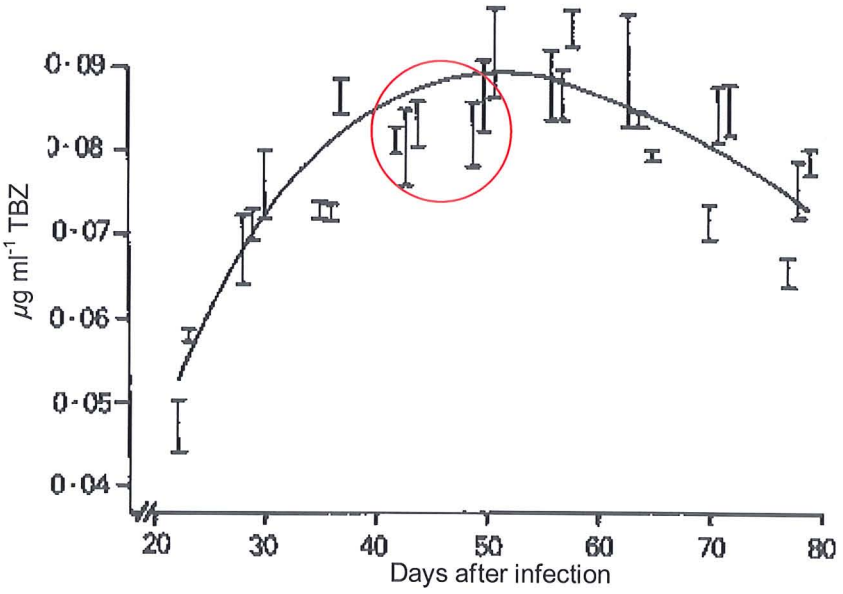


FIG 2: Patterns of LC50 of the susceptible strain of *Haemonchus contortus* (sheep 1) with indication of the maximum and minimum values in each triple test. TBZ thiabendazole

C

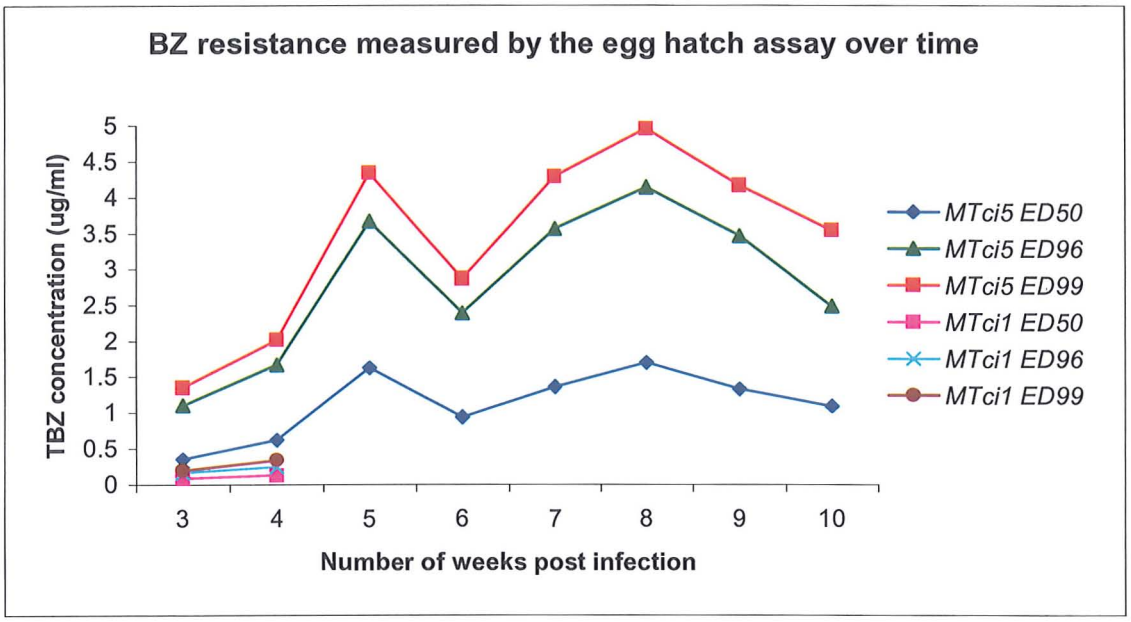
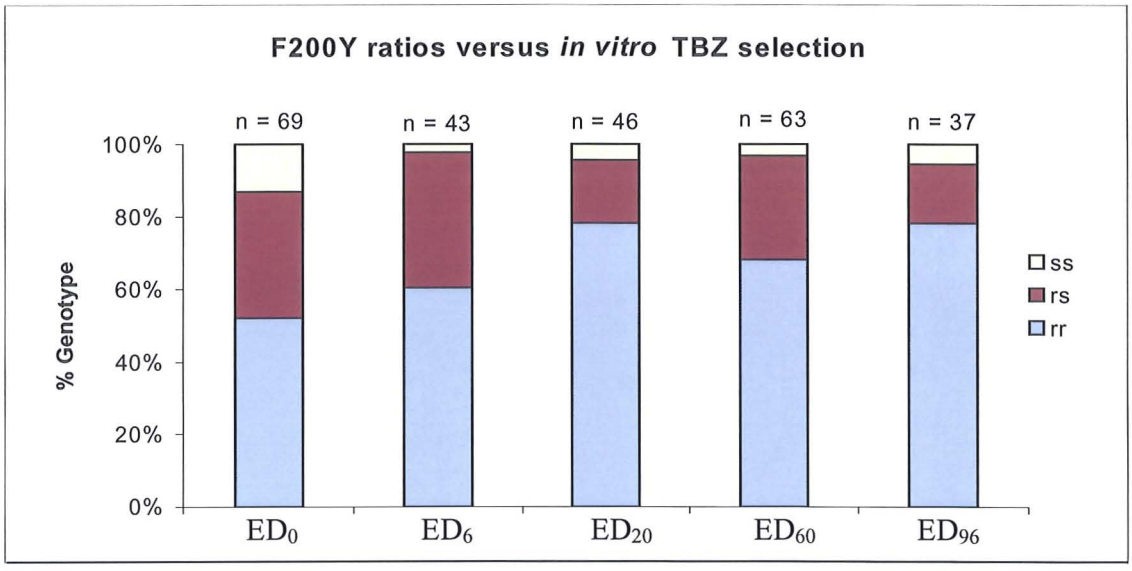


Figure 4.8.4: Graph showing the F200Y isotype I β-tubulin genotype ratios of L_1 survivors from the egg hatch assay in the *MTci5* isolate. N refers to the total sample size of L_1 collected from each TBZ concentration. The egg development values of the population shown (ED_0, ED_6, ED_{20}, ED_{60} and ED_{96}) equate to the following inaccurate BZ concentrations: 0, 0.19, 0.5, 1 and 2μg/ml TBZ. The rr, rs and ss legends refer to $P200^{Tyr/Tyr}$, $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes.



5.0 Chapter 5 Origins and diversity of isotype I β-tubulin alleles of *T. circumcincta* and the effects of anthelmintic selection upon genetic diversity

5.1 Introduction

5.1.1 The requirement for population genetics studies of UK *T. circumcincta* isolates

There have been relatively few population genetics studies of nematodes to date (reviewed by Anderson *et al.,* 1998). Chapter 3 presented work using five neutral microsatellite markers to analyse the genetic structure of the *MTci5* isolate and to investigate the potential effects of anthelmintic selection across these loci. In this Chapter, a different population genetics approach is described, whereby a single locus, which is known to be involved in BZ resistance, will be characterised in detail. This characterisation will examine the genetic diversity of the isotype I β-tubulin gene within and between six UK *T. circumcincta* isolates, each with varying histories of anthelmintic selection, geographical origin and gene flow. The effects of anthelmintic selection upon the allele frequencies at this locus will also be examined. This type of research can provide valuable insights into the origins and spread of BZ resistance alleles. An understanding of how resistance alleles arise in a population is a prerequisite for the control of nematode populations. This type of study has only been conducted once before (Silvestre & Humbert, 2002); hence, it will be useful to compare the current findings with those observed previously to give a clearer idea of the origins of BZ resistance.

Specifically, this research aims to determine how BZ resistance alleles can arise in a population and there are three possibilities to consider. The first is the spread of resistance alleles via gene flow. Indeed, the extensive movement of animals and lack of adequate quarantine measures in the UK have been highlighted as factors which may have contributed to the spread of anthelmintic resistance in the UK (Sargison *et al.,* 2004). Thus, a study of the

number and frequency of BZ resistance alleles within and between populations is one way of assessing the impact of gene flow upon the development of BZ resistance. Secondly, in order for alleles to spread via migration, we must determine how they first arose. Some suggest that BZ resistance is a pre-adaptation, with the necessary resistance allele(s) already occurring at a low frequency within a population, which then come to the fore with continued anthelmintic selection (Jackson & Coop, 2000). The third possibility is that BZ resistance can arise as a spontaneous new mutation and continues to do so under selection. A comparison of isolates with varying degrees of resistance is also useful in determining how BZ resistance develops; for instance, certain alleles may increase in frequency as resistance progresses. Despite the fact that BZ resistance is already widespread across the globe, our understanding of how resistance alleles arise and spread throughout populations can be applied to other drug classes in areas where resistance has not yet developed.

The work in Chapters 3 and 4 indicated that the F200Y isotype I β-tubulin mutation was under strong selection following BZ treatment. This study also aims to investigate the effect of anthelmintic selection upon the isotype I β-tubulin locus in more detail by assaying sequence variation surrounding the F200Y mutation. In previous studies, the development of BZ resistance has been attributed to the loss of variation across both isotype I and II genes (Roos *et al.,* 1990; Kwa *et al.,* 1993; Beech *et al.,* 1994; Grant & Mascord, 1996; Otsen *et al.,* 2001). In particular, a loss of susceptibility (rather than an accumulation of resistance) was observed and in some cases, the complete deletion of the isotype II locus occurred (Kwa *et al.,* 1993; Roos *et al.,* 1995). However, there may be other ways of attaining resistance without losing genetic variation and this will be investigated amongst UK isolates of *T. circumcincta.*

5.1.2 Use of single strand conformation polymorphism in analysis of population genetic diversity

A number of techniques are available for analysing sequence variation at a single locus, however, single strand conformation polymorphism (SSCP) analysis was considered the most appropriate method for this study due to its simple, rapid, and cost-effective nature. SSCP does not involve a complex assay design or the use of radioisotopes, it is very sensitive to mutations and it highlights variation at the individual worm level. Whilst sequencing is required at first to confirm the identity of alleles constituting each haplotype, thereafter SSCP

can be used as a simple, rapid diagnostic assay for the presence or absence of particular alleles. The methods and attributes of SSCP are described in detail in Chapter 1, Section 1.5.6.

5.1.3 Previous studies of the effects of selection upon the isotype I β-tubulin gene

A number of studies have been conducted in recent years which have assessed the impact of anthelmintic selection upon the genetic diversity of isotype I β-tubulin alleles using a population genetics approach (Roos *et al.,* 1990; Kwa *et al.,* 1993; Beech *et al.,* 1994; Silvestre & Humbert, 2002; Otsen *et al.,* 2001; Eng & Prichard, 2005). Roos *et al.,* (1990) used RFLP to study the effects of BZ selection upon the variation of α and β-tubulin genes in *H. contortus*. Prior to selection, the susceptible populations commonly showed between two and six hybridising RFLP fragments when genomic Southern blots were probed with β-tubulin. However, selected (and phenotypically BZ resistant) populations showed a maximum of two fragments, suggesting a loss of polymorphism at this locus was associated with the development of BZ resistance. Moreover, only two rounds of selection were required to generate resistance, leading the authors to conclude that the alleles responsible for resistance must have been relatively common within the population prior to selection. Similarly, Kwa *et al.,* (1993) used RFLP to study the effects of gradual BZ selection upon the isotype I and II β-tubulin genes of *H. contortus* and found a reduction in genetic diversity associated with resistance. The number of hybridising isotype I fragments was reduced from four to only one. Another study using RFLP and sequencing was used to compare the genetic variability of isotype I and II β-tubulin genes amongst two independently derived BZ resistant *H. contortus* isolates and a BZ susceptible isolate (Beech *et al.,* 1994). Genetic variation was greatly reduced as a consequence of BZ selection across both isotype I and II β-tubulin loci in terms of the number of alleles observed and the nucleotide diversity estimates. Again, selection for two particular alleles of the isotype I gene was apparent.

There may also be effects of other anthelmintics upon the isotype I β-tubulin locus, besides BZ. For example, Eng & Prichard, (2005) employed SSCP and RFLP techniques to compare the genetic diversity of a number of candidate resistance genes amongst IVM-selected and -naïve *Onchocerca volvulus* worms. Due to the difficulties of resistance diagnosis in this

species, this study could only compare worms from hosts, which had either been treated with IVM a number of times versus those which had never received treatment. Since worms were collected from human hosts in a number of locations in the same region, Founder effects could not be held responsible for any observed differences between isolates. The authors reported significant differences in the allele frequencies of the isotype I β-tubulin gene between the unselected and the IVM-selected specimens. The consistent nature of these differences between selected and unselected worms, led the authors to propose that the isotype I β-tubulin gene (or a gene that segregates with it) was under selection by IVM. Moreover, this selection resulted in a change in the amino acid sequence between alleles of IVM-treated and naïve worms. In particular, Met117Leu, Val120Iso and Val124Ala, which are located in the H3 helix of the proposed β-tubulin secondary structure, were observed. An intron deletion (24bp) was also apparent in IVM-selected worms. Subsequent studies have reported a strong association of this selected allele with the (crude) phenotypic estimation of IVM resistance in this species* (Eng *et al.,* 2006). Similarly, these authors have reported an effect of IVM selection upon the isotype I β-tubulin gene of *H. contortus*. SSCP analysis of the coding region (amino acid positions 195 to 235), displayed evidence of IVM selection upon allele frequencies. Furthermore, effects upon the F200Y isotype I β-tubulin mutation of this gene have been implied as a consequence of IVM selection. An increase in the frequency of $P200^{Phe/Tyr}$ (but not $P200^{Tyr/Tyr}$) genotypes at this locus was apparent. This did not alter the phenotypic level of BZ resistance observed in this isolate, however, the authors point out that an increase in the frequency of the resistant allele could obviously lead to BZ resistance if the F200Y isotype I β-tubulin locus is the sole determinant of BZ resistance. Whether the effects of IVM selection upon this locus are due to genetic hitchhiking or potential cross-resistance mechanisms remain to be determined. Thus, there is plenty of evidence to suggest that the isotype I β-tubulin gene is under selection by one or more broad spectrum anthelmintics. Hence, the effects of drug selection upon this gene in the *MTci5* isolate will be analysed using SSCP. However, this chapter aims to do more than just examine the effects of selection at this gene in one population. The SSCP technique will also be used to characterise alleles from this locus in a number of other *T. circumcincta* isolates with varying levels of anthelmintic resistance in order to examine the origins and diversity of resistance alleles.

* This involves analysing the number of circulating microfilariae following IVM treatment, a stage of the worm which is thought to be most sensitive to the effects of IVM exposure.

5.1.4 Previous study of the origins and diversity of BZ resistance alleles

The only previous study which has conducted extensive sequencing of alleles to investigate the origin and diversity of BZ resistance alleles amongst trichostrongylid nematode populations was conducted upon nematode communities in closed goat farms in Central and South-Western France (Silvestre & Humbert, 2002). In studying closed populations, the possibility of the spread of resistance alleles via gene flow was excluded. Hence, their findings would be used to determine which of the two remaining possible mechanisms was responsible for the creation of BZ resistance alleles. These possibilities were that a) resistance alleles were pre-existing, i.e. occurring at low frequencies in the population prior to selection or b) that alleles have arisen via spontaneous mutation in response to selection. In essence, the authors reported a total of eight different BZ resistance alleles in *T. circumcincta* across all farms, with each farm only showing a maximum of two resistance alleles. Some alleles were common amongst farms of the same region and given that the goat herds were closed prior to the introduction of BZ to the market, the conclusion was reached that BZ resistance alleles were pre-existing. However, resistance alleles were also specific to the region they were found in, suggesting two separate origins of resistance. The authors concluded that BZ resistance arose in France by two mechanisms: by the presence of pre-existing resistance alleles and by the occurrence of spontaneous mutations leading to resistance.

This study design sampled few individuals from many farms, and in doing so, identified the most common resistance alleles in each population. However, the research described in this Chapter will adopt a different approach, whereby one population, which has been subject to the influence of gene flow throughout its development of BZ resistance, will be characterised in great detail. Subsequently, it is hoped that the information gained via SSCP and sequencing of selected alleles from the *MTci5* isolate will allow some basic characterisation of the genetic diversity of the other UK isolates; thus negating the requirement for mass sequencing.

5.1.5 History of UK *T. circumcincta* isolates to be characterised by SSCP

Six UK *T. circumcincta* isolates were selected for analysis using SSCP. These isolates vary in their resistance status from wholly susceptible to multiple drug resistant, however, all are likely to have been exposed to BZ anthelmintics in the field at some stage in their history. Their geographical origins are confined to the Central and Southern mainland and to an island

off the North West coast of Scotland. The geographical origins of each isolate are shown in Figure 5.1.

5.1.5.1 Resistant mainland isolates (MTci3, MTci4 and MTci5)

All resistant isolates have been maintained at MRI and passaged at least twice annually. *MTci3* is a BZ resistant isolate which was originally isolated from sheep on Firth Mains farm (near Penicuik, Central Scotland) in 1983.

The origins of the *MTci4* isolate can be traced back to the 1980s. Some feral goats were captured at Glen Saugh (near Aberdeen, Scotland) and transferred to Sourhope Farm (near Kelso in the Scottish Borders) in 1985. These animals had been treated with BZ prior to their transferral to Sourhope Farm, but the extent of this treatment is unknown. They were treated at four week intervals with BZ from April to October from 1985 to 1988 whilst at Sourhope Farm. In the summer of 1988, some goats were diagnosed with severe parasitic gastroenteritis and it was eventually confirmed that BZ was ineffective in treating this condition. From late autumn 1988, IVM was used to drench the goats at five week intervals and a controlled efficacy test that was conducted during this time suggested that IVM was effective with the exception of some low-grade early L_4 survival (94.4% efficacy). In the spring of 1991, after two full seasons of IVM use, some reduced efficacy in lactating does was apparent. A CET determined the efficacy of IVM at 67.5% and at this stage, the population, now referred to as *MTci4* was isolated (Jackson *et al.*, 1992a, b, c). It is estimated that seven generations of BZ-resistant *T. circumcincta* were exposed to IVM before resistance became apparent (F. Jackson, personal communication).

5.1.5.2 Susceptible mainland isolates (MTci1 and MTci2)

All susceptible isolates have been maintained at MRI and passaged at least twice annually. *MTci1* is a drug susceptible isolate, which was isolated in 1979 from lambs grazing on pastures within MRI. *MTci2* was obtained from the Central Veterinary Laboratories (Weybridge) in 2000 (exact field origin unknown). *MTci2* is known to be susceptible to IVM and LEV, however, mild BZ resistance is suspected from previous egg hatch assays.

5.1.5.3 Susceptible geographically isolated population (ScKiTc)

This isolate is represented by a number of adult worm lysates comprising a mixture of *Teladorsagia* spp, (supplied by V. Grillo, J. Gilleard, B. Craig and J. Pemberton, Universities of Glasgow and Edinburgh). The classification of *Teladorsagia* spp remains contentious, whether this complex represents morphological variants or cryptic species. Evidence was provided for the former theory in a study of the ITS-2 region, which failed to highlight any significant differences between the sequences of different morphs (Stevenson *et al.,* 1996). Furthermore, no genetic differentiation was detected between these apparent variants using a panel of microsatellite markers (Grillo, V., Craig, B., Pemberton, J. and Gilleard, J., unpublished data).

These worms were isolated from feral Soay sheep on the island of Hirta (St Kilda) and these sheep are known to have periodic population crashes as a result of starvation or parasitism (Gulland, 1992). The flock of 107 animals was originally brought from the island of Soay in 1932 and it has been a 'closed flock' ever since (Jewell *et al.,* 1974). Whilst *ScKiTc* is classed as a susceptible isolate, it is possible that some sheep may have been dosed with anthelmintic for research purposes in the past. Therefore, it is uncertain how much selection these parasites have encountered, but it is likely to be far less than their mainland counterparts.

5.1.6 Aims of study

This Chapter aims to answer the following questions about the *MTci5* isolate in particular:

- *How diverse are the isotype I β-tubulin alleles in the unselected MTci5 isolate?*
- *Are there any effects of drug selection upon SSCP allele frequencies in MTci5? This will indicate whether the isotype I β-tubulin alleles are under selection by BZ, IVM or LEV.*
- *How many haplotypes carry the F200Y isotype I β-tubulin mutation? If several haplotypes carry the resistance allele, this suggests that the F200Y isotype I β-tubulin mutation is an "ancient" pre-existing polymorphism or that there has been a migration of resistance alleles from multiple sources. If only one resistance allele is found, this would be more consistent with the F200Y mutation being of more recent origin.*

The following questions are posed regarding the characterisation of the remaining five isolates:

- *What is the frequency of the F200Y isotype I β-tubulin mutation amongst other isolates?*
- *Are the same or similar isotype I β-tubulin resistance alleles found in each of the different isolates?*
- *Do the isotype I β-tubulin haplotypes in the ScKiTc population differ from those in the other populations e.g. due to lack of gene flow or population bottlenecks?*
- *Are there differences in the isotype I β-tubulin haplotypes between the two mainland BZ susceptible isolates (MTci1 & MTci2)?*
- *Could this analysis be used as a diagnostic tool for the presence of BZ resistance alleles in a population?*

5.2 Characterisation of β-tubulin isotype I alleles from MTci5 isolate

5.2.1 SSCP assay design

The region of the isotype I β-tubulin gene selected for SSCP analysis was a 276bp fragment that included part of two exons extending from codons 151 to 216 (see Figure 5.2). This region was selected to include the F167Y and F200Y mutations due to the evidence in the literature that these amino acid substitutions may confer BZ resistance (Elard *et al.,* 1998; Elard & Humbert, 1999; Silvestre & Cabaret, 2002). The chosen sequence also contained a 120bp intron, since it was considered that more polymorphism would reside in non-coding sequence. Furthermore, the choice of fragment size for SSCP genotyping to a large extent determines the level of polymorphism that is likely to be seen. A smaller fragment will generally produce simple SSCP patterns, but will show little polymorphism. A larger fragment will tend to have more complex patterns that are more difficult to resolve, but will contain much greater polymorphic information content. The fragment analysed in this study was selected as it encompassed an intron to maximise the level of polymorphism being assayed. The following primers were used in PCR: Forward 5'-CCAAAATTCGCGAGGAGTA-3' and Reverse 5'-TTTCAAGGTGCGGAAGCAGA-3' under stringent conditions to prevent generation of PCR artefacts. The quality and efficiency of the PCR was estimated by running a random subset of the samples on a 1.5% agarose gel. If single strong bands were apparent, the samples were then used for SSCP analysis.

SSCP analysis was first used to characterize isotype I β-tubulin alleles from the unselected *MTci5* population in order to create a frame of reference for the drug-selected *MTci5* populations and the other isolates.

5.2.2 Approach to allele characterisation using SSCP and sequencing

5.2.2.1 Step One: classification of SSCP genotypes

As an initial step, 72 adult male worms from the unselected *MTci5* isolate were genotyped by SSCP. Each novel banding pattern encountered was given an arbitrary identification number

(see Figure 5.3). In the unselected *MTci5* population, 14 different banding patterns (or genotypes) could be discerned[Ψ]. These genotype banding patterns were then "decoded" into their constitutive individual alleles, by cloning individual alleles from a representative worm. The SSCP patterns of the single cloned alleles were then compared back to the SSCP patterns of the original worm genotype. This is now explained in more detail.

5.2.2.2 Step Two: classification of SSCP alleles

Two representatives of each of the 14 genotype classes in the unselected *MTci5* population were selected and the PCR products were cloned into the PGEM-T Easy vector. Eight transformed colonies from each cloned genotype were archived, each in 50µl of distilled water per well of a 96-well plate. Four individual colonies derived from each individual worm were immediately re-amplified by PCR using the same primers. These amplified clones were subsequently run on an SSCP gel adjacent to the worm genotype from which they were derived (see Figure 5.4). This allowed the SSCP patterns of the individual allele that comprised each of the 14 SSCP genotype classes to be determined. Each novel allele encountered was assigned an arbitrary identification (a letter). In cases where the four clones derived from a single worm all produced identical patterns which exactly matched the corresponding worm genotype, it was concluded that the worm was homozygous for that allele. In cases where all four colonies derived from a single worm produced identical patterns but these did not account for all of the bands in the corresponding worm genotype, the remaining four archived colonies were amplified by PCR to check for the presence of an additional allele. This process resulted in the identification of five SSCP allele classes that could be reliably and consistently differentiated and these were designated A, B C, D and E (Figure 5.5). The genotyping was extended into the drug-selected populations of *MTci5* worms (72, 76 and 69 worms from the BZ-, IVM- and LEV-selected populations, respectively) and any new genotype which could not be matched to the existing allele 'library' was itself cloned and checked as previously described.

It is important to emphasise that there is a limit of the ability of SSCP to clearly differentiate all polymorphisms in a PCR fragment. Hence each allele class is likely to contain a number of different sequence variants. This is not a problem for genetic analysis as it is equivalent to

[Ψ] The arbitrary identification of genotype profiles was only necessary at this stage and this type of classification was not used later in the analysis. Some profiles labelled as unique genotypes were later found to comprise the same alleles, however, this system was over-cautious and did not underestimate the genetic variation at this locus.

"binning alleles" as is routinely done for many types of genetic marker, such as microsatellite alleles for example. Sequence variation within an allele class was suspected by the fact that there was some variation in the precise SSCP pattern of those alleles designated as class D and E (Figure 5.6). No attempt was made to place these subtle variants into separate allele classes as the resolution of the SSCP did not allow them to be consistently differentiated. The variation within each of the allele classes was investigated by sequencing.

5.2.2.3 Step Three: characterisation of SSCP alleles by sequencing

A number of clones from each allele class were selected for sequencing in order to assess the extent of sequence variation within each allele class. These clones were derived from all four *MTci5* populations (unselected and drug-selected) and were, for the most part, selected at random. However, due to the high level of variation witnessed amongst class D alleles, as many different clones for this allele class were selected for sequencing as possible, in order to obtain a good measure of the sequence variation in the population. Cloned alleles for sequencing were derived from a total of 28 *MTci5* adult worms. Worms which were clearly heterozygous for each allele class were generally chosen. In total, eight representative alleles of class A, six of class B, six of class C, nine of class D and five of class E were sequenced. Details of these alleles will be discussed later in this Chapter.

5.2.3 Analysis of SSCP allele data from the unselected *MTci5* population

5.2.3.1 SSCP Allele and genotype frequencies

The frequency of each allele class in the unselected *MTci5* population is displayed in Figure 5.7. There was a high frequency of allele class A (49%) in the unselected *MTci5* isolate, followed by class D at 26%. Allele classes B, C and E all had frequencies of less than 12%. Observed genotype frequencies are displayed in Figure 5.8. There were 11 different genotypes in total and the most common genotypes were A/A (n=18) and A/D (n=15). Genotypes with counts of between five and ten individuals included: A/B, A/C and C/D. Rarer genotypes (fewer than five) included: A/E, B/C, B/D, C/E, D/D and D/E; and those not found in the population were B/B, B/E, C/C and E/E.

5.2.3.2 Test for Hardy-Weinberg Equilibrium in the unselected MTci5 population

Chi-square analysis was performed to test whether the different SSCP genotype frequencies were in Hardy-Weinberg Equilibrium (HWE) in the unselected *MTci5* population. This analysis was conducted using the GenAlEx add-in software (Version 6, Peakall & Smouse, 2006) for Microsoft Excel (see Figure 5.8). The analysis showed no significant deviation from HWE ($p>0.05$).

5.2.3.3 Relationship between F200Y isotype I β-tubulin mutation and SSCP haplotype

The relationship between the SSCP allele classes and the F200Y isotype I β-tubulin mutation was investigated since the SSCP profiling and F200Y genotyping data were obtained from the same individuals. Whilst the homozygous resistant ($P200^{Tyr/Tyr}$) and homozygous susceptible ($P200^{Phe/Phe}$) individuals could be included in the analysis, the heterozygous individuals ($P200^{Phe/Tyr}$) had to be discounted as it would not be possible to determine the relationship between the SSCP alleles and the F200Y isotype I β-tubulin mutation, i.e. which SSCP allele was associated with $P200^{Tyr}$ or $P200^{Phe}$. A sample size of 9 $P200^{Phe/Phe}$ worms and 32 $P200^{Tyr/Tyr}$ worms was available. It is unfortunate that the pool of $P200^{Phe/Phe}$ genotypes was so small, however, it was important to identify which SSCP allele classes carried resistance. As an initial step, the numbers of each SSCP genotype were counted per $P200^{Tyr/Tyr}$ and $P200^{Phe/Phe}$ groups (Figure 5.9a) and then this was used to calculate the numbers of each allele class which were resistant or susceptible at the F200Y isotype I β-tubulin locus (Figure 5.9b). The frequencies are expressed as percentages in Figure 5.9c. This revealed that SSCP allele classes A, B, and D contained a mixture of both resistant and susceptible alleles confirming the previous suggestion that there is sequence variation within each class. Nevertheless, the most common SSCP allele class in the *MTci5* isolate (A) predominantly consists of F200Y isotype I β-tubulin resistance alleles. The striking conclusion of this analysis is that the F200Y isotype I β-tubulin mutation is present on at least four different haplotype backgrounds.

5.2.4 Analysis of SSCP allele data from the drug-selected *MTci5* populations

5.2.4.1 Allele and genotype frequencies

The same classification system described in Section 5.222 was used to identify alleles from each drug-selected population. The allele frequencies from the four *MTci5* populations are shown in Figure 5.10. The allele frequencies appeared broadly similar amongst the four *MTci5* populations. However, the frequency of class A alleles appeared greater in the BZ- and IVM-selected populations, and the frequency of class B alleles was highest in the unselected population. BZ selection increased the overall frequency of the class A alleles by 13% (48.6% to 61.8%). Class D alleles were the second most common alleles whereas class B and E alleles were relatively rare in the *MTci5* isolate. However, there were no significant differences between the allele frequencies of each population, as determined by multiple 'tests of two proportions' conducted in Minitab v 14 (data not shown).

The genotype frequencies were calculated per population (Figure 5.11) and tests of two proportions were conducted in Minitab v14. The frequency of A/B genotypes was significantly lower in the BZ-selected ($z = -3.16$, $p = 0.002$) and IVM-selected populations ($z = -3.16$, $p = 0.002$) when compared with the unselected population. Furthermore, there was a significantly higher frequency of A/D genotypes in the BZ-selected population compared with the unselected ($z = 2.49$, $p = 0.013$) and IVM-selected populations ($z = -2.33$, $p = 0.02$). There was also a significant decrease in the frequency of C/D genotypes in the BZ-selected population ($z = -3.41$, $p = 0.001$) compared with the unselected population. Hence, there were significant effects of BZ and IVM selection upon the isotype I β-tubulin locus, which were detectable at the genotype level, but not at the level of allele frequencies.

5.2.4.2 Test of Hardy-Weinberg Equilibrium amongst the drug-selected MTci5 populations

Chi-square analysis was performed to test whether the SSCP genotype frequencies were in Hardy-Weinberg Equilibrium (HWE) in the drug-selected *MTci5* populations. This analysis was conducted using the GenAlEx add-in software (Version 6, Peakall & Smouse, 2006) for Microsoft Excel (see Figure 5.12a-c). The analysis showed no significant deviation from HWE in any of the populations ($p>0.05$).

5.2.4.3 Relationship between F200Y isotype I β-tubulin mutation and SSCP allele class in MTci5 isolate

The relationship between the SSCP allele class and the F200Y isotype I β-tubulin genotype was re-investigated when all *MTci5* populations were considered. This provided more $P200^{Tyr/Tyr}$ and $P200^{Phe/Phe}$ worms for analysis. The same process described in Section 5.2.3.3 of determining what proportion of genotypes and alleles carried susceptibility or resistance was used and the results are shown in Figures 5.13 & 5.14, respectively. From these results, we can see that not just three allele classes, but all five are represented by both susceptible and resistance alleles. Therefore, at least five different resistance alleles exist amongst all *MTci5* populations.

5.2.5 Preliminary analysis of isotype I β-tubulin allele sequences

In the previous Section, all individuals in the four *MTci5* populations were genotyped based on the secondary conformation of the isotype I β-tubulin alleles. The effects of drug selection upon these SSCP-defined alleles were also investigated. We sequenced a number of cloned alleles from each SSCP allele class to gain more information about the extent of sequence diversity at this locus in the *MTci5* isolate. Furthermore, the sequence information would give a clearer indication of how many BZ resistance alleles and how many susceptible alleles were present in the *MTci5* isolate. Eight alleles from allele class A, six from class B, six from class C, nine from class D and five from class E were selected for sequencing. All allele clones were profiled using SSCP prior to sequencing to ensure they had been identified correctly. Clones were derived from 28 *MTci5* adults in total. Up to four independent clones for each allele were sequenced from each individual. Generally heterozygous worms were chosen for sequencing of alleles. This was to ensure that duplicate clones of the same allele class could be guaranteed to be from the same allele. For example, if two A-type clones were sequenced from an A/D heterozygote then both clones must be derived from the same A-type allele. However, if two A-type clones were sequenced from an A/A worm, these clones could potentially be from two different A-type alleles. This approach allowed the comparison of the duplicate clones to be used to distinguish PCR induced mutations and sequencing errors from true polymorphisms.

5.2.5.1 Allele class A sequences

Sixteen sequences from eight individuals were obtained for allele class A. The alignment was identical, except for two errors, showing the presence of one resistance haplotype (Figure 5.15). These errors were either sequencing- or PCR- induced as they were not common to the duplicate sequences from that allele. Hence, only one sequence type was found and it carried the F200Y isotype I β-tubulin mutation. Three A/A homozygotes were used: PI02; PT02 and PT03. Homozygotes were used in this case because one of the worms (PT03) was genotyped as $P200^{Phe/Tyr}$ at the F200Y isotype I β-tubulin locus (whereas PI02 and PT02 were $P200^{Tyr/Tyr}$), therefore, 2 different A-type alleles (which were indistinguishable by SSCP) must exist. However, the susceptible allele was not found by sequencing, despite amplifying, cloning and sequencing four times. This may be due to a cloning bias against the susceptible allele. It is likely that there are two alleles in this class, one resistant and one susceptible. The product length of the resistant A allele examined, including intron sequence, was 270bp.

5.2.5.2 Allele class B sequences

Fifteen sequences from a total of six (heterozygous) worms were obtained for allele class B (Figure 5.16). Three different haplotypes were found in this group: two susceptible and one resistant. This variation was not detectable in the SSCP analysis. However, this is perhaps unsurprising since only two mutations distinguish these alleles, at 126 and 249 (the latter position is the site of the F200Y isotype I β-tubulin SNP). Interestingly, worm PB33 (which was the only surviving BZ-selected adult with a homozygous susceptible genotype, i.e. $P200^{Phe/Phe}$) showed a SNP at base 126, which was not found in any other SSCP allele. This was found in the intron region and, therefore, does not alter the translated coding sequence, however, unique SNPs may still be potential markers for resistance (perhaps if they are genetically linked changes in the same gene or to external loci). The product length of the class B alleles examined was 278 bases.

5.2.5.3 Allele class C sequences

Fourteen sequences from six (heterozygous) worms were included in the type C allele dataset (Figure 5.17). There were three different haplotypes found in total, one of which was resistant for the F200Y isotype I β-tubulin mutation (PT05, PT11, PT12 & PT15) and two which were susceptible (worms PT20 and PT19). One of the susceptible alleles (from worm PT20) was found to have two deletions (one of 5bp and the other of 13bp) in the intron region, which

were not apparent in any of the other allele classes. It also had three unique SNPs in the intron region distinguishing it from the other C alleles. However, this allele was more closely related in sequence to this class, than any other. Despite the disparity in sequence, the SSCP profile of this allele looked very similar to the other examples of this class. However, on a gel which had particularly high resolution, it appeared as two bands placed very close together, although, most of the time it appeared as one strong band due to lack of separation and thus looked very similar to the other C alleles. The PT19 susceptible allele was identical in sequence to the resistant allele from worms PT11, PT12 & PT15, except for the F200Y isotype I β-tubulin SNP. The product lengths of the C alleles were either 260bp (PT20) or 278bp (all others).

5.2.5.4 Allele class D and E sequences

The sequencing of class D and E alleles identified a total of nine D-type alleles and four E-type alleles. The differences between sequences within these classes were almost as great as those between classes, and thus, for the purpose of data analysis, the sequences of both D- and E-type alleles have been aligned together (Figure 5.18). Given the high level of variation amongst these alleles, it is unfortunate that they could not be discriminated more accurately and consistently by SSCP. The great diversity in sequence observed amongst D/E alleles is highlighted by the number of different SSCP profiles that were generated (as illustrated by Figure 5.5). There were a total of 15 SNPs, eight of which were found in the intron region. There were also three indels that were all found in the intron region. The product lengths varied between 275 and 276 bases. The D/E alleles were predominantly susceptible, with only four resistant alleles and nine susceptible alleles.

5.2.5.5 Test of Hardy-Weinberg Equilibrium when D and E alleles are grouped in one class

Chi-square analysis was performed again after D and E alleles were grouped together to test if this highlighted any deviation from Hardy-Weinberg Equilibrium (HWE) in any of the *MTci5* populations. This analysis was conducted using the GenAlEx add-in software (Version 6, Peakall & Smouse, 2006) for Microsoft Excel (see Figure 5.19a-d). The analysis showed no significant deviation from HWE in any of the populations ($p>0.05$), thus, the classification of D and E as separate allele classes did not affect the results of the Chi-square analysis.

5.2.5.6 Summary

The sequence information confirmed the classification system borne out of the SSCP analysis for three out of five allele classes. Alleles within classes A, B or C were more similar in sequence than those between classes. However, there was not a clear separation of D and E alleles based on sequence identity with some alleles from class D being more similar to those of class E (see Figure 5.18).

5.2.6 Phylogenetic analysis of allele sequences

5.2.6.1 General observations

The alignment of all SSCP alleles is shown in Figure 5.20. This was created using Genedoc (v 2.6003, Nicholas & Nicholas, 1997) and the consensus sequence for each allele was used. The main differences between the alleles are summarised in Tables 5.1 and 5.2. In these Tables, class A/B/C alleles and class D/E alleles are compared separately due to the large number of differences between these two groups. The sequences of the alleles, as generated by PCR using the same primers, were not all the same length, varying up to 18bp in some cases. For instance, the $C3^S$ allele was the shortest sequence at 260bp, followed by $A1^R$ at 270bp and the largest fragments were found in the B and C classes at 278bp. Whereas all of the D/E alleles were either 275 or 276bp long. The major (and consistent) differences between these two groups are summarised in Table 5.3. These differences are primarily located in the exon 2 region. However, all mutations were synonymous, that is, they did not cause a change in amino acid sequence except for position 249 corresponding to the F200Y substitution. There were nine transversions and ten transitions between these groups, which is perhaps surprising as transitions are thought to occur far more frequently in nature (approximately every two out of three SNPs are usually transitions). There was one consistent indel distinguishing the A/B/C alleles from the D/E alleles, in the intron 1 region.

From this analysis, it appears that there at least seven resistant alleles and 13 susceptible alleles in the *MTci5* population. Despite 28 individuals being a modest sample size, the SSCP technique acted as a pre-screening method to select alleles for sequencing. More of the D and E alleles were sequenced (than any other class) due to the high level of variation apparent from the gel profiles. Thus, whilst it is likely that there are more alleles in the *MTci5*

population which were not characterised by this study, this analysis probably accounts for most of the variation present.

5.2.6.2 Descriptive statistics

Using the program DnaSP version 4.0 (Rozas *et al.,* 2003), some descriptive statistics were carried out upon the consensus sequences for each novel allele. The sequences were aligned first using Genedoc (v 2.6003, Nicholas & Nicholas, 1997) as DnaSP requires all sequences to be of the same length. The total length of the sequence analysed was 278bp but only 248 bases were considered due to alignment gaps. Of these sites, there were 207 monomorphic sites (invariable), and 41 polymorphic sites. Of these polymorphisms, 10 were singletons (or non-informative polymorphisms found in one allele), 31 were parsimony informative sites (those that that have a minimum of two nucleotides that are present at least twice in the sequence). There was one non-synonymous mutation at base position 249 (site of F200Y isotype I β-tubulin mutation) and 13 synonymous changes found in the exon regions (see Table 5.3).

5.2.6.3 Nucleotide diversity (π)

The DnaSP version 4.0 program (Rozas *et al.,* 2003) was used to estimate the nucleotide diversity (π) amongst the alleles, including variance and standard deviation. The calculation of π was carried out both including and excluding the Jukes-Cantor correction (Jukes & Cantor, 1969). The program has an option to define coding and non-coding regions to enable such estimates to be calculated for each region of the sequence: the entire length (bases 1-278); the exon 1 region (bases 1-49); the intron 1 region (bases 50-175) and the exon 2 region (bases 176-278).

The nucleotide diversity estimates are displayed in Table 5.4. The Jukes-Cantor correction did not have a very large influence on the estimate of nucleotide diversity in both exons (correct to two decimal places), suggesting that even though there is a large amount of variation amongst alleles, the probability of multiple hits influencing the analysis is low. The sampling variance and standard deviations (square root of the variance) for these regions were also encouragingly small. However, there was a larger difference between π and π (J-C corrected) in the intron region and this was accompanied by a larger standard deviation value. Thus, the true nucleotide diversity of intron 1 can be said to be somewhere between 0.086 and 0.095 average

differences per site. As one would expect from the higher mutation rate of non-coding DNA, the nucleotide diversity of the intron region was much higher than that of the exon regions. This is very similar to that estimated by Beech *et al.,* (1994) for the same intron I region of isotype I β-tubulin from an unselected isolate of *H. contortus*, which displayed a π value of 0.094. This gives confidence in the analysis and suggests that BZ selection has not affected the rate of divergence of the intron region. Interestingly, the estimate of π for exon 2 was double that of exon 1, and this concurs with the observation that BZ selection is strongly applied upon codon 200, which resides in exon 2.

5.2.6.4 Haplotype diversity

The DnaSP version 4.0 program (Rozas *et al.,* 2003) was used to estimate haplotype diversity (Nei, 1987). Table 5.5 displays the number of haplotypes and estimates of Hd for each region of the sequence, as well as the overall estimate for the entire length. Again, it is apparent that the intron region is responsible for generating the largest number of haplotypes (n = 15), and also that exon 2 explains a greater amount of haplotype diversity (n = 6) than exon 1 (n = 3). The Hd estimates suggest that exon 1 is most highly conserved amongst haplotypes (0.511), whereas intron 1 shows a very high Hd value (of 0.974) suggesting that this region diverged some time ago. To a lesser extent, exon 2 seems to have undergone divergence (Hd value of 0.789) and this value is much higher than one would expect if recent divergence had occurred, for instance in response to BZ selection. However, the positioning of the F200Y SNP in exon 2 may account for this variation in haplotype diversity.

5.2.6.5 Haplotype neutrality

The DnaSP version 4.0 program (Rozas *et al.,* 2003) was used to conduct neutrality tests. As described previously, each region was tested individually, since it is possible that the entire region shows haplotype neutrality, but that parts of the sequence deviate from neutrality, due to recombination events. Such haplotype blocks can result from positive selection upon single codon substitutions, such as the F200Y isotype I β-tubulin mutation*. The results of the neutrality tests are shown in Table 5.6 and include estimates of: Tajima's D statistic (1989); Fu & Li's D statistic (1993); Fu & Li's F statistic (1993); Fu's F Test (1997) and Strobeck's (S) probability statistic (1987). The expectation of Tajima's D statistic under the neutral

* There was an example of such a case in a study of the 'white gene' amongst populations of *Drosophila melanogaster* (Kirby & Stephan, 1995) whereby part of the gene showed a highly significant deviation from neutrality, but analysis of the entire region failed to highlight this.

model is zero. When D is positive, as it is in this case, then it suggests that balancing selection has created an excess of alleles in intermediate frequencies. However, these were not significant positive values ($p>0.05$); therefore, this suggests that all regions of the sequence do not deviate significantly from haplotype neutrality. Similarly, there was no indication of a deviation from neutrality across any region from Fu & Li's D statistic (1993), Fu & Li's F test (1993) or Fu's F test (1997), since all estimates of all regions indicated non-significant results ($p>0.05$). Strobeck's S statistic also showed no significant S values in any region of the sequence; thus, the haplotype frequency distribution follows a neutral model. However, the sequences examined do not represent a random sample of alleles from the *MTci5* population, and the F200Y isotype I β-tubulin mutation is already present at a high frequency in this sample, hence, it is not surprising that these tests do not show a deviation from haplotype neutrality.

5.2.6.6 Clustal W tree analysis

Phylogenetic analysis was used to reveal the relationship between the allele classes and is shown in Figure 5.21. Consensus sequences were first generated for each allele in each individual using Seqman (DNA star v5.08, 2004) and then aligned via the Clustal W method (Higgins, 1994) using MegAlign software (DNA star v5.08, 2004). The alleles separated well into A, B and C classes, whereas the D and E alleles were not well separated. This indicated that the D/E alleles should not have been classified separately by SSCP and from this point forward, will be treated as one class of alleles. As suspected from the preliminary examination, the Clustal W analysis revealed two distinct lineages: one leading to A, B and C alleles and the other leading to D/E alleles.

5.2.6.7 Comparison with published French T. circumcincta isotype I β-tubulin alleles

Similar findings have been reported previously, whereby sequence analysis of this gene has revealed two distinct types of isotype I β-tubulin (Leignel *et al.*, 2002; Silvestre & Humbert, 2002). Silvestre & Humbert (2002) termed these as *'Type I'* and *'Type II'* based on the synonymous mutations and indel listed in Table 5.3. Each type represents a number of different alleles (including both resistant and susceptible alleles) found within and between populations of *T. circumcincta* from farms in Central and Southern France (Silvestre & Humbert, 2002). The published consensus sequences of these fragments were compared with the alleles characterised by this study using Clustal W (Higgins, 1994) tree analysis

(MegAlign; DNA star version 5.08, 2004) and it was apparent that the same classification of allele type can be applied here. Not only are these published sequences (Silvestre & Humbert, 2002) homologues of those characterised in this study, but some are identical to those found in the *MTci5* isolate. For example, allele $A1^R$ shares 100% homology with allele *Type II_tcr7* and allele $C1^R$ shares 100% homology with *Type II_tcr6* alleles (see Figure 5.22). Whilst the other alleles were not identical, they still shared high homology, for example: $B1^R$, $B2^S$ and $B3^S$ all share 98-99% homology with '*Type II_tcr8*' and alleles $C2^S$ and $C3^S$ share between 93 and 98% homology with *Type II_tcr6, tcr7 & tcr8*. Similarly, the D/E alleles are more closely related to the *Type I* sequences (see Figure 5.22), and share 97-98% homology with *Type I_ tcr1, tcr2, tcr3, tcr4 & tcr5*.

5.3 Diversity of β-tubulin alleles amongst other UK T. circumcincta isolates and relationship with the F200Y isotype I β-tubulin mutation

There are very few characterization studies which take into account multiple isolates from multiple sources, with such varying degrees of resistance. Despite the limitations of the SSCP technique as discussed earlier, this study will provide valuable clues as to the variation amongst six UK *T. circumcincta* isolates. The Pyrosequencing assay for the F200Y isotype I β-tubulin mutation and the SSCP technique described earlier were applied to four other mainland *T. circumcincta* isolates and one geographically isolated population (St Kilda Soay sheep isolate). The Pyrosequencing assay only worked successfully for the mainland populations; whereas, the SSCP technique provided data for all populations.

5.3.1 Frequency of the F200Y isotype I β-tubulin mutation

The Pyrosequencing assays (described Sections 3.3 and 4.4) were used to determine the frequency of three isotype I mutations (F167Y, E198A & F200Y) amongst the four mainland *T. circumcincta* isolates. This was carried out to provide a means of a comparison with the *MTci5* isolate and also to compare with the results of the SSCP analysis for each isolate. The genotype frequencies of the F200Y isotype I β-tubulin mutation are displayed in Figure 5.23. Attempts to genotype worms from the *ScKiTc* isolate were unsuccessful and this is thought to be due to either sequence variation at the primer sites or poor template quality. Time constraints prevented troubleshooting, thus no data were obtained for the frequency of the F200Y isotype I β-tubulin mutation of this isolate.

The sample sizes were modest for the other isolates (~ 40 individual L3), however, this was expected to be sufficiently representative of the populations. The *MTci1* isolate showed no resistance alleles whatsoever, whereas the other susceptible isolate (*MTci2*) displayed a few heterozygotes with an overall frequency of the resistant allele of 7.5%. The *MTci3* isolate, known to be BZ resistant from previous phenotypic characterisation, displayed frequencies of 41% and 59% of the resistant and susceptible alleles, respectively. The *MTci4* isolate was highly resistant with overall frequencies of 90% for the resistant allele and only 10% for the

susceptible allele. These findings were similar to the *MTci5* isolate following BZ selection (frequencies of 89% for the resistant allele and 11% for the susceptible allele).

5.3.2 Frequency of the E198A and F167Y mutations of isotype I β-tubulin

As discussed in Chapter 4 (Section 4.4), there was no reason to believe the E198A mutation had occurred amongst any of these isolates due to the stringency of the F200Y isotype I β-tubulin genotyping assay. The Pyrosequencing assay for the isotype I β-tubulin F167Y mutation (described in Chapter 4, Section 4.4) was also carried out upon all four mainland isolates as part of the BZ resistance characterisation. All worms were genotyped successfully as $P167^{TTC/TTC}$ homozygotes; therefore, there was no evidence of this mutation in any of the isolates.

5.3.3 Hardy-Weinberg Equilibrium analysis: F200Y isotype I β-tubulin locus

Chi-square analysis was performed to test whether the F200Y isotype I β-tubulin genotype frequencies were in Hardy-Weinberg Equilibrium (HWE) for each isolate. This analysis was conducted using the GenAlEx add-in software (Version 6, Peakall & Smouse, 2006) for Microsoft Excel (see Figure 5.24a-c). The analysis showed no significant deviation from HWE in any of the populations ($p>0.05$). The *MTci1* isolate could not be analysed as it was monomorphic at this locus (i.e. 100% $P200^{Phe/Phe}$).

5.3.4 Use of SSCP in analysis of diversity of isotype I β-tubulin alleles

5.2.4.1 Allele frequencies amongst isolates

SSCP was used to classify alleles in these populations and all genotypes could be resolved into the same five classes that were described from the *MTci5* isolate (A to E). Due to time constraints, no sequence analysis was performed on alleles from these other isolates. D/E alleles were grouped together due to the previous finding that the SSCP classification system did not relate to the extent of sequence identity within these classes. The allele frequencies for each isolate are displayed in Figure 5.25. It seems that the allele class A group is more common in resistant isolates: *MTci3*, *MTci4* and *MTci5*, showing frequencies of 29%, 51% and 53%, respectively, whereas class A allele frequencies are less than 7% amongst the

susceptible isolates (*MTci1, MTci2* and *ScKiTc)*. Allele class B is a less common group amongst most of the isolates (all less than 5%), but is present at a high frequency in the *ScKiTc* isolate (33%). Allele class C is present at a high frequency in the susceptible *MTci1* isolate (21%) and the highly resistant *MTci4* isolate (30%), and thus, does not appear to be strongly associated with either susceptibility or resistance. Allele classes D and E are present at a higher frequency in the susceptible isolates *MTci1* and *MTci2* (75% and 93%, respectively) than in the resistant isolates or amongst *ScKiTc* worms (all less than 56%).

5.3.4.2 Test for Hardy-Weinberg Equilibrium of SSCP genotypes

Chi-square analysis was performed to test whether the SSCP genotype frequencies were in Hardy-Weinberg Equilibrium (HWE) amongst the various *T. circumcincta* populations. This analysis was conducted using the GenAlEx add-in software (Version 6, Peakall & Smouse, 2006) for Microsoft Excel (see Figure 5.26a-e). There was no significant deviation from HWE ($p>0.05$) in any of the mainland populations, however, there was a highly significant deviation ($p<0.01$) from HWE in the *ScKiTc* isolate. This suggests that geographical isolation has influenced the allele frequencies at the isotype I β-tubulin locus.

5.3.4.3 Relationship between the F200Y isotype I β-tubulin mutation and SSCP haplotype

It was decided to analyse the relationship between the SSCP genotypes and the F200Y isotype I β-tubulin mutation amongst these isolates. The *ScKiTc* isolate had to be excluded from this analysis due to the lack of information regarding the F200Y isotype I β-tubulin locus for these worms. As an initial step, the number of $P200^{Tyr/Tyr}$ and $P200^{Phe/Phe}$ homozygotes with each possible SSCP genotype was counted. Then the proportion of SSCP haplotypes* (i.e. each allele class) carrying either resistance or susceptibility in terms of the F200Y isotype I β-tubulin SNP was calculated (see Figures 5.27a-e). As stated earlier, the $P200^{Phe/Tyr}$ heterozygotes could not be included in this analysis since it could not be determined which SSCP haplotype carried the resistant or the susceptible allele. Isolates *MTci1* and *MTci2* (Figures 5.27a and b) were not helpful in this analysis either, since there were no $P200^{Tyr/Tyr}$ genotypes found in either population, thus all haplotypes appear susceptible. The *MTci4* isolate, whilst it failed to provide any $P200^{Phe/Phe}$ homozygotes for analysis, (Figure 5.27d) was

* Note that each allele class is likely to be represented by a number of alleles, therefore this calculation attempts to determine the proportion of alleles within each class carrying either resistance ($P200^{Tyr}$) or susceptibility ($P200^{Phe}$).

shown to display at least three different resistance alleles. Furthermore, the *MTci3* isolate (and *MTci5* isolate as previously mentioned, see Figure 5.27e) also showed multiple resistance alleles (Figure 5.27c). Whilst we did not sequence alleles from the *MTci3* or *MTci4* isolates, we know that these populations carry similar alleles to those found in the *MTci5* isolate, due to similarity in SSCP profiles. Therefore, the phenomenon of the F200Y isotype I β-tubulin mutation being present on multiple haplotype backgrounds has been demonstrated from three mainland UK *T. circumcincta* isolates.

Chapter 5 Tables & Figures

Figure 5.1: Map showing the approximate geographical locations of the origin of each isolate studied in this Chapter. The most recent locations for all isolates are displayed, for instance, *MTci4* was isolated from a farm in the Scottish borders but was known to have originated in Aberdeenshire.

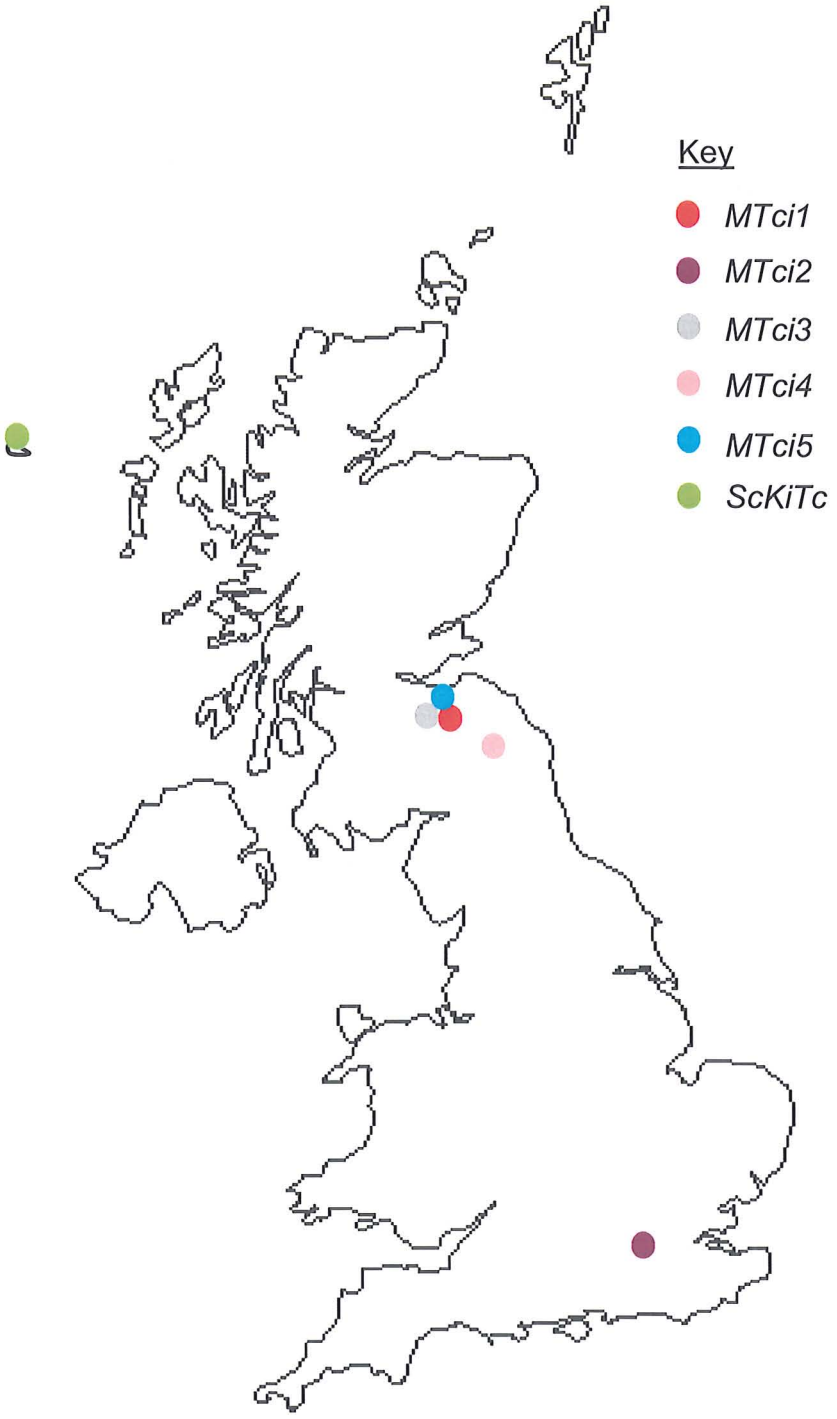


Figure 5.2: Full length cDNA of *T. circumcincta* isotype I β-tubulin gene (accession number Z96258) displaying the region analysed by SSCP. The primer sites are highlighted in pink and the position of the intron is boxed in black. The positions of the F167Y and F200Y isotype I β-tubulin mutations are highlighted in red.

MREIVHVQAGQCGNQIGSKFWEVISDEHGIQPDGTYKGESALQLEFTRINVYYNEA

HGGKYVPRAVLVDLEPGTMDSVRSGPYGQLFRPDNYVFGQSGAGNNWAKGFTHYTE

GAELVDNVLDVVRKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYPDRIF

F167Y F200Y

TMASFSVVPSPKVSDTVVEPYNATLSVHQLVENTDETFCIDNEALYDICFRTLKLT

NPTYFTGDLNHLVSVTMSGVTTCLRFPGQLNADLRKLAVNMVPFPRLHFFMPGFAP

LSAKGAQAYFTRASTVAELTQQMFDAKNMMAACDPRHGRYLTVAAMFRGRMSMREV

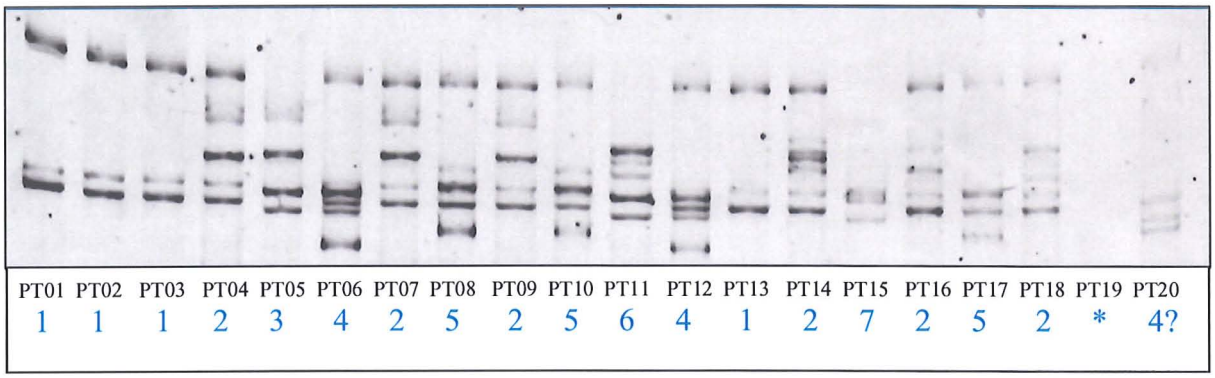
DDQMMSVQNKNSSYFTFVEWIPNNVKTAVCDIPPRGLKMAATFVGNSTAIQELFKR

ISEQFTAMFRRKAFLHWYTFTGEGMDEMEFTEAESNMNDLISEYQQYQEATADDMG

DLDAEGAEEPYPEE

Figure 5.3: Two sample SSCP gel photographs are displayed. These gels show the initial profiles of forty unselected *MTci5* adults. At this stage, it is unclear which are homozygotes and which are heterozygotes, therefore, each novel banding pattern (genotype) is first assigned an arbitrary identity of 1 to 14 (these are shown in blue). PT refers to the unselected *MTci5* adults numbers 01 to 20 (Figure 5.3a) and 41 to 60 (Figure 5.3b). An asterisk* denotes a failed PCR, which would be repeated until a clear profile was achieved. The last genotype displayed on Figure 5.3a was faint, therefore, it was also repeated to confirm the profile identity assigned to it. All bands in a profile, however faint in comparison to the rest of the bands, were taken into account. Several representatives from each profile were then cloned to separate out the alleles (see Figure 5.4).

3a



3b

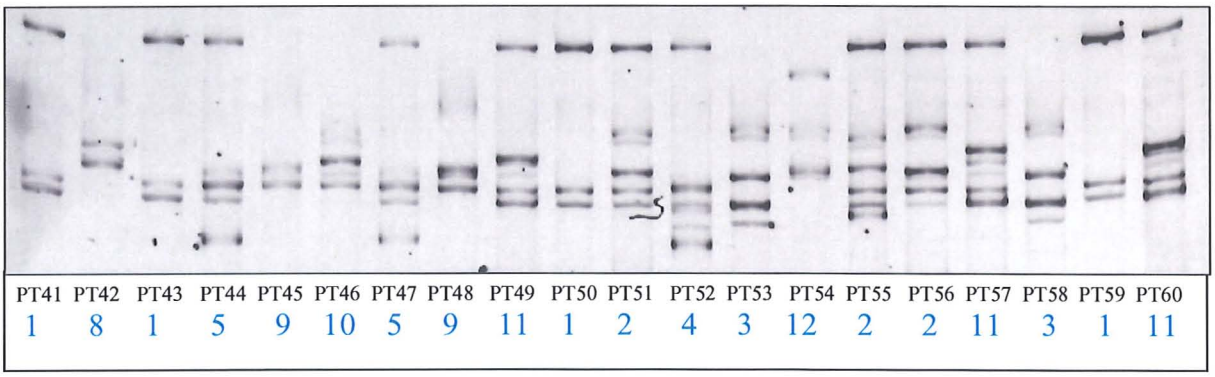
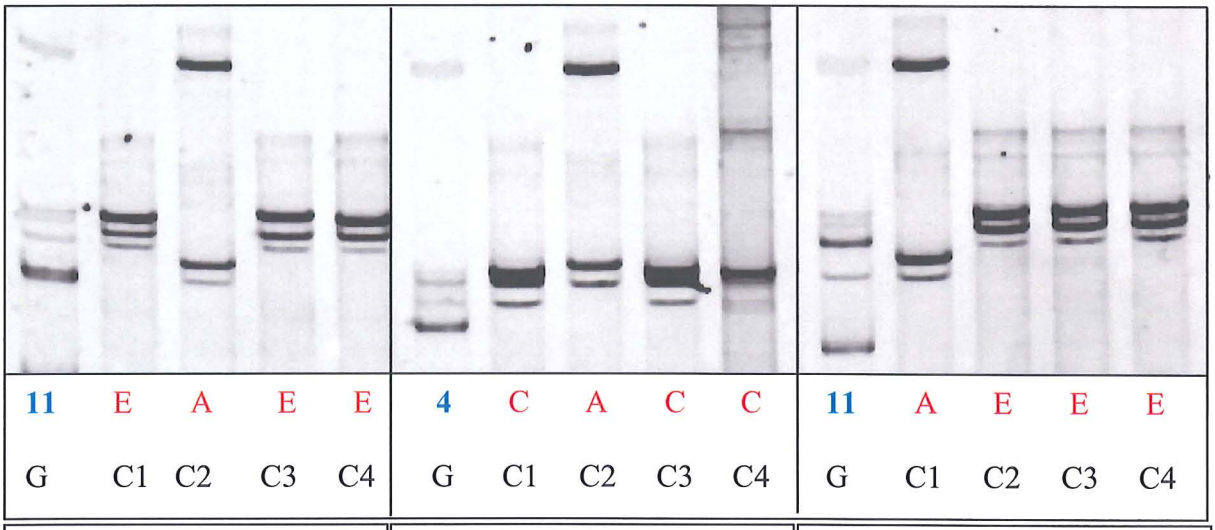
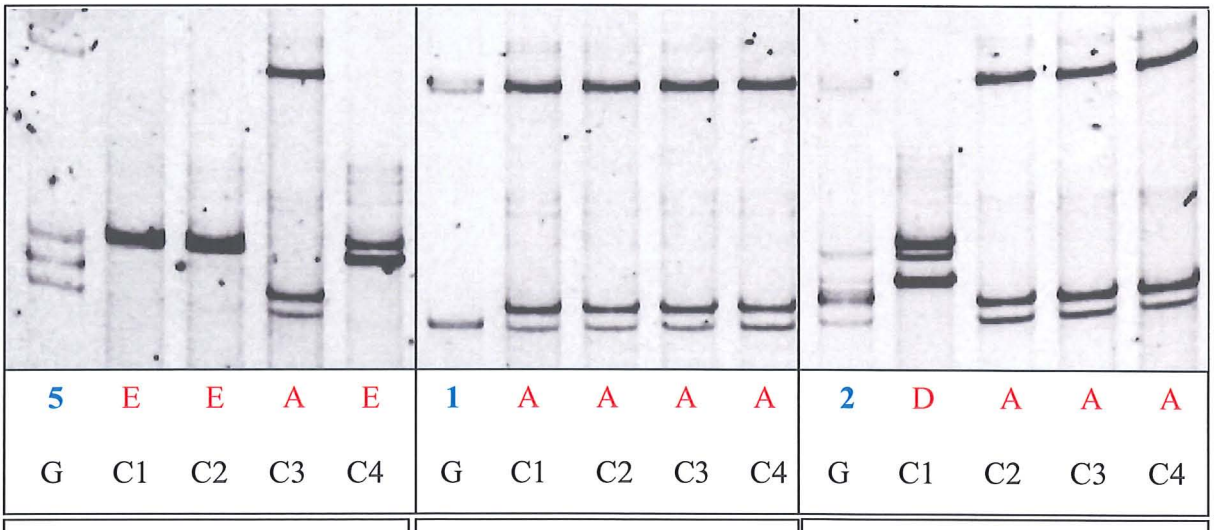


Figure 5.4: These sample gels display the cloned alleles (C1 to C4) which were run alongside the single worm genotypes (or profiles, G) from which they originated. The purpose of this exercise was to confirm that true alleles (and not PCR artefacts) had been cloned from the correct individuals and also to identify the banding pattern of each allele for classification. In order to classify these, each novel banding pattern was assigned an arbitrary identity (a letter, shown in red below). The blue numbers denote the original profile identity assigned (see previous Figure 5.3).



Worm ID: PT49 Decoded genotype: A/E heterozygote	Worm ID: PT52 Decoded genotype: A/C heterozygote	Worm ID: PT60 Decoded genotype: A/E heterozygote



Worm ID: PT44 Decoded genotype: A/E heterozygote	Worm ID: PT01 Decoded genotype: A/A homozygote	Worm ID: PT51 Decoded genotype: A/D heterozygote

Figure 5.5: This gel shows the standard form and positioning of each allele class. Most of the genotyping was conducted using these examples of alleles as 'standards' on a gel.

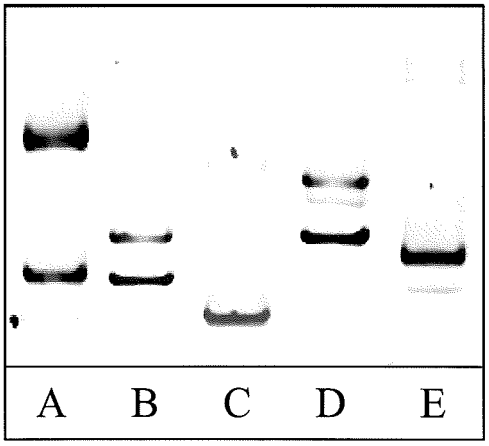


Figure 5.6: This gel demonstrates the high level of variation in secondary conformation amongst the group D alleles. The differences are mostly detectable between neighbouring lanes, however, they are very difficult to discriminate on different gels. The worm identity is denoted by PXXX. PT, PB, PI and PL refer to unselected, BZ-selected, IVM-selected and LEV-selected *MTci5* adults from trials 1 and 2 (see Chapter 3). Worms PL12 and PB33 are later shown to be different in primary sequence (see Figure 5.18) despite the similarity shown here. This is the reason for grouping alleles into a class, to prevent misidentification.

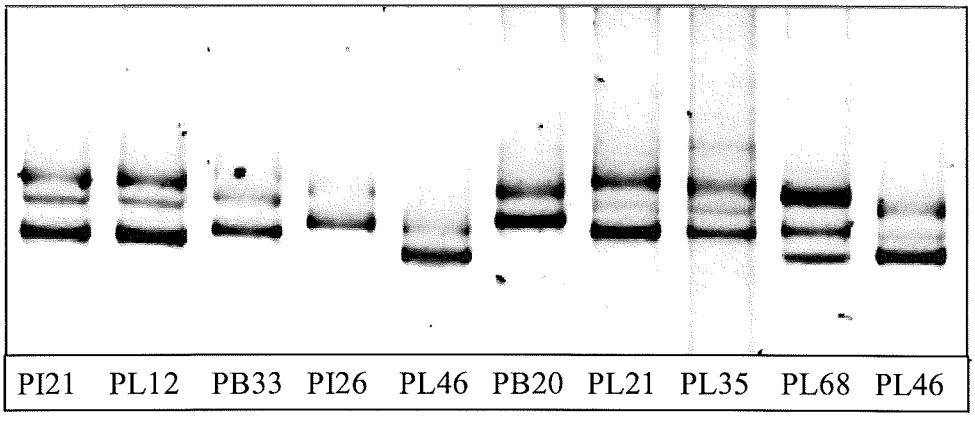


Figure 5.7: Observed SSCP allele frequencies of the unselected *MTci5* population. The actual percentages are displayed on the chart.

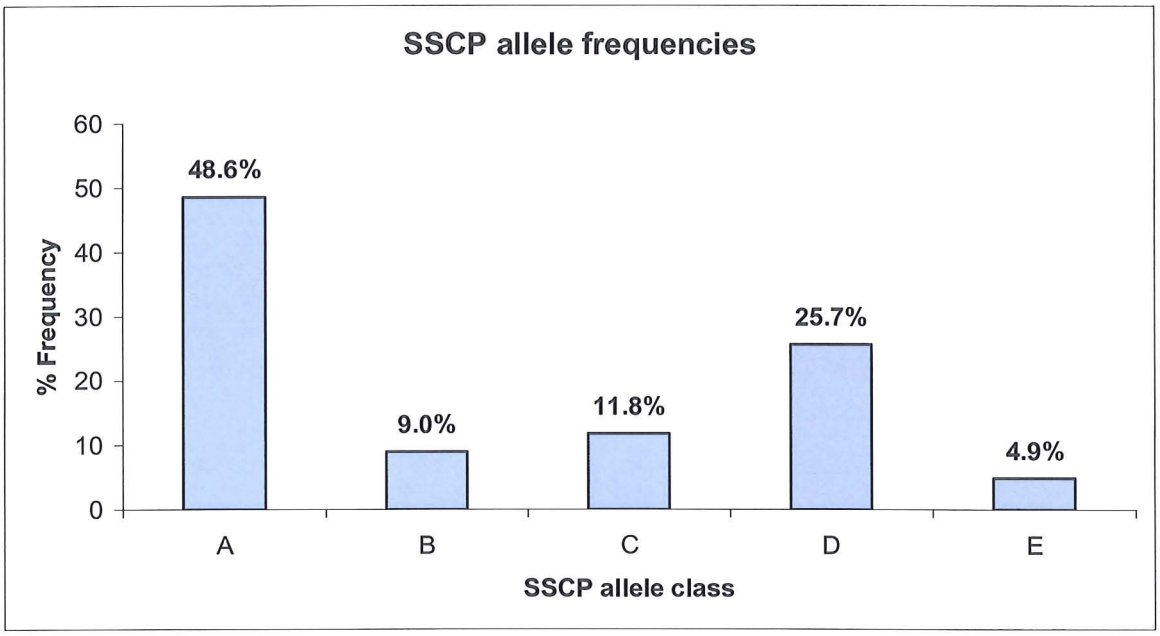


Figure 5.8: Hardy Weinberg Equilibrium Chi-square statistics calculated in GenAlEx version 6 (Peakall & Smouse, 2006) add-in for Microsoft Excel. The graph shows observed versus expected genotype counts in the unselected *MTci5* isolate. There was no significant deviation from HWE (degrees of freedom = 10, Chi-square = 8.523, p = 0.578).

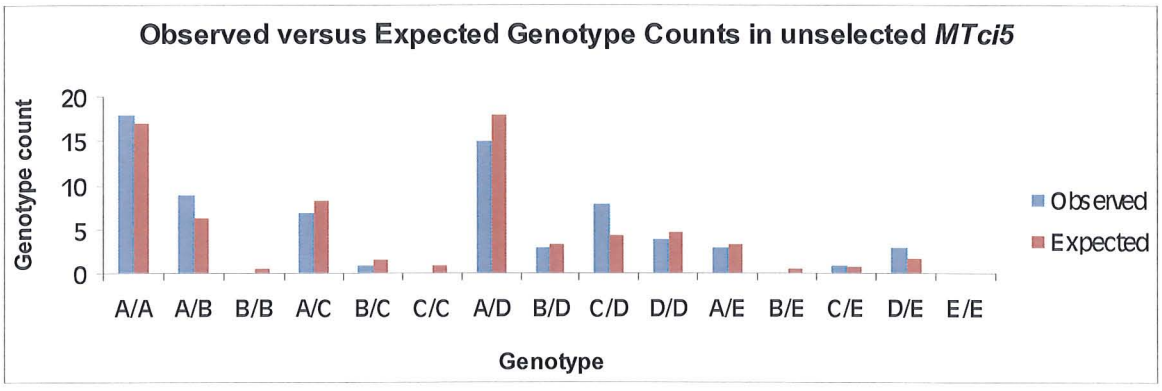


Figure 5.9: To establish the relationship between the isotype I β-tubulin F200Y mutation and the SSCP haplotype, the following steps were carried out.

Figure 5.9a: Step 1: The number of $P200^{Tyr/Tyr}$ (rr) and $P200^{Phe/Phe}$ (ss) individuals with every possible SSCP genotype was counted. The $P200^{Phe/Tyr}$ (rs) individuals were excluded from the analysis as it was not clear which SSCP haplotype would be resistant or susceptible. The total sample size was 41.

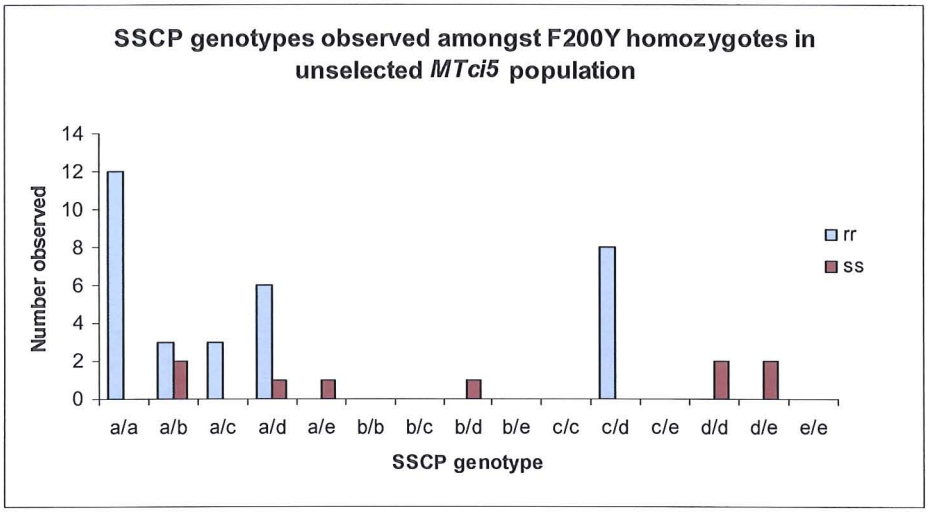


Figure 5.9b: Step 2: The number of SSCP haplotypes carrying either the $P200^{Tyr}$ resistant (r) or $P200^{Phe}$ susceptible (s) allele of the isotype I β-tubulin gene was counted e.g. the number of copies of allele class A carrying the resistant allele was calculated as: 2 x a/a (24) + 1 x a/b (3) + 1 x a/c (3) + 1 x a/d (6) + 1 x a/e (0) = 36. Hence, the total number of possible alleles was 82.

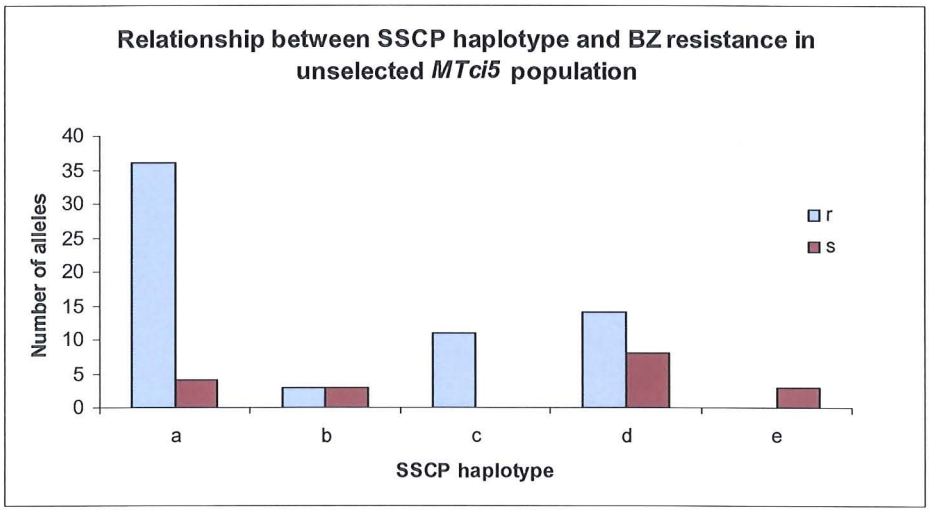


Figure 5.9c: Step 3: The number of SSCP haplotypes carrying either the P200^{Tyr} resistant (r) or P200^{Phe} susceptible (s) allele of the isotype I β-tubulin gene was calculated (the total counts from which the percentages were derived are shown on the graph). For example, 90% of class A alleles carried the P200^{Tyr} (r) allele etc.

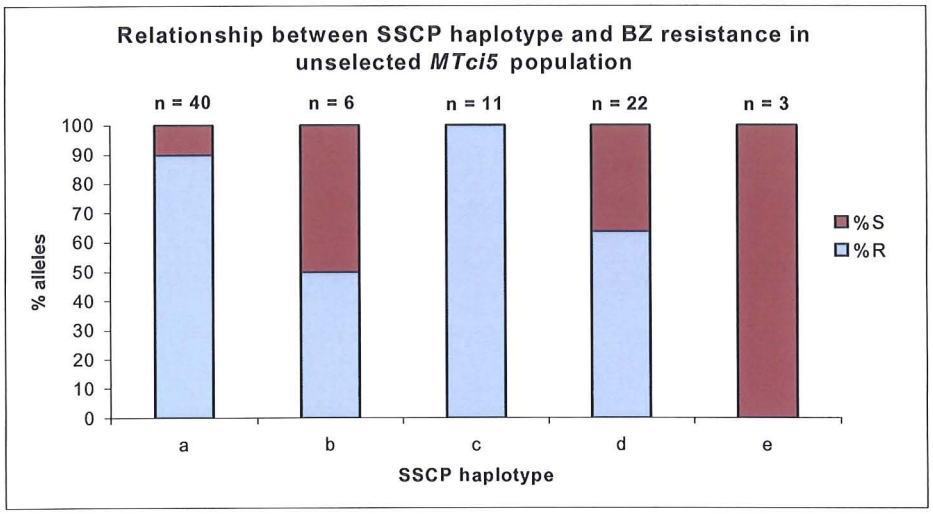


Figure 5.10: This graph shows the percentage allele frequencies of each class across the four *MTci5* populations.

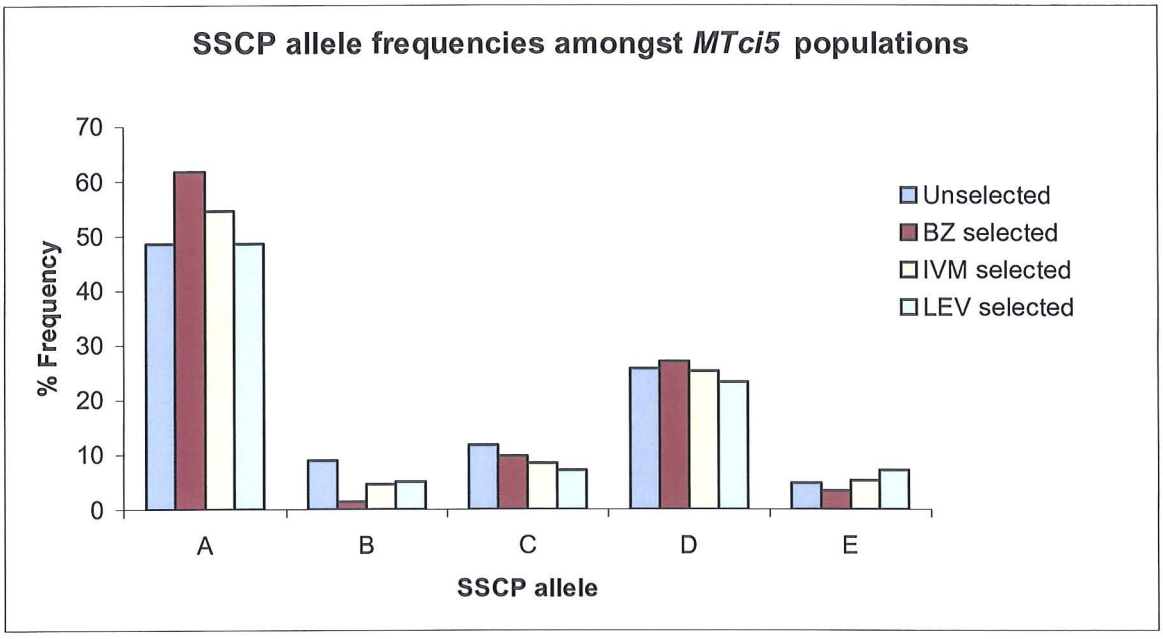


Figure 5.11: Graph showing the percentage genotype frequencies of each class across the four *MTci5* populations.

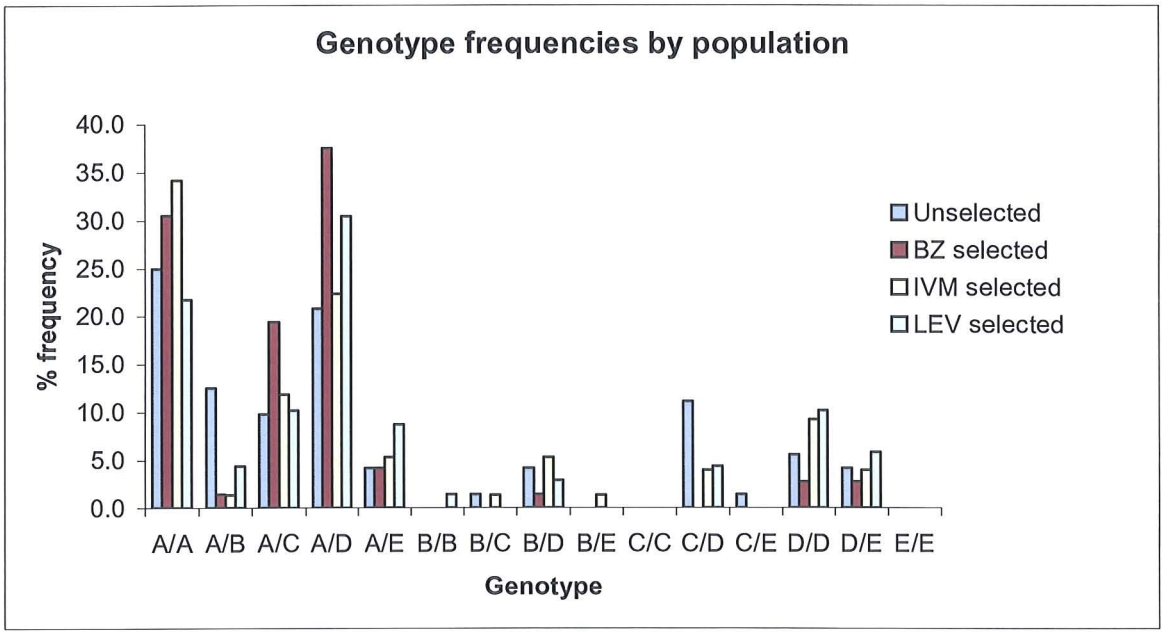
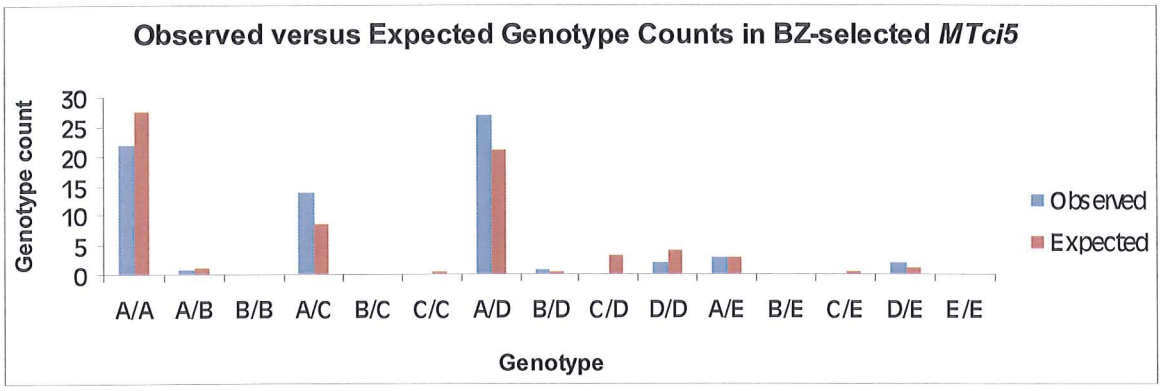
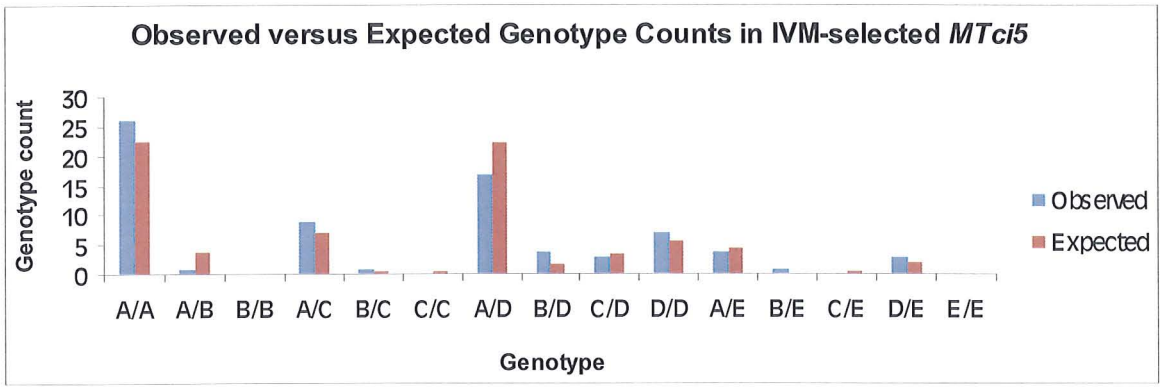


Figure 5.12: Chi-square (X^2) statistics (for Hardy Weinberg Equilibrium) calculated in GenAlEx version 6 (Peakall & Smouse, 2006) add-in for Microsoft Excel. The graphs show the observed versus expected genotype counts in the three drug-selected *MTci5* populations. There was no significant deviation from HWE in any of the *MTci5* populations (see HWE statistics below). Df = degrees of freedom, X^2 = Chi-square statistic and p = probability value.

5.12a. Chi-square result for BZ-selected *MTci5* population: Df = 10, X^2 = 13.165, p = 0.215.



5.12b Chi-square result for IVM-selected *MTci5* population: Df = 10, X^2 = 10.531, p = 0.395.



5.12c Chi-square result for LEV-selected *MTci5* population: Df = 10, X^2 = 7.885, p = 0.640.

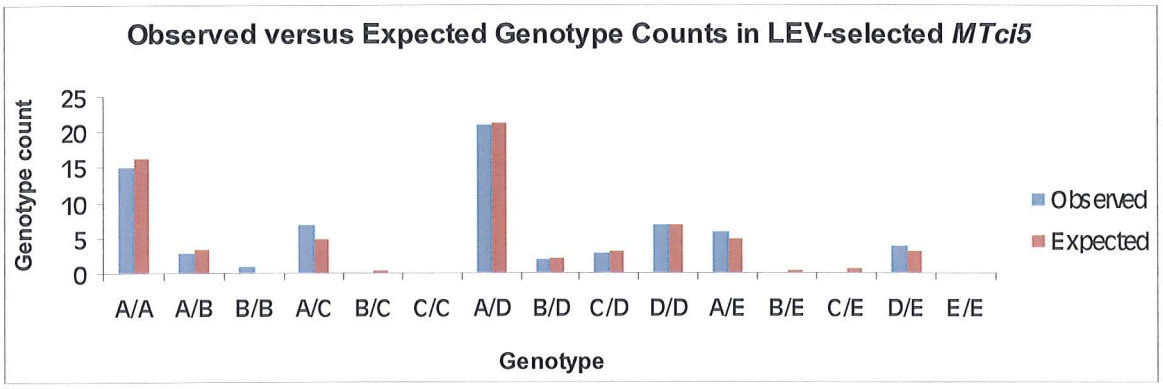


Figure 5.13: This chart displays the proportion of $P200^{Tyr/Tyr}$ (rr) and $P200^{Phe/Phe}$ (ss) genotypes for each possible SSCP genotype from the *MTci5* isolate (all four populations have been pooled). Sample sizes of five or fewer were available for A/E, B/B, B/C and B/D genotypes. All other genotypes had sample sizes of between 7 and 63.

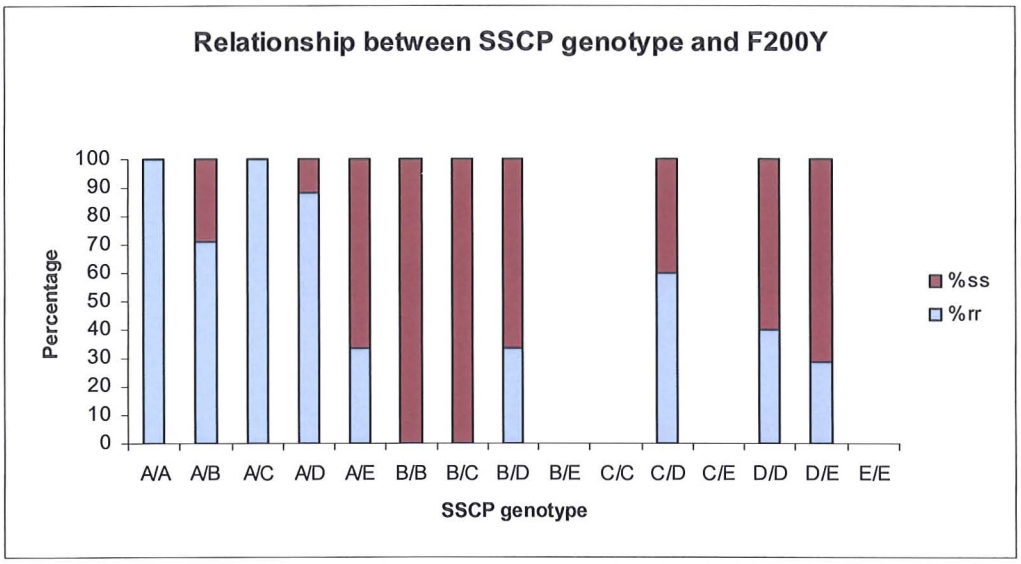


Figure 5.14: From the information gained about the frequency of $P200^{Tyr}$ (r) and $P200^{Phe}$ (s) alleles amongst SSCP genotypes (see Figure 5.13), the number and proportion of $P200^{Tyr}$ and $P200^{Phe}$ alleles per SSCP haplotype could then be calculated. This is displayed in the chart below. Sample sizes for each haplotype class were 141, 12, 32, 67 and 10 for A, B, C, D and E, respectively.

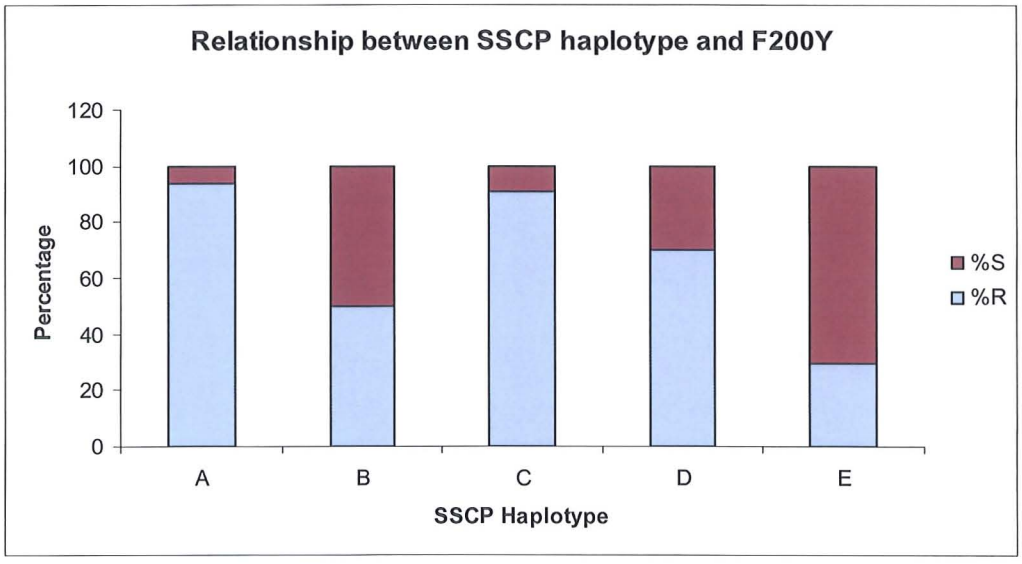


Figure 5.15: Alignment of allele class A sequences created in GeneDoc version 2.6 (Nicholas & Nicholas, 2006). The consensus sequence is given at the top and dots indicate agreement with consensus. Dashes indicate areas which failed to be sequenced (only at the start and end of sequences) and indel positions. The black outline denotes the position of the intron region and the red outline indicates the position of the F200Y isotype I β-tubulin mutation. The primer sites have been removed from the alignment as they do not represent 'true sequence' obtained *de novo*. The nomenclature used refers to the worm identity, allele class and clone number. For instance, PB15_A_1 refers to BZ-selected *MTci5* worm number 15, allele class A, clone number 1. PT, PI and PL refer to unselected, IVM-selected and LEV-selected populations, respectively.

```
                    *         20         *         40         *         60         *
Consensus : TCCGGATAGAATCATGGCTTCATTCTCAGTTGTTCCATCGCCTAAGGTAATTTATCTCTGACAGTCGTCC : 70
PB15_A_1  : ..............................................................................  : 70
PB15_A_2  : ..............................................................................  : 70
PB71_A_1  : ..............................................................................  : 70
PB71_A_2  : ..............................................................................  : 70
PI02_A_1  : -----------------------------------.........................................  : 35
PI02_A_2  : ------------------------------------.......................................  : 31
PL44_A_1  : ..............................................................................  : 70
PL44_A_2  : ..............................................................................  : 70
PT02_A_1  : -----------------------------------.........................................  : 35
PT02_A_2  : ..............................................................................  : 70
PT03_A_1  : ..............................................................................  : 70
PT03_A_2  : ..............................................................................  : 70
PT10_A_1  : ..............................................................................  : 70
PT10_A_2  : ..............................................................................  : 70
PT20_A_1  : ..............................................................................  : 70
PT20_A_2  : ..............................................................................  : 70

                   80         *        100         *        120         *        140
Consensus : TTTTTCGAGATCGTATGTACAGGTTCTCGAGGTGCAACCCATTTTCCGAACTCTTTACACCAATGCTTTA : 140
PB15_A_1  : ..............................................................................  : 140
PB15_A_2  : ..............................................................................  : 140
PB71_A_1  : ..............................................................................  : 140
PB71_A_2  : ..............................................................................  : 140
PI02_A_1  : ..............................................................................  : 105
PI02_A_2  : ..............................................................................  : 101
PL44_A_1  : ..............................................................................  : 140
PL44_A_2  : ..............................................................................  : 140
PT02_A_1  : ..............................................................................  : 105
PT02_A_2  : ..............................................................................  : 140
PT03_A_1  : ..............................................................................  : 140
PT03_A_2  : ..............................................................................  : 140
PT10_A_1  : ..............................................................................  : 140
PT10_A_2  : ..............................................................................  : 140
PT20_A_1  : ..............................................................................  : 140
PT20_A_2  : ..............................................................................  : 140

                    *        160         *        180         *        200         *
Consensus : CAGTGAAGTGTGTTATGTTTATAGGTATCCGACACCGTTGTGGAACCTTACAATGCCACTCTTTCTGTTC : 210
PB15_A_1  : ..............................................................................  : 210
PB15_A_2  : ..............................................................................  : 210
PB71_A_1  : ..............................................................................  : 210
PB71_A_2  : ..............................................................................  : 210
PI02_A_1  : ..............................................................................  : 175
PI02_A_2  : ..............................................................C...............  : 171
PL44_A_1  : ..............................................................................  : 210
PL44_A_2  : ..............................................................................  : 210
PT02_A_1  : ..............................................................................  : 175
PT02_A_2  : ..............................................................................  : 210
PT03_A_1  : ..............................................................................  : 210
PT03_A_2  : ..............................................................................  : 210
PT10_A_1  : ..............................................................................  : 210
PT10_A_2  : ..............................................................................  : 210
PT20_A_1  : ..............................................................................  : 210
PT20_A_2  : ..............................................................................  : 210

                                        F200Y
                   220         *        240         *        260         *
Consensus : ACCAATTGGTAGAAAACACCGATGAAACGTACTGTATCGATAATGAGGCTCTGTACGATA : 270
PB15_A_1  : ..............................................................  : 270
PB15_A_2  : ..........................G...................................  : 270
PB71_A_1  : ..............................................................  : 270
PB71_A_2  : ..............................................................  : 270
PI02_A_1  : ..............................................................  : 235
PI02_A_2  : ..............................................................  : 231
PL44_A_1  : ..............................................................  : 270
PL44_A_2  : ..............................................................  : 270
PT02_A_1  : ..............................................................  : 235
PT02_A_2  : ..............................................................  : 270
PT03_A_1  : ..............................................................  : 270
PT03_A_2  : ..............................................................  : 270
PT10_A_1  : ..............................................................  : 270
PT10_A_2  : ..............................................................  : 270
PT20_A_1  : ..............................................................  : 270
PT20_A_2  : ..............................................................  : 270
```

Figure 5.16: Alignment of allele class B sequences created in GeneDoc version 2.6 (Nicholas & Nicholas, 2006). The consensus sequence is given at the top and dots indicate agreement with consensus. Dashes indicate areas which failed to be sequenced (only at the start and end of sequences) and indel positions. The black outline denotes the position of the intron region and the red outline indicates the position of the F200Y isotype I β-tubulin mutation. The primer sites have been removed from the alignment as they do not represent 'true sequence' obtained *de novo*. The nomenclature used refers to the worm identity, allele class and clone number. For instance, PB33_B_1 refers to BZ-selected *MTci5* worm number 33, allele class B, clone number 1. PT, PI and PL refer to unselected, IVM-selected and LEV-selected populations, respectively.

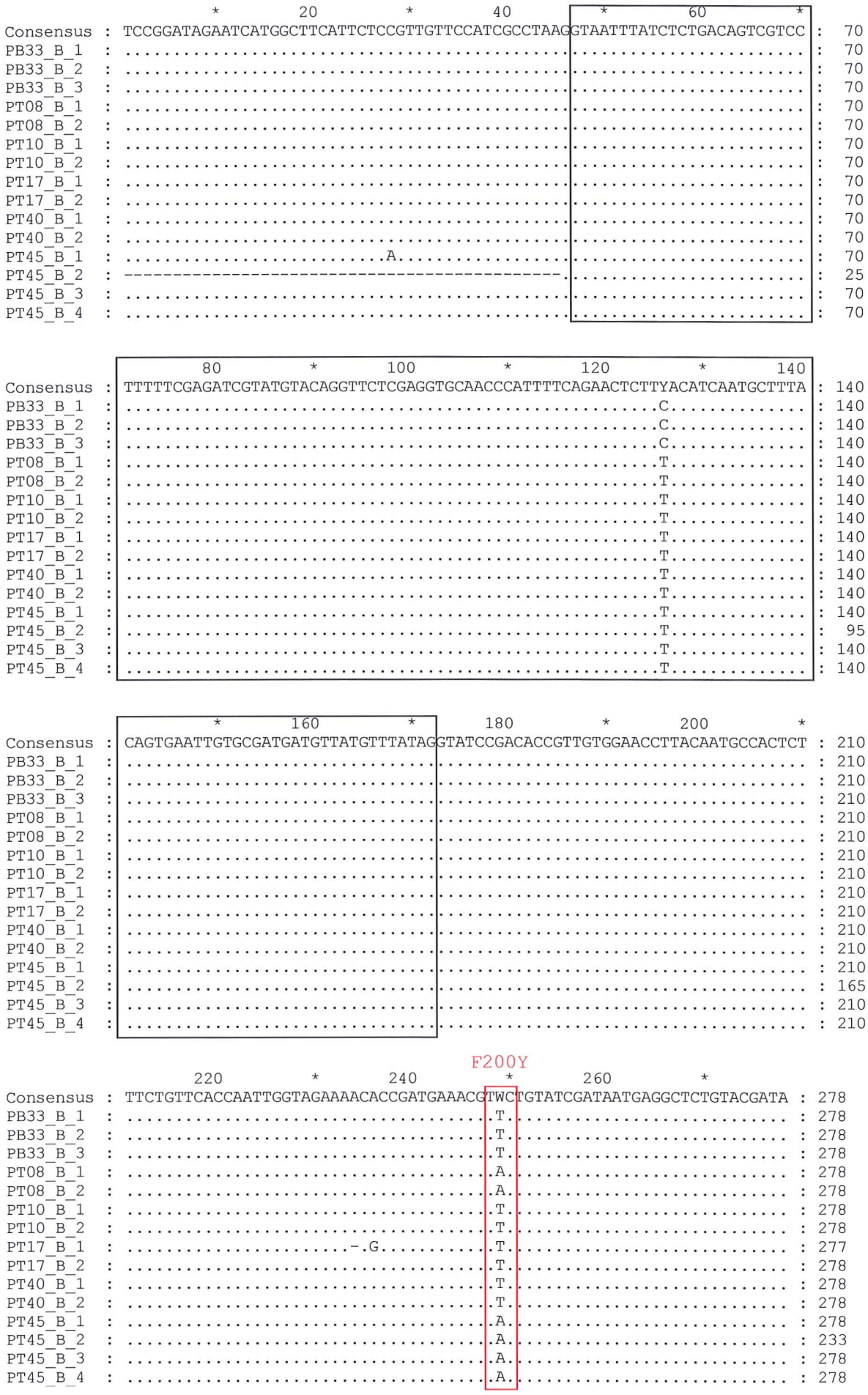

F200Y

Figure 5.17: Alignment of allele class C sequences created in GeneDoc version 2.6 (Nicholas & Nicholas, 2006). The consensus sequence is given at the top and dots indicate agreement with consensus. Dashes indicate areas which failed to be sequenced (only at the start and end of sequences) and indel positions. The black outline denotes the position of the intron region and the red outline indicates the position of the F200Y isotype I β-tubulin mutation. The primer sites have been removed from the alignment as they do not represent 'true sequence' obtained *de novo*. The nomenclature used refers to the worm identity, allele class and clone number. For instance, PT05_C_1 refers to unselected *MTci5* worm number 5, allele class C, clone number 1. PB, PI and PL refer to BZ-selected, IVM-selected and LEV-selected populations, respectively.

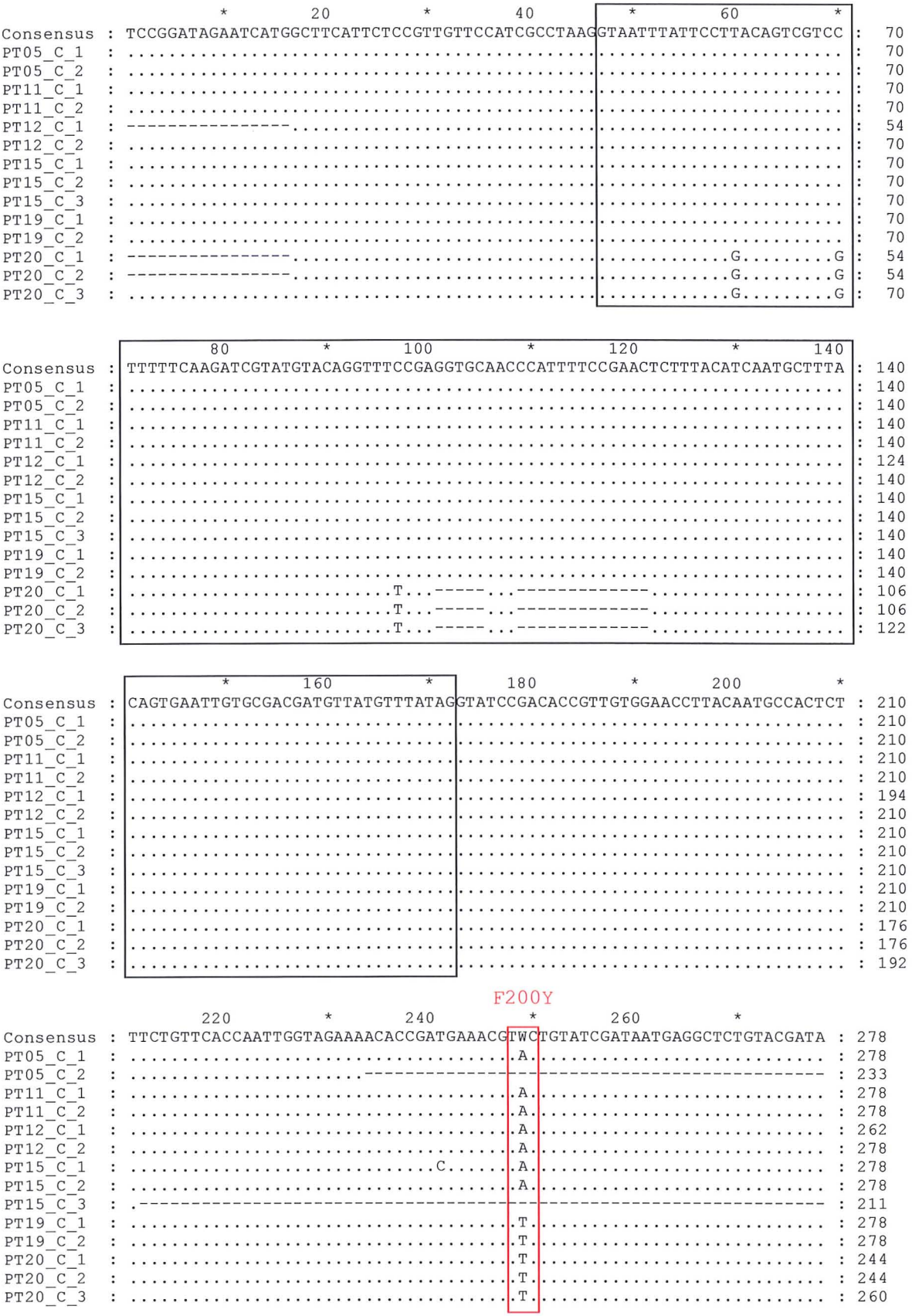
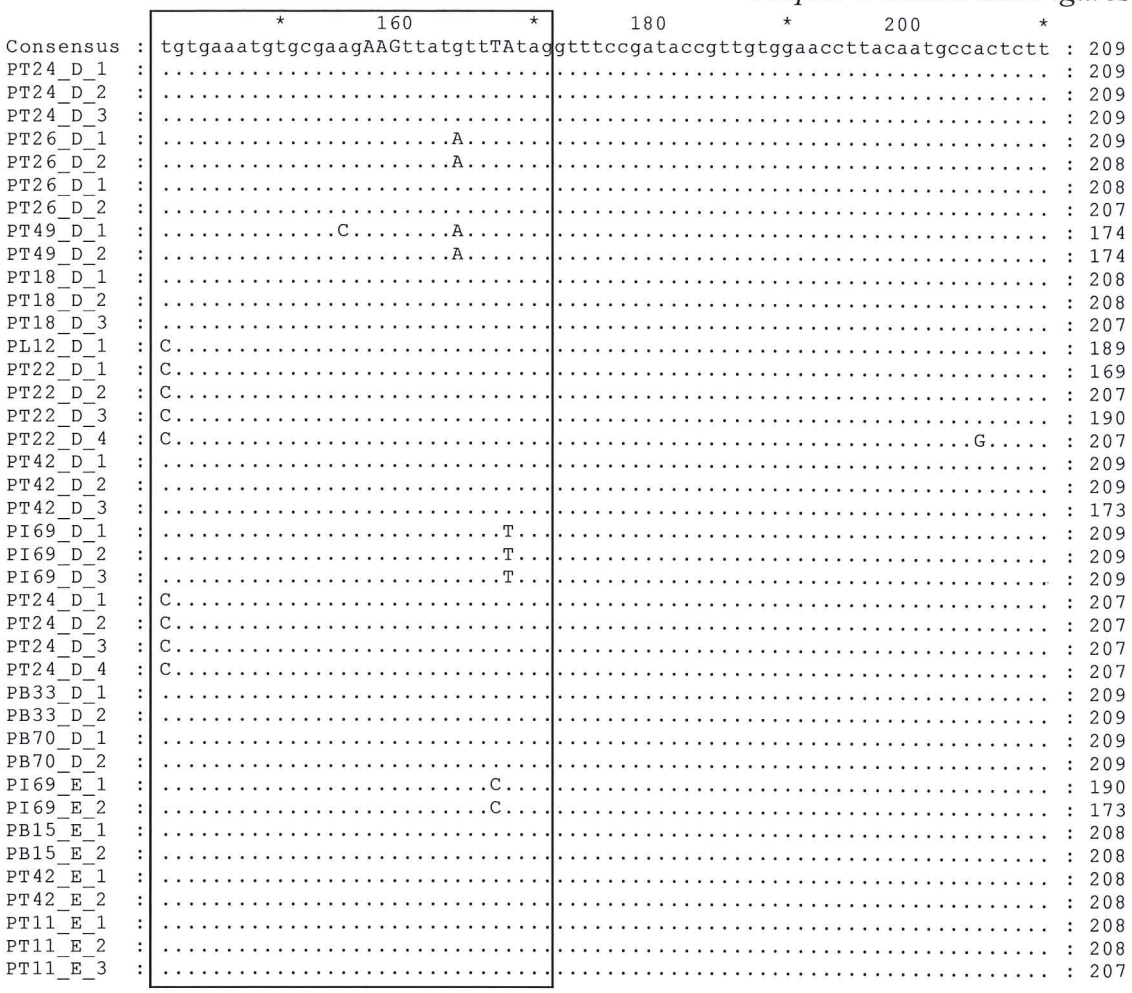

F200Y

Figure 5.18: Alignment of allele class D and E sequences created in GeneDoc version 2.6 (Nicholas & Nicholas, 2006). The consensus sequence is given at the top and dots indicate agreement with consensus. Dashes indicate areas which failed to be sequenced (only at the start and end of sequences) and indel positions. The black outline denotes the position of the intron region and the red outline indicates the position of the F200Y isotype I β-tubulin mutation. The primer sites have been removed from the alignment as they do not represent 'true sequence' obtained *de novo*. The nomenclature used refers to the worm identity, allele class and clone number. For instance, PT24_D_1 refers to unselected *MTci5* worm number 24, allele class D, clone number 1. PB, PI and PL refer to BZ-selected, IVM-selected and LEV-selected populations, respectively.

```
                    *         20         *         40         *         60         *
Consensus : tccggatagaatcatggcttcattctccgttgttccatcaccaaaggtaatgtatccctaacagtagtcc : 70
PT24_D_1  : ............................................................... : 70
PT24_D_2  : ............................................................... : 70
PT24_D_3  : ............................................................... : 70
PT26_D_1  : ............................................................... : 70
PT26_D_2  : ............................................................... : 70
PT26_D_1  : ..............................................G.............. : 70
PT26_D_2  : ..............................................G.............. : 69
PT49_D_1  : --------------------------------.......-.....-.............. : 35
PT49_D_2  : --------------------------------.....-.....-.............. : 35
PT18_D_1  : ..............................................C.............. : 70
PT18_D_2  : ..............................................C.............. : 70
PT18_D_3  : ..............................................C.............. : 70
PL12_D_1  : ----------------.....-.....-.....-.....-.....--....C..... : 50
PT22_D_1  : ------------------------------------.....-.....--....C..... : 30
PT22_D_2  : ..............................-.....-.....-.....--....C..... : 68
PT22_D_3  : ----------------.....-.....-.....-.....-.....--....C..... : 51
PT22_D_4  : ..................C...........-.....-.....-.....--....C..... : 68
PT42_D_1  : ..............................-.....-.....-.....-..... : 70
PT42_D_2  : ..............................-.....-.....-.....-..... : 70
PT42_D_3  : --------------------------------.....-.....-...-..... : 34
PI69_D_1  : ..............................-.....-.....-.....-..... : 70
PI69_D_2  : ..............................-.....-.....-.....-..... : 70
PI69_D_3  : ..............................-.....-.....-.....-..... : 70
PT24_D_1  : ..............................-.....-.....-.....--..... : 68
PT24_D_2  : ..............................-.....-.....-.....--..... : 68
PT24_D_3  : ..............................-.....-.....-.....--..... : 68
PT24_D_4  : ..............................-.....-.....-.....--..... : 68
PB33_D_1  : ..............................-.....-.....-.....-..... : 70
PB33_D_2  : ....................A.....-.....-.....-.....-..... : 70
PB70_D_1  : ..............................-.....-.....-.....-..... : 70
PB70_D_2  : ..............................-.....-.....-.....-..... : 70
PI69_E_1  : ----------------.....-.....-.....-...-.....-..... : 52
PI69_E_2  : --------------------------------.....-.....-.....C..... : 35
PB15_E_1  : ..............................-.....-.....-.....-..... : 70
PB15_E_2  : ..............................-.....-.....-.....-..... : 70
PT42_E_1  : ..............................-.....-.....-.....-.....C.. : 70
PT42_E_2  : ..............................-.....-.....-.....-.....C.. : 70
PT11_E_1  : ..............................-.....-.....-.....-..... : 70
PT11_E_2  : ..............................-.....-.....-.....-..... : 70
PT11_E_3  : ..............................-.....-.....-.....-..... : 70
```

```
                80         *         100        *         120        *         140
Consensus : tttttgagatcgTatgtacaggttctcgaggtgcaatccattt-acgaGctcttcacattgatgcgcaaC : 139
PT24_D_1  : ..............................-.....-.....-.....-.....T : 139
PT24_D_2  : ..............................-.....-.....-.....-.....T : 139
PT24_D_3  : ..............................-.....-.....-.....-.....T : 139
PT26_D_1  : ..............................-.....-.....-.....-..... : 139
PT26_D_2  : ....-.....-.....-.....-.....-.....-.....-.....-..... : 138
PT26_D_1  : ..............................-.....-.....-....-..... : 138
PT26_D_2  : ..............................-.....-.....-....-..... : 137
PT49_D_1  : ..............................-.....-.....-.....-..... : 104
PT49_D_2  : ..............................-.....-.....-.....-..... : 104
PT18_D_1  : ..............................-.....-.....-....-..... : 138
PT18_D_2  : ..............................-.....-.....-....-..... : 138
PT18_D_3  : ..............................-.....-.....-....--..... : 137
PL12_D_1  : ..............T...........T.....T.....T....-..... : 119
PT22_D_1  : ..............T...........T.....T.....T....-..... : 99
PT22_D_2  : ..............T...........T.....T.....T....-..... : 137
PT22_D_3  : ..............T...........T.....T.....T....-..... : 120
PT22_D_4  : ..............T...........T.....T.....T....-..... : 137
PT42_D_1  : ............C.....-.....-.....-.....-.....-.....T : 139
PT42_D_2  : ............C.....-.....-.....-.....-.....-.....T : 139
PT42_D_3  : ............C.....-.....-.....-.....-.....-.....T : 103
PI69_D_1  : ............C.....-.....-.....-.....-.....-.....T : 139
PI69_D_2  : ............C.....-.....-.....-.....-.....-.....T : 139
PI69_D_3  : ............C.....-.....-.....-.....-.....-.....T : 139
PT24_D_1  : ..............T...........T.....T.....T....-..... : 137
PT24_D_2  : ..............T...........T.....T.....T....-..... : 137
PT24_D_3  : ..............T...........T.....T.....T....-..... : 137
PT24_D_4  : ........G.....T...........T.....T.....T....-..... : 137
PB33_D_1  : ..............................-.....-.....-.....-.....T : 139
PB33_D_2  : ..............................-.....-.....-.....-.....T : 139
PB70_D_1  : ..............................-.....-.....-.....-.....T : 139
PB70_D_2  : ..............................-.....-.....-.....-.....T : 139
PI69_E_1  : ..............................-.....-.....-....-..... : 120
PI69_E_2  : ..............................-.....-.....-....-..... : 103
PB15_E_1  : ..............................-.....-.....-....-..... : 138
PB15_E_2  : ..............................-.....-.....-....-..... : 138
PT42_E_1  : ..............................-.....-.....-....-..... : 138
PT42_E_2  : ..............................-.....-.....-....-..... : 138
PT11_E_1  : ..............................-.....-.....-....-..... : 138
PT11_E_2  : ..............................-.....-.....-....-..... : 138
PT11_E_3  : ..............................-.....-.....-....--..... : 137
```


160
180
200
Consensus : tgtgaaatgtgcgaagAAGttatgttTAtaggtttccgataccgttgtggaaccttacaatgccactctt : 209
PT24_D_1 : ..... : 209
PT24_D_2 : ..... : 209
PT24_D_3 : ..... : 209
PT26_D_1 : .....A..... : 209
PT26_D_2 : .....A..... : 208
PT26_D_1 : ..... : 208
PT26_D_2 : ..... : 207
PT49_D_1 : .....C.....A..... : 174
PT49_D_2 : .....A..... : 174
PT18_D_1 : ..... : 208
PT18_D_2 : ..... : 208
PT18_D_3 : ..... : 207
PL12_D_1 : C..... : 189
PT22_D_1 : C..... : 169
PT22_D_2 : C..... : 207
PT22_D_3 : C..... : 190
PT22_D_4 : C.....G..... : 207
PT42_D_1 : ..... : 209
PT42_D_2 : ..... : 209
PT42_D_3 : ..... : 173
PI69_D_1 : .....T..... : 209
PI69_D_2 : .....T..... : 209
PI69_D_3 : .....T..... : 209
PT24_D_1 : C..... : 207
PT24_D_2 : C..... : 207
PT24_D_3 : C..... : 207
PT24_D_4 : C..... : 207
PB33_D_1 : ..... : 209
PB33_D_2 : ..... : 209
PB70_D_1 : ..... : 209
PB70_D_2 : ..... : 209
PI69_E_1 : .....C..... : 190
PI69_E_2 : .....C..... : 173
PB15_E_1 : ..... : 208
PB15_E_2 : ..... : 208
PT42_E_1 : ..... : 208
PT42_E_2 : ..... : 208
PT11_E_1 : ..... : 208
PT11_E_2 : ..... : 208
PT11_E_3 : ..... : 207

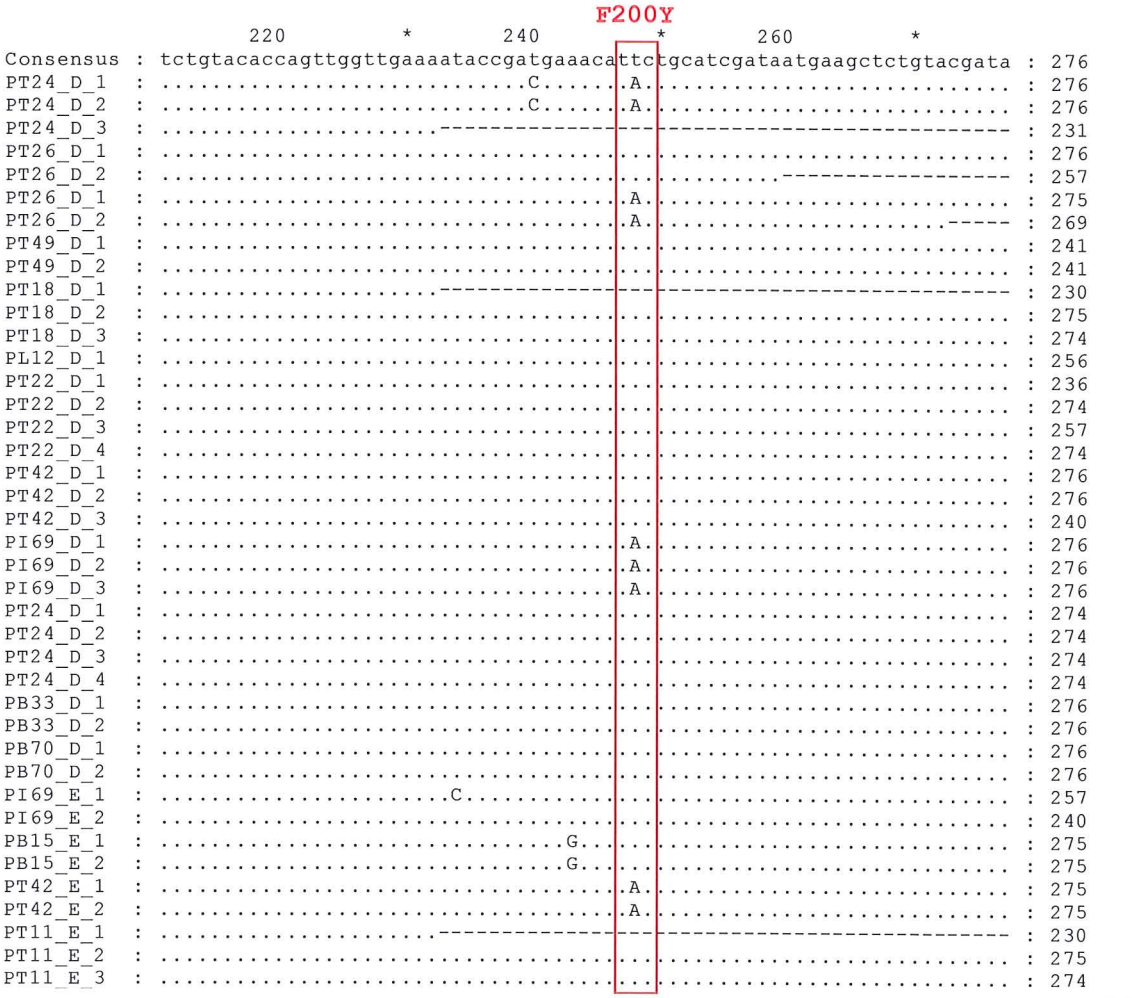
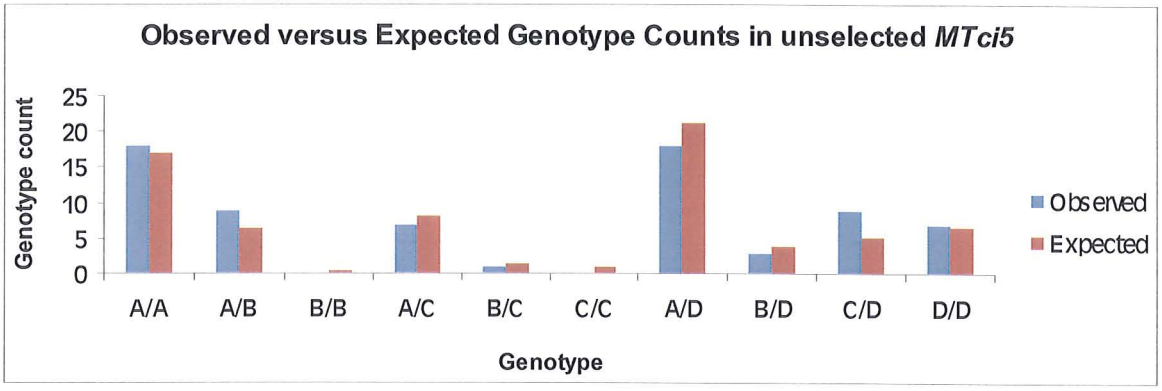
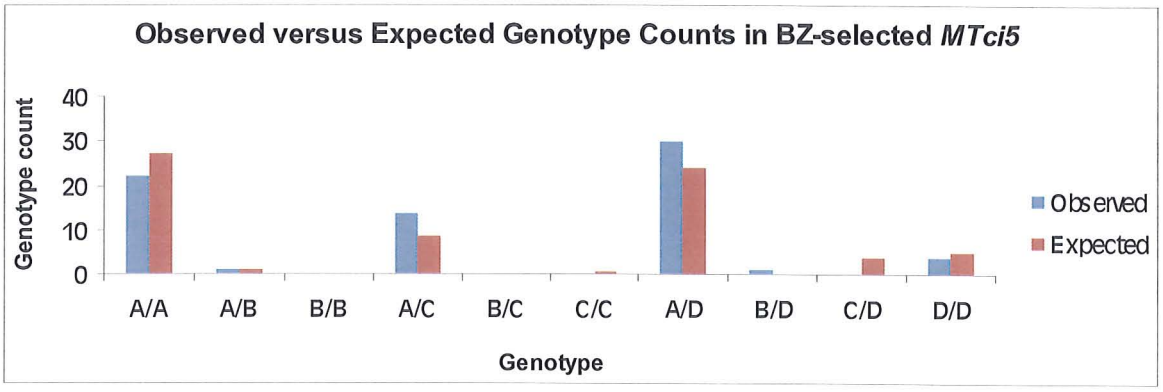

F200Y
220
240
260
Consensus : tctgtacaccagttggttgaaaataccgatgaaacattctgcatcgataatgaagctctgtacgata : 276
PT24_D_1 : .....C.....A..... : 276
PT24_D_2 : .....C.....A..... : 276
PT24_D_3 : .....---------- : 231
PT26_D_1 : ..... : 276
PT26_D_2 : .....---------- : 257
PT26_D_1 : .....A..... : 275
PT26_D_2 : .....A.....----- : 269
PT49_D_1 : ..... : 241
PT49_D_2 : ..... : 241
PT18_D_1 : .....---------- : 230
PT18_D_2 : ..... : 275
PT18_D_3 : ..... : 274
PL12_D_1 : ..... : 256
PT22_D_1 : ..... : 236
PT22_D_2 : ..... : 274
PT22_D_3 : ..... : 257
PT22_D_4 : ..... : 274
PT42_D_1 : ..... : 276
PT42_D_2 : ..... : 276
PT42_D_3 : ..... : 240
PI69_D_1 : .....A..... : 276
PI69_D_2 : .....A..... : 276
PI69_D_3 : .....A..... : 276
PT24_D_1 : ..... : 274
PT24_D_2 : ..... : 274
PT24_D_3 : ..... : 274
PT24_D_4 : ..... : 274
PB33_D_1 : ..... : 276
PB33_D_2 : ..... : 276
PB70_D_1 : ..... : 276
PB70_D_2 : ..... : 276
PI69_E_1 : .....C..... : 257
PI69_E_2 : ..... : 240
PB15_E_1 : .....G..... : 275
PB15_E_2 : .....G..... : 275
PT42_E_1 : .....A..... : 275
PT42_E_2 : .....A..... : 275
PT11_E_1 : .....---------- : 230
PT11_E_2 : ..... : 275
PT11_E_3 : ..... : 274

Figure 5.19: Chi-square (X^2) statistics (for Hardy Weinberg Equilibrium) calculated in GenAlEx version 6 (Peakall & Smouse, 2006) add-in for Microsoft Excel. The graphs show the observed versus expected genotype counts in all four *MTci5* populations when D and E alleles are treated as one class. There was no significant deviation from HWE in any of the *MTci5* populations (see HWE statistics below). Df = degrees of freedom, X^2 = Chi-square statistic and p = probability value.

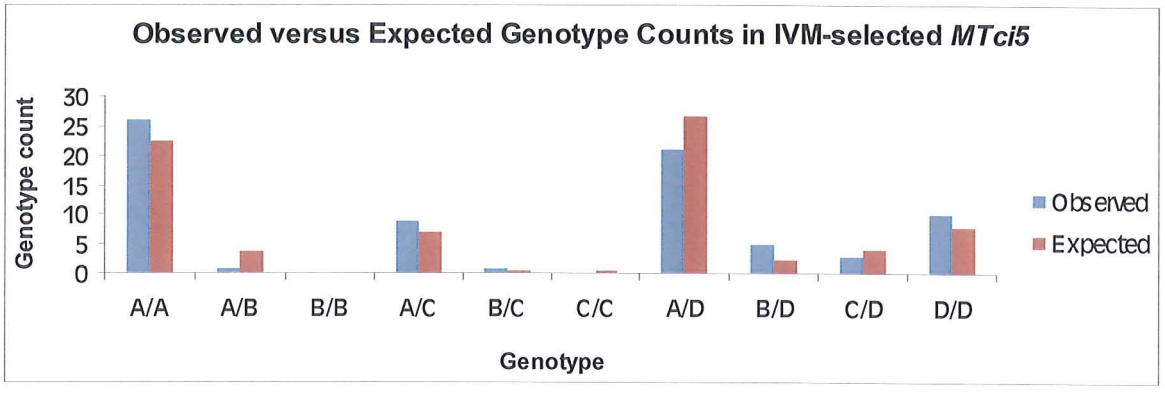
5.19a. Chi-square result for unselected *MTci5* population: Df = 6, X^2 = 6.738, p = 0.346.



5.19b Chi-square result for BZ-selected *MTci5* population: Df = 6, X^2 = 11.272, p = 0.080.



5.19c Chi-square result for IVM-selected *MTci5* population: Df = 6, X^2 = 9.573, p = 0.145.



5.19d Chi-square result for LEV-selected *MTci5* population: Df = 6, X^2 = 6.226, p = 0.398.

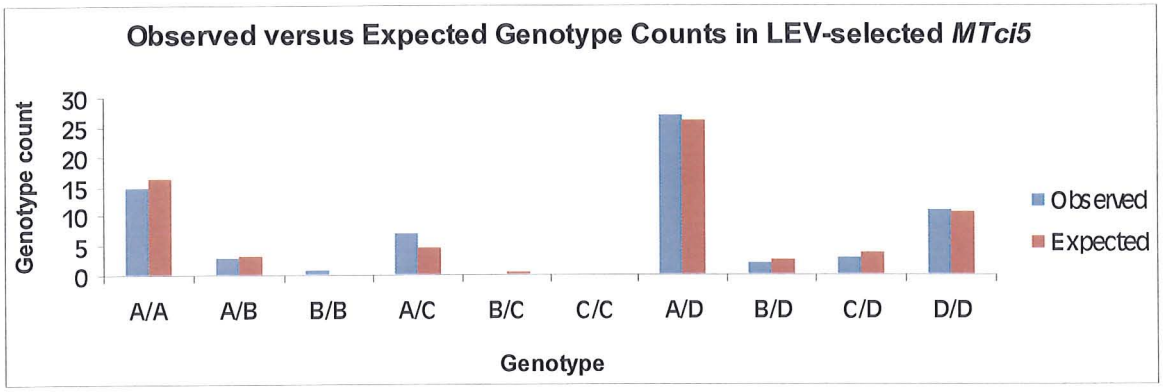


Figure 5.20: Alignment of the consensus sequences from each haplotype found in the *MTci5* isolate (created in GeneDoc version 2.6, Nicholas & Nicholas, 2006). Each haplotype has a new identity, for example, A1R refers to allele class A, haplotype number 1, which is resistant at the F200Y isotype I β-tubulin locus (an S refers to susceptible). The sequence positions in this Figure are referred to in Tables 5.1 and 5.2, which summarise the differences between haplotypes. Dots indicate agreement with the top sequence. Dashes indicate areas which failed to be sequenced (start of E1S sequence only) and indel sites. The black outline denotes the position of the intron region (positions 47 to 172) and the red outline indicates the position of the F200Y isotype I β-tubulin mutation (position 249). The primer sites have been removed from the alignment as they do not represent 'true sequence' obtained *de novo*. The nomenclature used refers to the worm identity, allele class and clone number. For instance, PT24_D_1 refers to unselected *MTci5* worm number 24, allele class D, clone number 1. PB, PI and PL refer to BZ-selected, IVM-selected and LEV-selected populations, respectively.

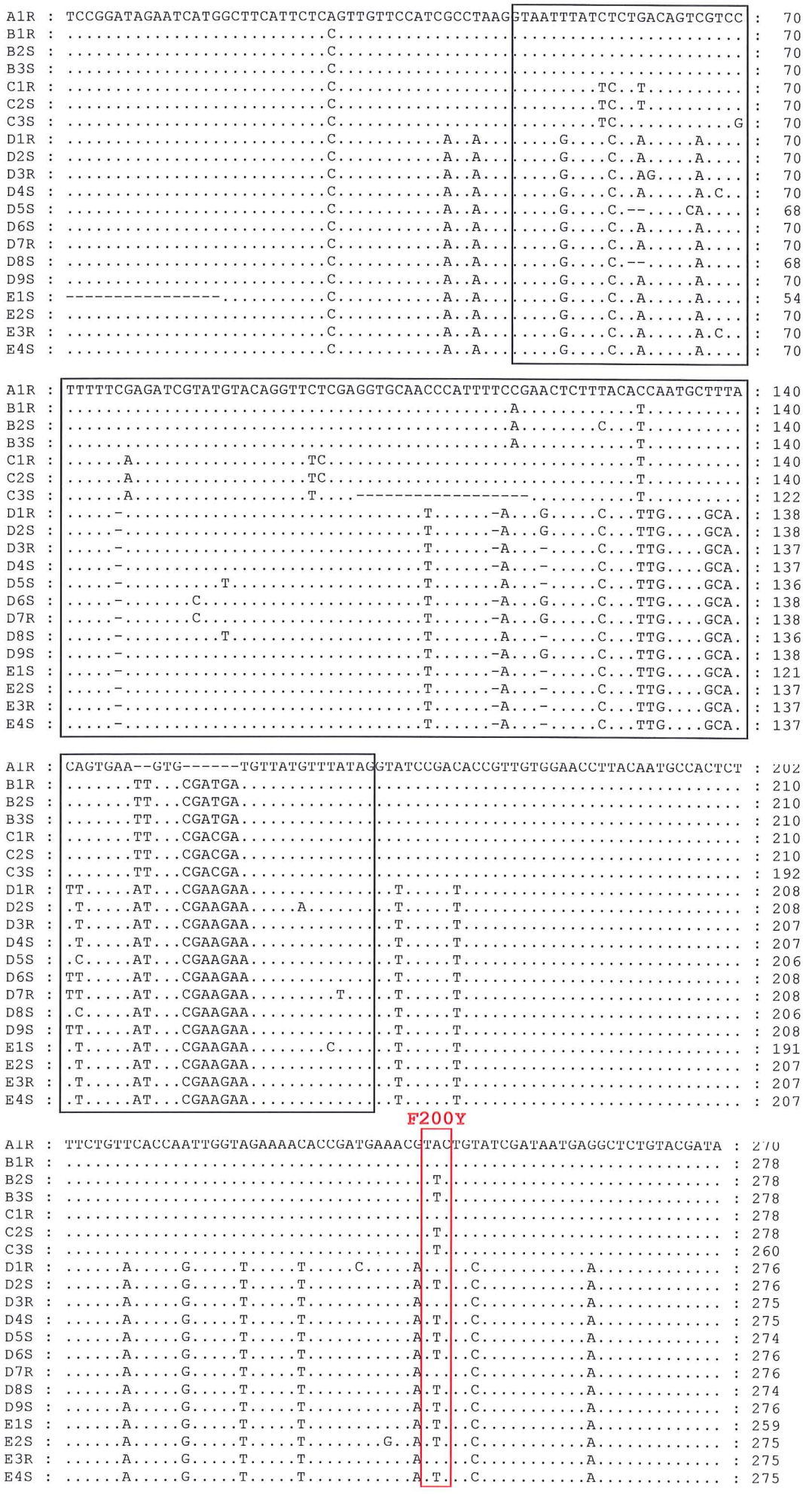

F200Y

Table 5.1: Differences within the A/B/C alleles group sequenced from the *MTci5* isolate. The length of the consensus sequence was 278bp and different lengths from this have also been highlighted below. The superscript R or S refers to $P200^{Tyr}$ and $P200^{Phe}$ alleles, respectively, of the isotype I β-tubulin gene, which determines BZ resistance or susceptibility (position 249 is the site of F200Y isotype I β-tubulin SNP).

Worm ID	Allele ID	Position of haplotype-defining SNP in sequence alignment													Position of indel in sequence			Sequence length (bp)
		28	56	57	60	70	77	96	97	117	126	130	156	249 (F200Y)	101-118	148-149	153-158	
ALL	$A1^R$	A	C	T	G	C	G	C	T	C	T	C	-	A	NO	YES	YES	270
PT 08 & PT45	$B1^R$	C	C	T	G	C	G	C	T	A	T	T	T	A	NO	NO	NO	278
PB33	$B2^S$	C	C	T	G	C	G	C	T	A	C	T	T	T	NO	NO	NO	278
PT 10, PT17 & PT40	$B3^S$	C	C	T	G	C	G	C	T	A	T	T	T	T	NO	NO	NO	278
PT 05, PT11,PT12 & PT15	$C1^R$	C	T	C	T	C	A	T	C	C	T	T	C	A	NO	NO	NO	278
PT19	$C2^S$	C	T	C	T	C	A	T	C	C	T	T	C	T	NO	NO	NO	278
PT20	$C3^S$	C	T	C	G	G	A	T	T	C	T	T	C	T	YES	NO	NO	260

Table 5.2: Differences within the D/E alleles group sequenced from the *MTci5* isolate. The length of the aligned consensus was 278bp and sequences which differed from this are also detailed in this Table. The superscript R or S refers to $P200^{Tyr}$ and $P200^{Phe}$ alleles, respectively, of the isotype I β-tubulin gene, which determines BZ resistance or susceptibility (position 249 is the site of F200Y isotype I β-tubulin SNP).

Worm ID	Allele ID	Position of haplotype-defining SNP in sequence alignment													Position of indel in sequence			Sequence length (bp)
		61	65	68	84	87	141	142	165	168	169	241	244	249 (F200Y)	59-60	115	120	
PT24	$D1^R$	A	T	T	T	G	T	T	G	T	A	C	A	A	TA	-	G	276
PT26, PT49	$D2^S$	A	T	T	T	G	C	T	A	T	A	T	A	T	TA	-	G	276
PT26	$D3^R$	G	T	T	T	G	C	T	G	T	A	T	A	A	TA	-	-	275
PT18	$D4^S$	A	T	C	T	G	C	T	G	T	A	T	A	T	TA	-	-	275
PL12, PT22	$D5^S$	A	C	T	T	T	C	C	G	T	A	T	A	T	-	T	-	274
PT42	$D6^S$	A	T	T	C	G	T	T	G	T	A	T	A	T	TA	-	G	276
PI69	$D7^R$	A	T	T	C	G	T	T	G	T	T	T	A	A	TA	-	G	276
PT24	$D8^S$	A	T	T	T	T	C	C	G	T	A	T	A	T	-	T	-	274
PB33, PB70	$D9^S$	A	T	T	T	G	T	T	G	T	A	T	A	T	TA	-	G	276
PI69	$E1^S$	A	T	T	T	G	C	T	G	C	A	T	A	T	TA	-	-	275
PB15	$E2^S$	A	T	T	T	G	C	T	G	T	A	T	G	T	TA	-	-	275
PT42	$E3^R$	A	T	C	T	G	C	T	G	T	A	T	A	A	TA	-	-	275
PT11	$E4^S$	A	T	T	T	G	C	T	G	T	A	T	A	T	TA	-	-	275

Table 5.3: Consistent differences between the A/B/C allele and D/E allele groups are shown in the Table below. Only those mutations which are present in all sequences of a group have been highlighted here. The letters (TV) and (TS) indicate a transversion or transition event, respectively. A dash (-) denotes an indel.

Allele group	Position of group-defining SNP in sequence alignment																		
	Exon 1		Intron 1									Exon 2							
	40 (TS)	**43** (TV)	**52** (TV)	**66** (TV)	**76** (-)	**131** (TS)	**132** (TS)	**137** (TV)	**138** (TS)	**139** (TV)	**159** (TV)	**175** (TV)	**181** (TS)	**217** (TS)	**223** (TV)	**229** (TV)	**235** (TS)	**247** (TS)	**253** (TS)	**265** (TS)
A/B/C	G	T	T	C	C	C	A	T	T	T	T	A	C	A	A	A	C	G	T	G
D/E	A	A	G	A	-	T	G	G	C	A	A	T	T	G	T	T	T	A	C	A

Table 5.4: Estimates of nucleotide diversity, π (Nei, 1987), sampling variance and standard deviation and π with Jukes-Cantor (1969) correction. Estimates have been made for exon 1 (bases 1 to 49), intron 1 (bases 50 to 175), exon 2 (bases 176 to 278) and the entire length of the sequence region (bases 1 to 278). These were calculated using DnaSP software version 4.0 (Rozas *et al.*, 2003).

Estimate	Exon 1	Intron 1	Exon 2	Overall
π	0.02159	0.08629	0.04379	0.05586
Sampling variance of π	0.0000148	0.0001125	0.0000312	0.0000477
Standard deviation of π	0.00385	0.01061	0.00559	0.00691
(π) Jukes-Cantor correction	0.02225	0.09520	0.04626	0.05959

Table 5.5: Estimates of haplotype diversity, Hd (Nei, 1987), with accompanying sampling variance and standard deviation values. Estimates have been made for exon 1 (bases 1 to 49), intron 1 (bases 50 to 175), exon 2 (bases 176 to 278) and the entire length of the sequence region (bases 1 to 278). These were calculated using DnaSP software version 4.0 (Rozas *et al.*, 2003).

Estimate	Exon 1	Intron 1	Exon 2	Overall
Number of haplotypes	3	15	6	20
Haplotype diversity (Hd)	0.511	0.974	0.789	1.000
Sampling variance of Hd	0.00824	0.00048	0.00411	0.00025
Standard deviation of Hd	0.091	0.022	0.064	0.016

Table 5.6: Estimates of haplotype neutrality. These were calculated using DnaSP software version 4.0 (Rozas *et al.*, 2003). Estimates have been made for exon 1 (bases 1 to 49), intron 1 (bases 50 to 175), exon 2 (bases 176 to 278) and the entire length of the sequence region (bases 1 to 278). These were calculated using DnaSP software version 4.0 (Rozas *et al.*, 2003). The probability of each test statistic (where applicable) is shown in brackets (p). Significant values at the 5% level are denoted by * and those at 1% level are denoted by **.

Estimate of neutrality	Exon 1	Intron 1	Exon 2	Overall
Tajima's D statistic	0.67051	0.19373	1.61280	0.67942
(p)	(>0.1)	(>0.1)	(>0.1)	(>0.1)
Fu & Li's D statistic	-0.12425	0.25891	0.55648	0.33633
(p)	(>0.1)	(>0.1)	(>0.1)	(>0.1)
Fu & Li's F statistic	0.10726	0.27885	0.99812	0.51142
(p)	(>0.1)	(>0.1)	(>0.1)	(>0.1)
Strobeck's probability, S (that nH < observed)	0.470	0.993	0.215	1.000

Figure 5.21: Phylogenetic relationship between SSCP allele classes. The consensus sequences for each allele clone per worm were created and compared using a Clustal W alignment (Higgins, 1994) in MegAlign, (DNA star version 5.08, 2004). The allele classes are colour coded to highlight the relationship between them more clearly. The letters R and S indicate whether the resistant ($P200^{Tyr}$) or susceptible ($P200^{Phe}$) allele is carried by each haplotype in terms of the F200Y isotype I β-tubulin mutation.

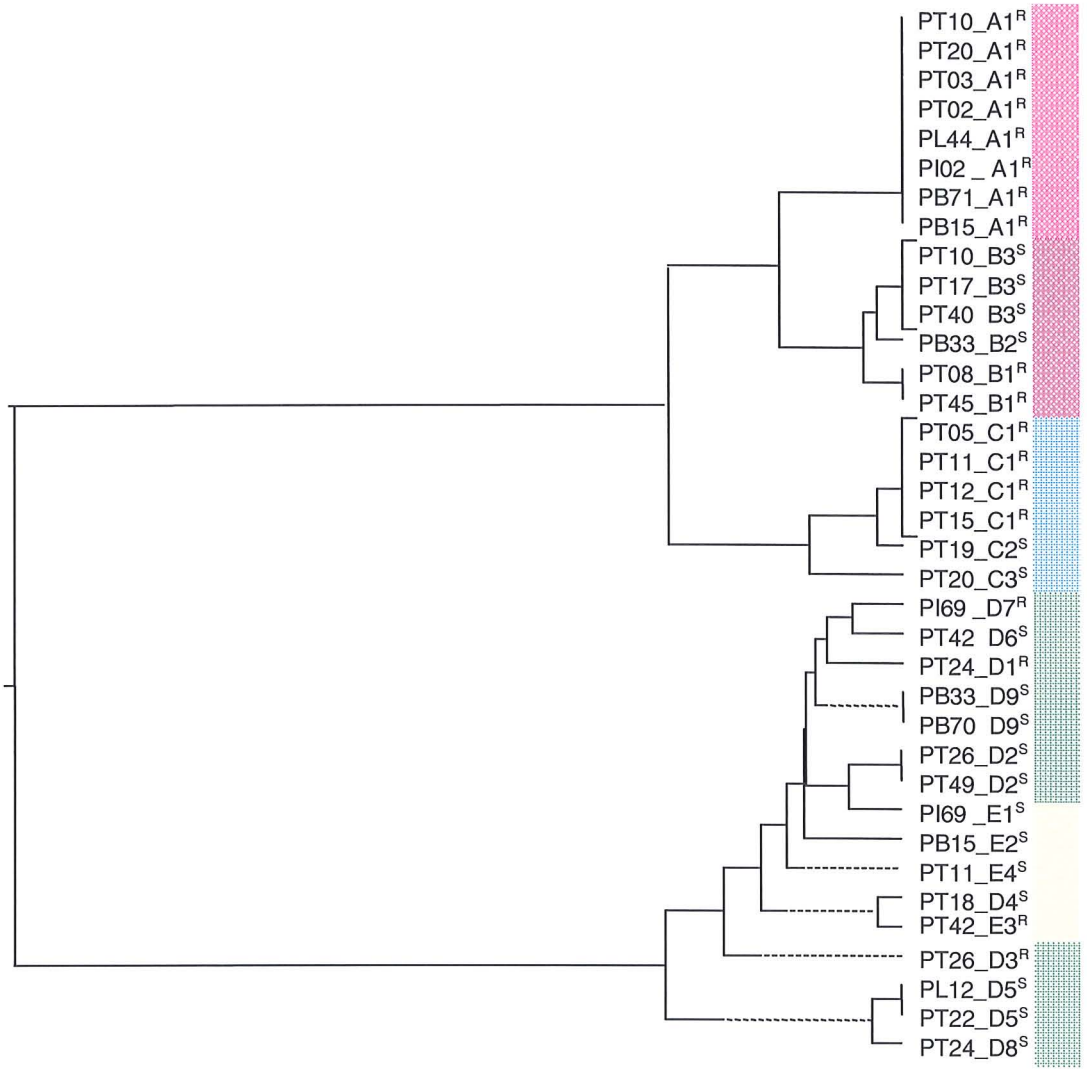


Figure 5.22: Phylogenetic relationship between SSCP alleles and French sequences published by Silvestre & Humbert (2002). The consensus sequences for each allele clone per worm were created and compared using a Clustal W alignment (Higgins, 1994) in MegAlign, (DNA star version 5.08, 2004). The allele classes are colour coded to highlight the relationship between them more clearly. Silvestre & Humbert's (2002) alleles are shaded in grey. The letters R and S indicate whether the resistant ($P200^{Tyr}$) or susceptible ($P200^{Phe}$) allele is carried by each haplotype in terms of the F200Y isotype I β-tubulin mutation.

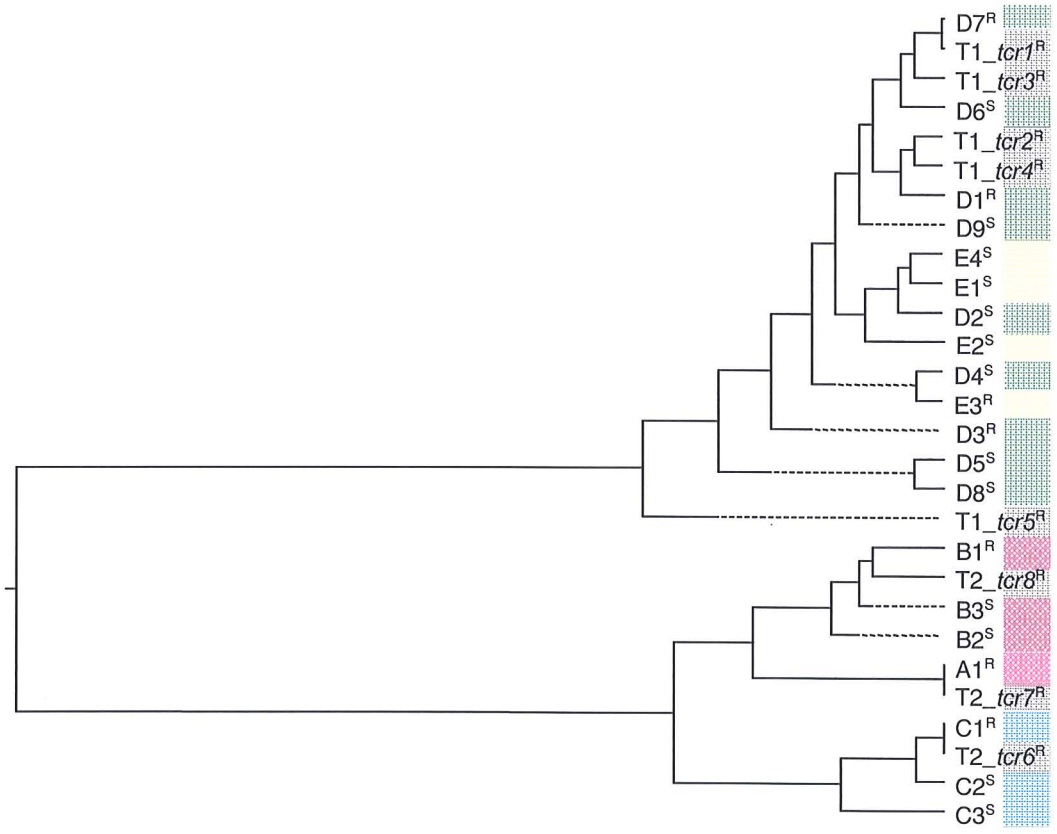


Figure 5.23: Graph showing the F200Y isotype I β-tubulin genotype ratios amongst all other isolates examined. The letters rr, rs and ss refer to genotypes $P200^{Tyr/Tyr}$, $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$, respectively. *MTci1* and *MTci2* are phenotypically susceptible, whilst *MTci3* and *MTci4* are phenotypically resistant to BZ. The sample size for each population was 40 (L_3).

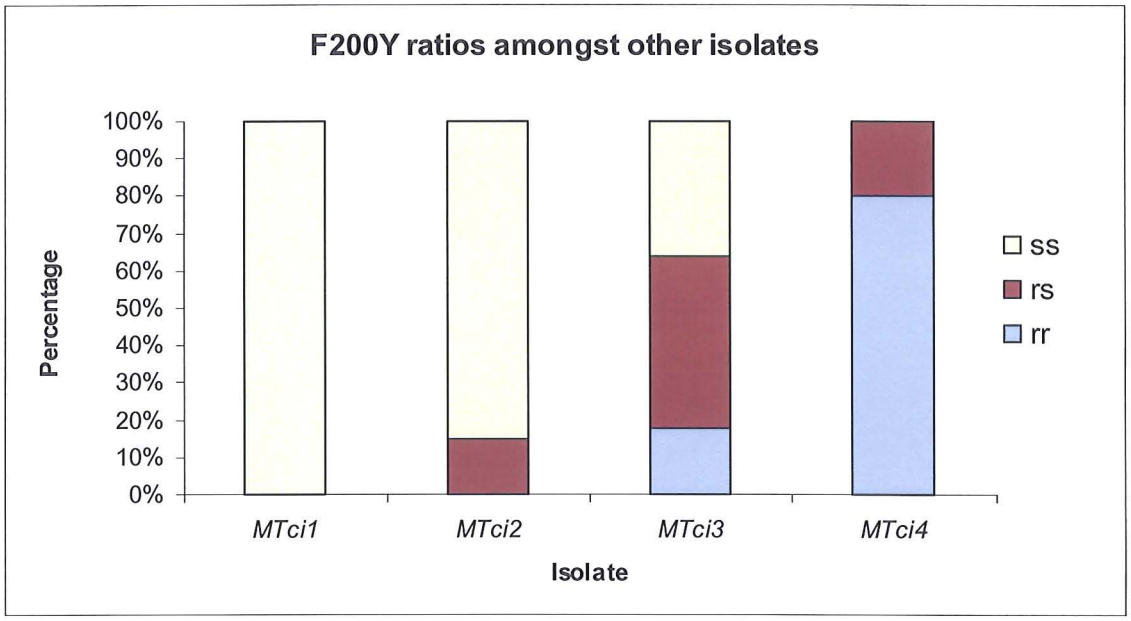
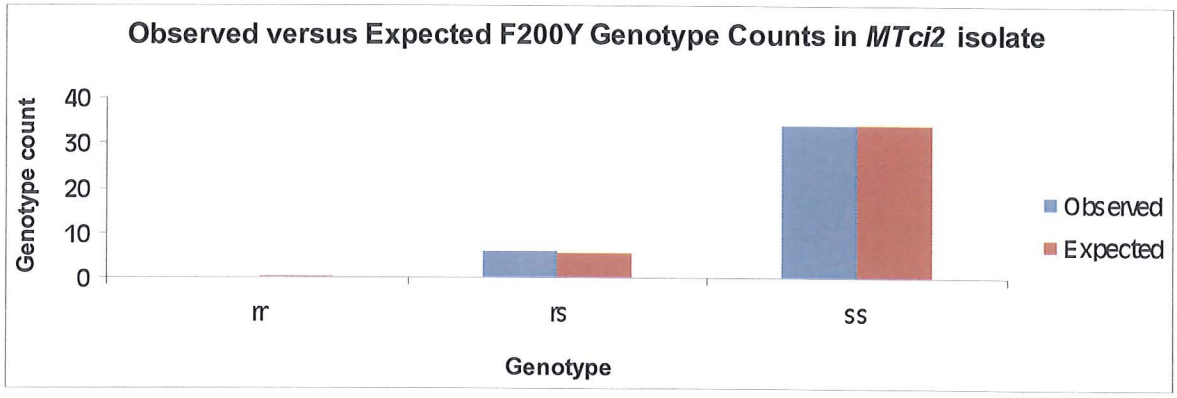
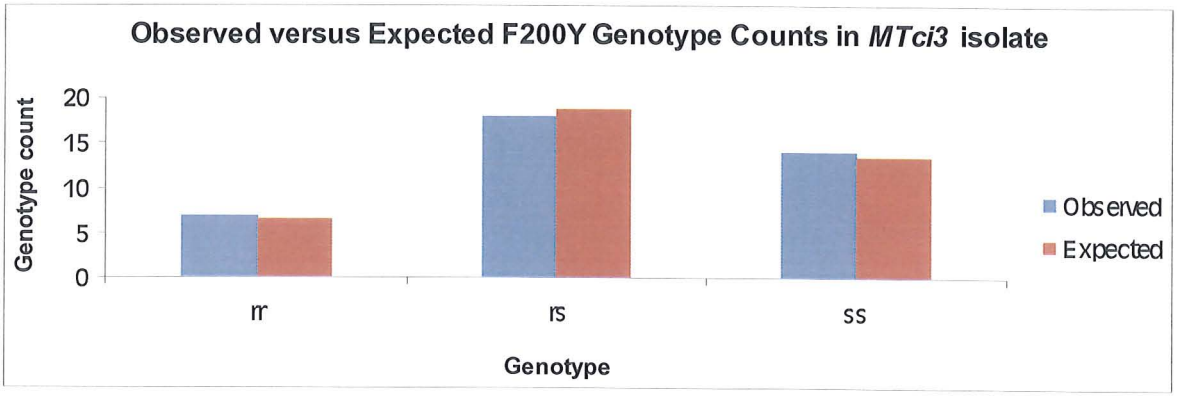


Figure 5.24: Chi-square (X^2) statistics (for Hardy Weinberg Equilibrium) calculated in GenAlEx version 6 (Peakall & Smouse, 2006) add-in software for Microsoft Excel. The graphs show the observed versus expected F200Y isotype I β-tubulin genotype counts amongst three mainland *T. circumcincta* populations. *MTci1* could not be analysed as it was monomorphic at this locus and *ScKiTc* could not be genotyped successfully. The rr, rs and ss refer genotypes refer to $P200^{Tyr/Tyr}$, $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$, respectively. There was no significant deviation from HWE in any of the isolates (see HWE statistics below). Df = degrees of freedom, X^2 = Chi-square statistic and p = probability value.

5.24a Chi-square result for *MTci2* isolate: Df = 1, X^2 = 0.263, p = 0.608.



5.24b Chi-square result for *MTci3* isolate: Df = 1, X^2 = 0.083, p = 0.773.



5.24c Chi-square result for *MTci4* isolate: Df = 1, X^2 = 0.494, p = 0.482.

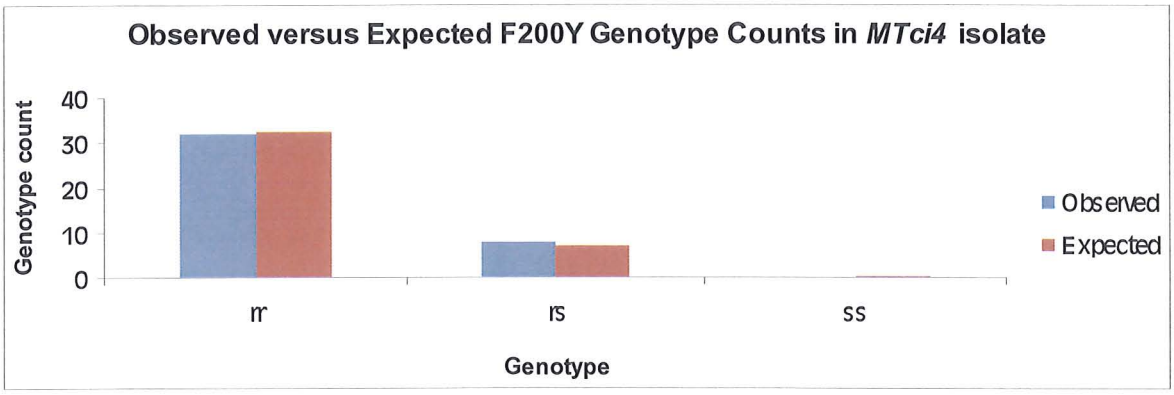


Figure 5.25: Graph showing the SSCP allele frequencies amongst all isolates examined. *MTci1* and *MTci2* are phenotypically susceptible, whereas *MTci3* and *MTci4* are phenotypically resistant to BZ. There was a total of 80 alleles examined for isolates *MTci1, MTci2, MTci3* and *MTci4* and 576 alleles for the *MTci5* isolate and 180 alleles for *ScKiTc* isolate. The allele frequencies for all four *MTci5* populations have been grouped together for this analysis. D and E alleles are also classed as one group here.

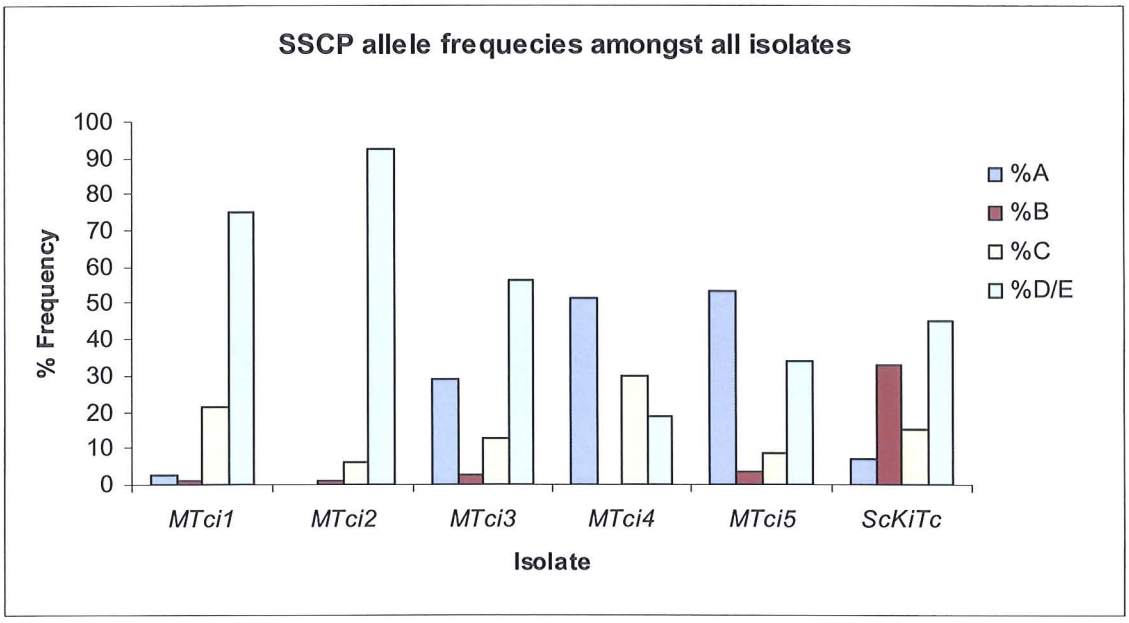
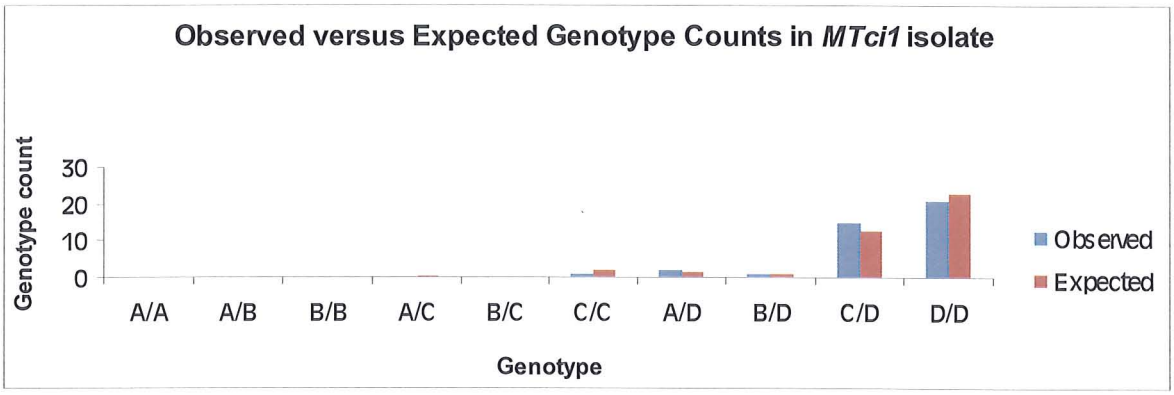
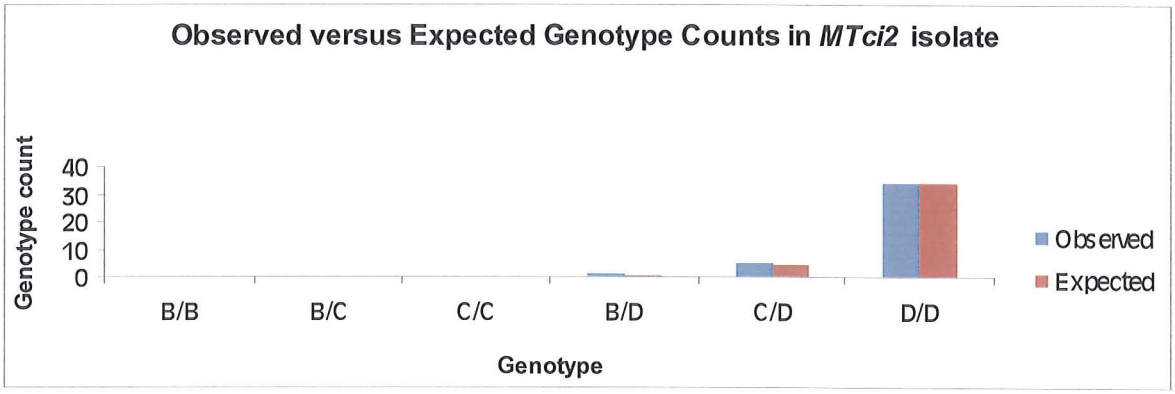


Figure 5.26: Chi-square (X^2) statistics (for Hardy Weinberg Equilibrium) calculated in GenAlEx version 6 (Peakall & Smouse, 2006) add-in for Microsoft Excel. The graphs show the observed versus expected genotype counts in the *MTci1, MTci2, MTci3, MTci4* and *ScKiTc* isolates (D and E alleles are treated as one class). There was no significant deviation from HWE in any of the mainland populations, however a highly significant deviation was observed from the *ScKiTc* isolate (see HWE statistics below). Df = degrees of freedom, X^2 = Chi-square statistic and p = probability value.

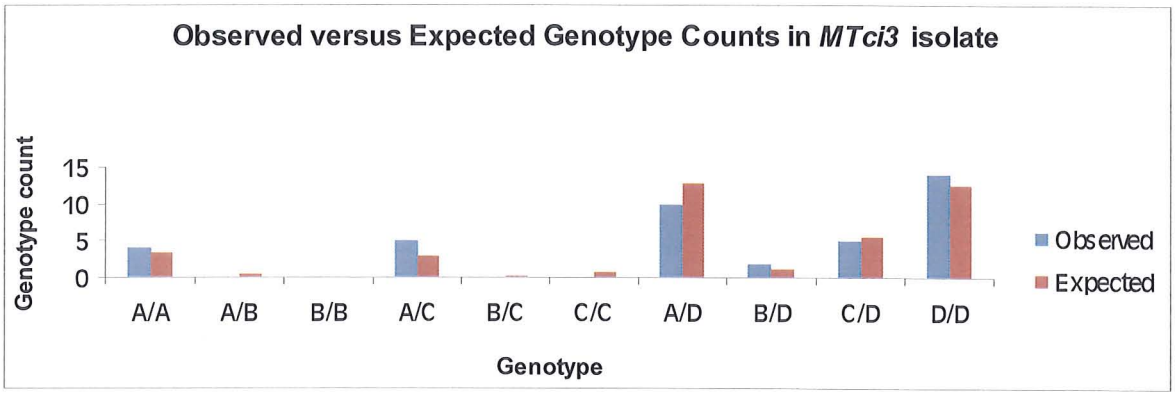
5.26a. Chi-square result for *MTci1* isolate: Df = 6, X^2 = 1.801, p = 0.937.



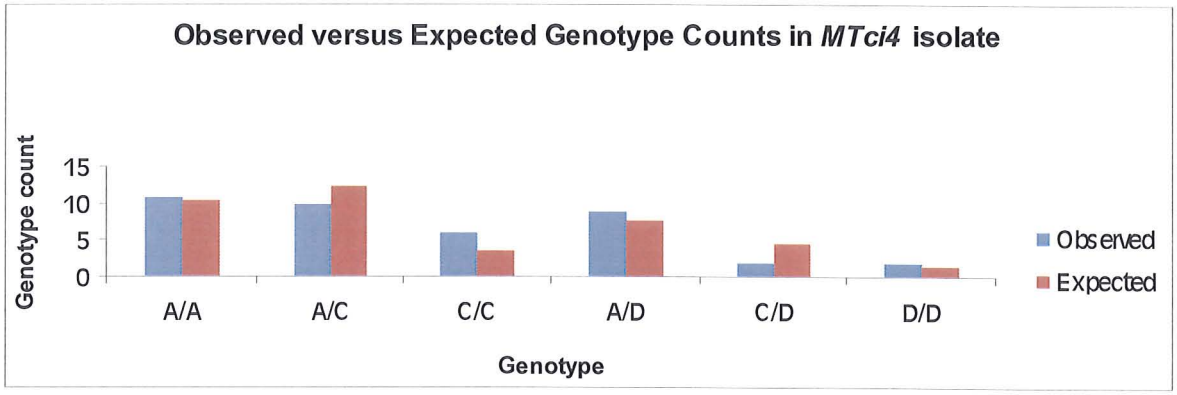
5.26b. Chi-square result for *MTci2* isolate: Df = 3, X^2 = 0.263, p = 0.967.



5.26c. Chi-square result for *MTci3* isolate: Df = 6, X^2 = 4.751, p = 0.576.



5.26d. Chi-square result for *MTci4* isolate: Df = 3, X^2 = 3.917, p = 0.271.



5.26e. Chi-square result for *ScKiTc* isolate: Df = 6, X^2 = 40.569, p < 0.001 (highly significant).

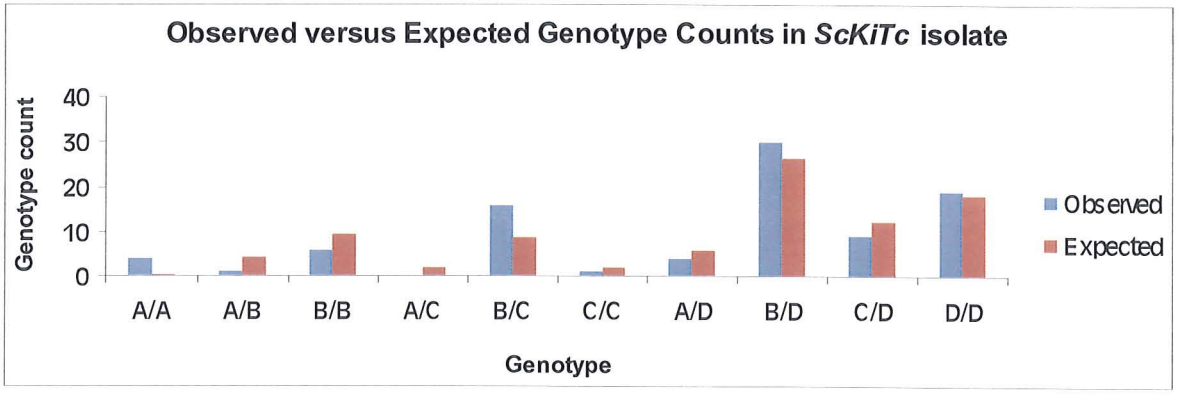
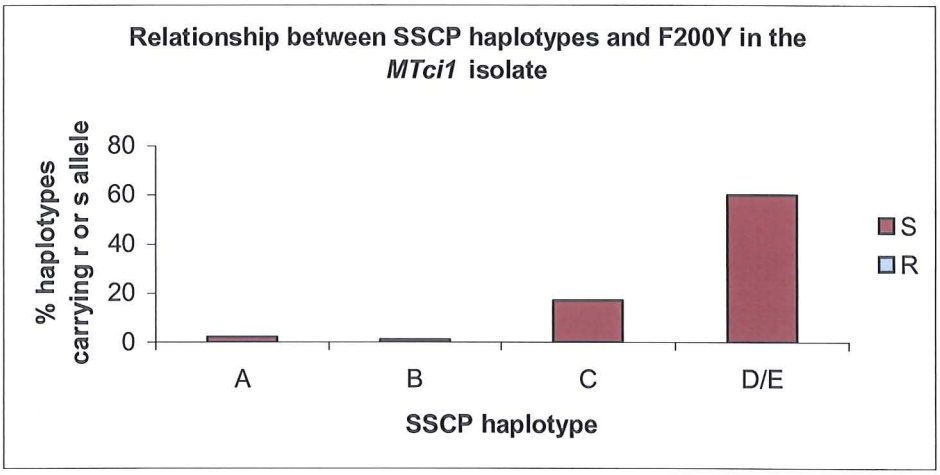
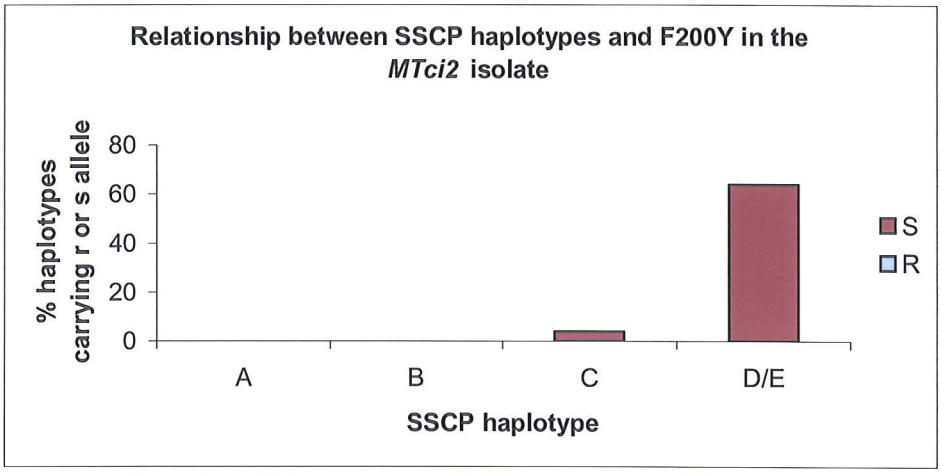


Figure 5.27: In the same way as Figure 5.9 shows, the relationship between SSCP haplotype and the P200^{Tyr} resistant (r) and P200^{Phe} susceptible (s) allele of the isotype I β-tubulin gene was explored in each isolate. Thus, the following charts show the proportion of each SSCP haplotype which carry resistance or susceptibility as defined by the F200Y isotype I β-tubulin mutation.

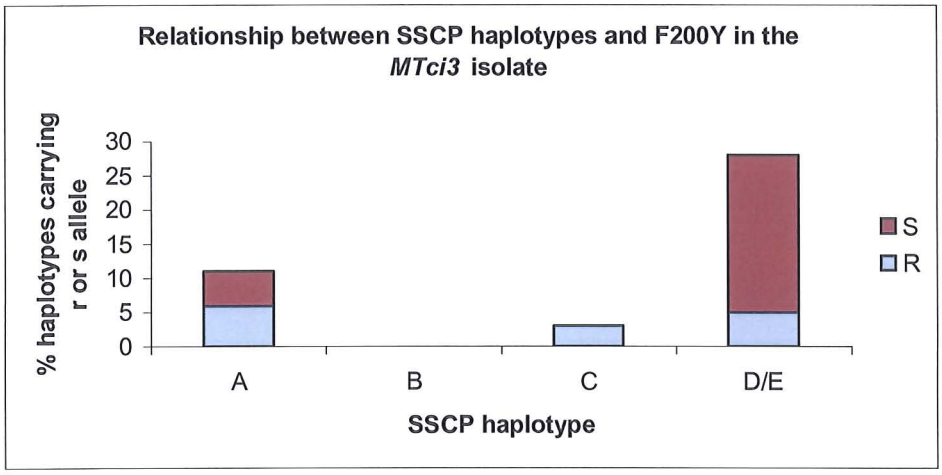
5.27a:



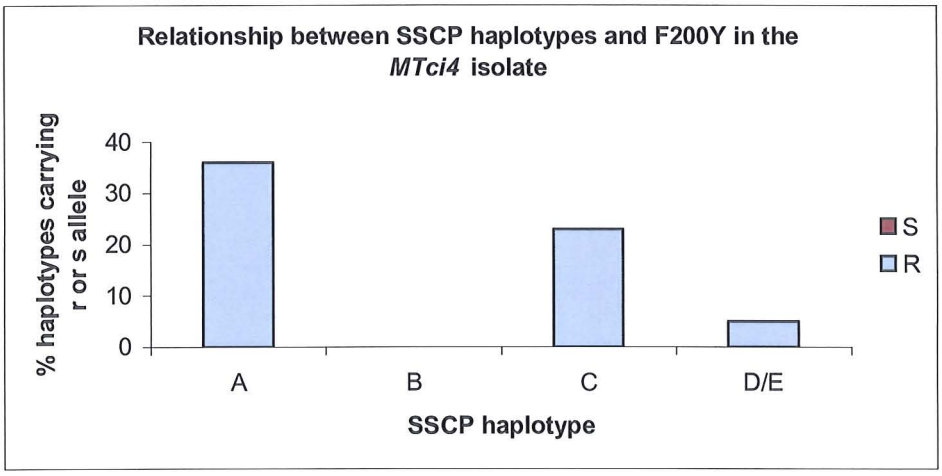
5.27b:



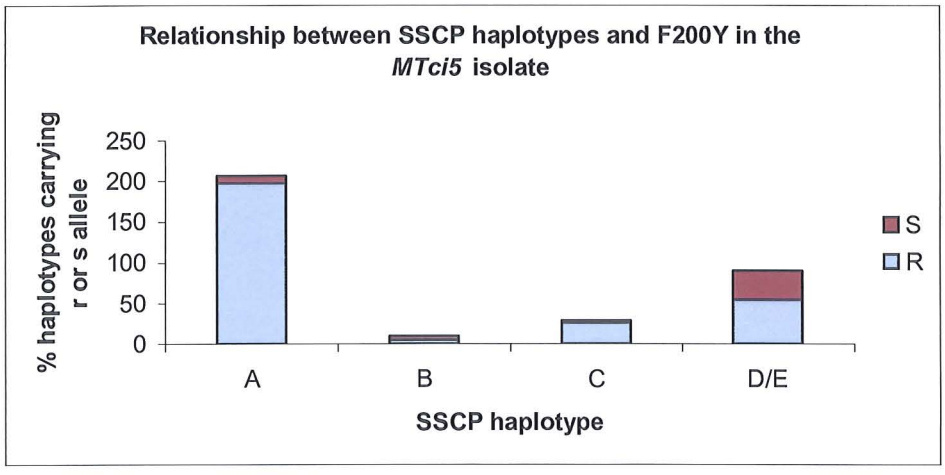
5.27c:



5.27d:



5.27e:



5.6 Discussion

This Chapter involved a survey of the diversity of isotype I β-tubulin alleles amongst a number of UK *T. circumcincta* isolates with varying levels of resistance and gene flow. Specifically, this research concentrated upon detailed characterisation of these alleles in the *MTci5* isolate. The isotype I β-tubulin gene has a well-documented involvement with BZ resistance and possibly IVM resistance amongst nematodes of veterinary and medical importance. Hence, studies such as this are necessary to aid our understanding of the origins and spread of resistance alleles amongst populations. A combined SSCP and sequencing approach was adopted to estimate the genetic diversity of the *MTci5* population prior to and following anthelmintic selection. This approach was chosen due to the rapid, simple and cost-effective nature of SSCP, which could be used to select representatives of all novel haplotypes for direct sequencing. This selective method was desirable as sequencing is expensive, time-consuming and often requires replicate samples due to errors. Furthermore, the SSCP screening method allows some genetic analysis, it removes the likelihood of sequencing PCR artefacts and it allows cloning bias to be identified. This Chapter involved analysing one isolate in great detail (*MTci5*) and using the information gained from it to interpret the genetic diversity of five other UK *T. circumcincta* populations in lesser detail.

In light of previous studies, this chapter initially aimed to answer the following questions regarding the *MTci5* isolate:

- *How diverse are the isotype I β-tubulin alleles in the unselected MTci5 isolate? This will provide a useful comparison to previous studies in the literature regarding resistant isolates. For instance, does MTci5 fit with the expectation that anthelmintic-selected populations show a reduced level of polymorphism?*
- *Are there any effects of drug selection upon SSCP allele frequencies in MTci5? This will indicate whether the isotype I β-tubulin alleles are under selection by BZ, IVM or LEV. From the previous chapters, we have shown that F200Y is under selection by BZ in this isolate, but are there other changes in the isotype I β-tubulin gene in response to drug selection? This may also indicate if the F200Y mutation is linked to other regions of the isotype I β-tubulin gene, which may be important in BZ resistance.*

- *How many haplotypes carry F200Y? If several haplotypes carry the resistance allele, this suggests that the F200Y mutation is an "ancient" pre-existing polymorphism or that there has been a migration of resistance alleles from multiple sources. If only one resistance allele is found, this will suggest that F200Y is a recently selected mutation.*

5.6.1 Diversity of alleles at the isotype I β-tubulin locus

There have been a number of studies which have assessed the genetic diversity of the isotype I β-tubulin gene and the effects of anthelmintic selection upon allele frequencies at this locus, however, most of these have used *H. contortus* as a model species (Roos *et al.,* 1990; Kwa *et al.,* 1993; Beech *et al.,* 1994; Otsen *et al.,* 2001, Eng *et al.,* 2006). Furthermore, very few studies have undertaken an investigation of the sequence diversity of the isotype I β-tubulin gene amongst trichostrongylid nematodes, with a view to assessing the diversity of resistance alleles in particular. The only study of this sort was conducted by Silvestre & Humbert (2002). The authors analysed the diversity of (isotype I β-tubulin) BZ resistance alleles amongst a number of field populations of *T. circumcincta* from goat farms in France (Silvestre & Humbert, 2002). These farms represented closed nematode communities which were established prior to the introduction of anthelmintics to the market; hence, resistance developed *in situ* without the influence of gene flow. In contrast, the *MTci5* isolate which was derived from a farm which was recently established (1997), and thus, is likely to represent a mixed population from different regions.

The French study aimed to determine whether the F200Y mutation of the isotype I β-tubulin gene was a pre-existing resistance allele or a spontaneous, commonly occurring mutation. A portion of this gene (extending across F200Y) was amplified and sequenced from adult *T. circumcincta* collected from a total of 14 farms (eight in Central France and six in SW France). Of these, ten farms were classed as BZ resistant via the EHA/FECRT methods (four in Central France and all six farms in SW France). A maximum of two resistance alleles were found on any one farm and the majority of farms (seven out of ten) displayed only one resistance allele. In total, Silvestre & Humbert (2002) reported eight novel resistance alleles. There was an important proviso of the latter study, however, in that there were very few individuals sequenced from any one population. The majority of populations were only represented by a sample size of between one and six worms to enable a large number of

populations to be sampled. Therefore, it is highly possible, that more resistance alleles were present in each population than were indicated by this study; if a similar sample size had been used to study the *MTci5* isolate, it is possible that only the $A1^R$ allele would have been found, due to its high frequency. However, it is worth noting that two farms were represented by 15 worms and only a single resistance allele was identified in each case. Hence, one can assume that the most common resistance alleles constituting each population have been identified and for the latter two farms, a single resistance allele appears to be present.

In contrast, the present study revealed dramatic diversity of BZ resistance alleles within the *MTci5* isolate. Alleles sequenced from 28 (predominantly heterozygous) worms from this population revealed 20 different isotype I β-tubulin alleles. Of these, there were seven BZ resistance alleles. This is a striking level of diversity and previous studies have not shown this level of variation. For example, only nine alleles were found representing isotype I β-tubulin from a population of *Cooperia oncophora* and this was a susceptible isolate, thus likely to show greater variation (Njue & Prichard, 2003). Furthermore, it is likely that there are additional alleles which have not been characterised by this study, since 16 of the unique *MTci5* alleles were sequenced from 13 worms from the unselected population alone. Indeed, it is known that a susceptible allele must exist in the allele class A, which was not characterised by this study. For instance, worm PT03 was genotyped as an A/A homozygote but had a $P200^{Phe/Tyr}$ genotype. This susceptible A allele could not be identified despite numerous sequencing attempts. However, by using SSCP as a pre-screening method, attempts were made to sequence all novel examples of each allele class and thus the majority of alleles have probably been identified.

Previous studies which have used non-sequencing techniques to assess the level of variation in a population have reported that much of the genetic diversity at the isotype I β-tubulin locus is lost following BZ selection (Roos *et al.,* 1990; Kwa *et al.,* 1993; Beech *et al.,* 1994; Otsen *et al.,* 2001). Furthermore, the *MTci5* isolate is known to have been under considerable anthelmintic selection in the field and has been selected further in this study, yet a high level of diversity remained. It seems likely that this field isolate has a history of multiple immigrant populations each bringing novel resistance alleles from different backgrounds. Hence, the BZ selection events would not result in a loss of diversity if there were many haplotypes carrying the resistant mutation, F200Y. Whereas, the French goat farms represent closed populations

with only one or two resistance alleles, therefore, by applying BZ selection, most of the allelic diversity would be lost. This is supported by the work of Beech *et al.,* (1994) who suggested that a closed population under continual BZ selection eventually loses the ability to develop further mechanisms of resistance, due to the loss of variation experienced across β-tubulin loci.

The most common alleles in the *MTci5* isolate were the class A and D alleles (47 and 26% respectively). There was a large number of A/A homozygotes in the population, although this did not cause a deviation from HWE. It seems that the $A1^R$ haplotype is the most common resistance allele in the *MTci5* isolate.

5.6.2 Origins of BZ resistance

It is clear from previous experiments (Chapter 3) that the F200Y isotype I β-tubulin mutation is under strong selection by BZ. Furthermore, from the work of others (Kwa *et al.* 1994, 1995), it can be said this mutation is an important genetic determinant of BZ resistance. The question that remains, however, is how did this mutation arise? In terms of the *MTci5* isolate, it is highly unlikely that all seven resistance alleles arose *in situ,* since there would be no selective advantage of generating further resistance alleles after one had already arisen. Therefore, in terms of the *MTci5* isolate, there are two possible explanations for the origin of BZ resistance:

1. F200Y is an ancient (pre-existing) polymorphism and linkage disequilibrium has broken down the association of this SNP with the rest of the isotype I β-tubulin gene over time, leading to its presence on multiple haplotypes.
2. Multiple resistance alleles have been introduced to the *MTci5* isolate via gene flow. These alleles originally arose because F200Y is a spontaneous, commonly occurring mutation.

When the sequence analysis performed on the *MTci5* alleles is considered together with the data from the French study, it seems that the second explanation is more likely for the following reasons. If a resistance mutation was an ancient pre-existing polymorphism in a population, one would expect the same resistance allele to be present on different farms, since

there is little genetic differentiation between UK and French populations (Grillo *et al.,* 2007). However, this was not the case. In the French farm, often there was only a single allele present, whereas in the *MTci5* population there were at least seven resistance alleles. Given the fact that the key difference between the French and the Scottish situation is that the French farms are closed to animal movement but Scottish farms are generally subject to animal movement, then migration of alleles seems to be the most plausible explanation for the difference between the two studies. Indeed, the *MTci5* isolate is from a flock that was only established in 1997 and so is likely to comprise parasites of multiple origins. The fact that identical alleles were present in the *MTci5* isolate and the French populations might, at first sight, be taken to suggest the presence of an ancient pre-existing allele. However, when one considers the population genetic structure of *T. circumcincta,* there is another simple explanation for this phenomenon. Since there is little 'between population sub-structuring' between the *T. circumcincta* populations in France and those in Scotland (Grillo *et al.,* 2007), the same range and frequencies of haplotypes would be anticipated to be present at any particular locus. Hence, if the F200Y isotype I β-tubulin mutation arose, then it would be most likely to occur on the most frequent haplotypes in the population. It follows that these common haplotypes are likely to be the same in France and Scotland. Therefore, resistance alleles of identical sequence are highly possible in different populations, even if they have arisen following the separation of those populations.

Secondly, there were resistant and susceptible alleles found in the *MTci5* isolate that were identical in sequence apart from the F200Y mutation (e.g. $C1^R$ and $C2^S$). It is highly unlikely that linkage disequilibrium has broken down to the extent that this mutation is inherited as a single unit. Rather, if recombination was an important factor in the spread of BZ resistance, longer sections of the gene would be inherited. Furthermore, the sequence analysis would reveal intermediate alleles, which were clearly based on the original resistance allele and the susceptible alleles with which it had recombined. This was not apparent. Moreover, the 'phylogenetic network estimation' (see Figure 5.61) performed by V. Hypsa (University of Glasgow, Scotland), strongly suggests that the resistance allele has arisen spontaneously on multiple occasions and on multiple haplotype backgrounds and these alleles appear to be derived from a number of susceptible allele ancestors (note the positioning of the resistance alleles at the terminal branches). This analysis estimates the number of evolutionary steps separating each pair of sequences, i.e. the 'connection limit', based on the number of

mutational differences between them. Thus, Figure 5.61 shows the genealogy of all sequences including the proposed number of 'missing sequences' or 'steps' separating each haplotype. This is more powerful than conventional phylogenetic analyses, since the output shows the most parsimonious arrangement. Interestingly, this analysis places all class E alleles within the same lineage, suggesting that the SSCP analysis was more reliable in distinguishing allele classes than the Clustal W analysis (see Figures 5.21 & 5.22) indicated. The sequences published from the French study were also included in this analysis and fit well with the genealogy of the *MTci5* alleles.

The French study also examined two other trichostrongylid species comprising the same nematode communities as *T. circumcincta*. Similar findings were made amongst *H. contortus* populations. For instance, 19 *H. contortus* adults were sequenced from a total of seven farms all located in SW France. Six different resistance alleles were found amongst these populations and most populations displayed only one resistance allele (only two populations had two resistance alleles). Again, the presence of a significant number of different resistance alleles, which were found on different farms and which could not have been spread via gene flow, suggests that this is not a pre-adaptation. These results support a theory that F200Y is a commonly-arising spontaneous mutation. Hence, the results of the present study are consistent with that of Silvestre & Humbert (2002) and the large diversity of resistance alleles in *MTci5* can be explained by multiple independent mutations, which originally arose on different farms and were subsequently brought together by gene flow. Whilst it may seem difficult to accept that exactly the same mutation could occur independently across a wide number of populations, and even species, especially when a number of other isotype I β-tubulin mutations (F167Y and E198A) have been shown to confer BZ resistance; it is feasible for two reasons. The first is that nematodes have a very large effective population size and a high mutation rate. For instance, Denver *et al.,* (2004) predicted that two mutations occur per genome, per generation in *C. elegans* and assuming that the *T. circumcincta* genome is 100Mb, as in *C. elegans*, then every one worm in a population of 1×10^8 will have two mutations. It follows that, on a farm with 500 sheep, where each host has an average adult female worm burden of 1000, each producing 500 eggs per day, would constitute a daily output of 2.5×10^8 worms per day. Hence, resistance alleles could potentially arise on a daily basis under selection. Furthermore, *T. circumcincta* has a long potential period of reproduction (Kerboeuf, 1985) due to its tolerance of cold temperatures, and thus, has plenty

of opportunities to produce resistant mutants. Secondly, the heavy selection pressure applied upon these field populations will result in a number of mutations arising that confer full or partial resistance to BZ. Some of these mutations may have a fitness cost, unlike F200Y (Elard *et al.,* 1998), therefore, this mutation is perhaps one of few which has a selective advantage and consequently reaches a high frequency in the population.

5.6.3 Effects of selection upon the isotype I β-tubulin locus

Given the significance of BZ selection upon the F200Y mutation of the isotype I β-tubulin gene witnessed in Chapter 3, it was perhaps surprising that there was not stronger evidence of selection upon the allele frequencies of this gene. However, F200Y was represented by all five SSCP allele classes, and we know that at least seven haplotypes in this population will be under selection by BZ, since they carry the F200Y mutation. Hence, if BZ selection was only acting upon the F200Y mutation, the effect of this selection would be applied evenly across several SSCP allele classes in the population, thus, there would be no obvious change in the frequency of any one allele class. This also accounts for the finding that the *MTci5* isolate did not appear to lose genetic diversity following BZ selection. These results add further support to the functional significance of the F200Y mutation, since it appears that it is the F200Y mutation itself and not a particular haplotype that it is associated with, that is under selection.

There were some significant changes in the genotype frequencies following both BZ and IVM selection. For instance, the frequency of A/B genotypes was reduced in both the BZ- ($z = -3.16$, $p = 0.002$) and IVM-selected populations ($z = -3.16$, $p = 0.002$), the frequency of A/D genotypes increased following BZ selection ($z = 2.49$, $p = 0.013$) and there was also a significant decrease in the frequency of C/D genotypes in the BZ-selected population ($z = -3.41$, $p = 0.001$). The changes which occurred as a consequence of BZ selection might be explained by a situation whereby two susceptible alleles representing one genotype (i.e. $P200^{Phe/Phe}$ individuals) were lost. However, these results perhaps also indicate that there may be an effect of IVM selection upon the isotype I β-tubulin gene, even though the allele frequencies between IVM-selected and unselected populations were not significantly different. This may be a statistical anomaly, since one would expect changes at the level of allele frequency if IVM selection was acting upon the isotype I β-tubulin gene. The failure of the SSCP technique in identifying individual haplotypes has undoubtedly resulted in a loss of

information regarding the effects of drug selection upon this gene. For instance, if individual alleles had been reliably distinguished, those carrying the F200Y mutation would presumably have increased in frequency following BZ selection. Hence, an effect of IVM selection upon this gene cannot be ruled out and further investigation is necessary. Furthermore, there is evidence in the literature of an effect of IVM selection upon the isotype I β-tubulin gene in two parasite species. For instance, Eng & Prichard (2005) reported three amino acid substitutions (M117L, V120I and V124A) located in the H3 helix of the isotype I β-tubulin gene of *Onchocerca volvulus,* which were only found in IVM-selected worms. These were accompanied by a 24bp deletion in intron I. These mutations have been found consistently in association with suspected IVM resistant *O. volvulus* ever since (Eng *et al.,* 2006). Similarly, these authors have reported an effect of IVM selection across the region encoding amino acids 195 to 235* of the isotype I β-tubulin gene of *H. contortus*. Furthermore, an increase in the frequency of $P200^{Phe/Tyr}$ (but not $P200^{Tyr/Tyr}$) genotypes at the F200Y mutation was apparent following IVM selection. Despite the fact that there was no evidence of a change in the frequency of the F200Y allele in the *MTci5* isolate following IVM selection, this was the only *MTci5* population which showed a deviation from HWE at this locus. However, as the principle underlying the Bonferroni correction states, one out of every twenty results analysed at the 0.05 significance level will show statistical significance by chance alone. Indeed, the tests of HWE upon isotype I β-tubulin SSCP alleles showed no deviation in any of the *MTci5* populations, however, the inaccuracy of the method as mentioned above could account for this finding.

5.6.4 Phylogenetic analysis of *MTci5* sequence data

5.6.4.1 Two clades of isotype I β-tubulin alleles

The presence of two distinct phylogenetic clades in the *MTci5* isolate and other *T. circumcincta* populations is intriguing. This phenomenon was first described by Leignel *et al.* (2002) and has been observed since from populations in France (Silvestre & Humbert, 2002), indicating that this is not an isolated case. At first sight, it might look as if there were population sub-structuring, however, the microsatellite analysis of this population (as described in Chapter 3 and by Grillo *et al.,* 2007) refutes that possibility. Furthermore, the

* The present study analysed the region encoding amino acids 151 to 216.

fact that these two clades are represented at the individual worm level, i.e. within the same genotype suggests that *MTci5* and the French populations are freely interbreeding populations with no genetic sub-structuring. Furthermore, the *MTci5* SSCP genotype data show no deviation from HWE, which is inconsistent with the presence of genetic sub-structuring. Instead, what this may represent is a situation where long ago, two populations which were genetically differentiated, were mixed together to create the modern *T. circumcincta* species. Over time, Hardy-Weinberg equilibrium would have been restored so that no genetic sub-structuring is detectable, but the highly conserved nature of this gene has preserved the integrity of these allele types.

5.6.4.2 Sequence diversity in the MTci5 isolate

The nucleotide diversity estimates for *MTci5* were very similar to that described by Beech *et al.,* (1994) for a susceptible population of *H. contortus*. This further indicates that there is no apparent loss of genetic diversity in *MTci5* as a consequence of anthelmintic selection in the field. Slatkin (1982, 1989) and Strobeck (1987) suggest that in a situation where two populations have evolved separately and divergence has occurred, and if the alleles from these populations are subsequently mixed by migration/gene flow, then the resultant estimate of nucleotide diversity will be much higher. It is likely that *MTci5* has been subject to a high level of gene flow, from a number of different populations, therefore, this might explain the high levels of nucleotide diversity witnessed here. The nucleotide diversity estimate can be used to calculate the effective population size (*Ne*) of *MTci5* using the equation $\pi = 4Ne\mu$, where μ is the mutation rate per sequence per generation. The estimate of μ is expected to be the same across all nematode species, 10^{-8} (Li, 1997). This means that with an overall π of 0.05586 (or 0.05959 with Jukes-Cantor, [1969] correction), the *Ne* of *MTci5* is between 1,396,500 and 1,489,750. The intron I π estimates of *MTci5* seem reasonable when compared with the π estimate for the same region of this gene in a susceptible *H. contortus* population. For example, Beech *et al.,* (1994) proposed a π of 0.094, and this is very similar to the π estimate of 0.086 (or 0.095 with Jukes-Cantor, [1969] correction) observed in *MTci5*. This similarity is perhaps surprising given that *H. contortus* has a ten fold higher fecundity than *T. circumcincta*, however, *MTci5* is probably a poor comparison to this particular *H. contortus* isolate, since it is likely that the high levels of gene flow have increased the level of genetic diversity beyond that of an isolated susceptible population.

5.6.4.3 Haplotype diversity/ neutrality

None of the haplotypes showed a significant deviation from the neutral model; therefore it would appear that selection for rare alleles has not occurred. This is consistent with the balanced effects of BZ selection across all alleles due to the large number of haplotypes carrying F200Y. Unfortunately inadequate sampling from all four *MTci5* populations or of alleles from other isolates has prevented the use of haplotype neutrality tests in analysis of gene flow. This would be a useful approach for future studies and the current data for *MTci5* haplotypes could be used again for this purpose.

Haplotype diversity (Hd) estimates were highest for the intron region (Hd = 0.974, nH = 15), and second highest for the exon 2 region (Hd = 0.789, nH = 6). This suggests that the exon 1 region is more highly conserved than the former regions of the isotype I β-tubulin gene. The high Hd estimates of intron 1 and exon 2 also suggest that these regions diverged some time ago. This is consistent with the findings that close homologues of the *MTci5* alleles were described by Silvestre & Humbert (2002). For instance, the UK and French *T. circumcincta* metapopulations are likely to have been geographically isolated for some time thus, these alleles have been very highly conserved. This is perhaps surprising, since non-coding DNA is generally thought to be more rapidly evolving than coding DNA, however, perhaps the lower Hd estimate for exon 2 (which suggests more recent divergence than is estimated for the intron region) is a product of the recent BZ selection pressure. Moreover, the F200Y mutation lies within exon 2 of the isotype I β-tubulin gene and there is strong evidence for selection upon this locus, as we report in Chapter 3.

5.6.5 Characterisation of isotype I β-tubulin alleles of other UK *T. circumcincta* isolates

5.6.5.1 Relationship between SSCP haplotype and F200Y isotype I β-tubulin mutation

The finding that F200Y exists on multiple haplotype backgrounds in the *MTci3* and *MTci4* isolates was crucial. In the *MTci3* isolate, there were two haplotype classes that contained resistant and susceptible alleles (A and DE) and the class C alleles were found to be resistant. It is not known exactly how many resistance alleles exist in this population without sequencing, but this analysis shows that there are at least three. This is important as it

supports a hypothesis of multiple origins of resistance, followed by migration and mixing of alleles. It also suggests that multiple resistant alleles have co-existed in single populations in the field for many years. For example, *MTci3* was isolated in 1983 at MRI and the first case of BZ resistance in the UK was reported only a year earlier (Britt, 1982). However, it is likely that BZ resistance alleles had been in existence for many years prior to the first reports of phenotypic resistance. Moreover, the opportunity for the F200Y mutation to arise spontaneously on multiple occasions was there for 20 years after the introduction of BZ to the market. The geographical origins of the *MTci3* and *MTci5* isolates are very close, although, the farm from which *MTci5* was isolated was not established until 1997. However, it is not unlikely that resistance alleles from *MTci3* reached the *MTci5* location, perhaps via other farms. The *MTci4* isolate also carries at least three resistant alleles. Whilst no susceptible alleles could be identified in this population, due to the lack of $P200^{Phe/Phe}$ genotypes, they must exist as the frequency of the F200Y mutation was found to be 90%.

5.6.5.2 Frequency of F200Y isotype I β-tubulin mutation amongst UK T. circumcincta isolates

The low frequency of F200Y observed amongst the two mainland susceptible isolates (*MTci1* & *MTci2*) was unsurprising, given the previous phenotypic characterisation of BZ resistance. Similarly, the *MTci3* isolate did not produce surprising results. However, the frequency of the F200Y mutation in the *MTci4* isolate was very high, and no susceptible homozygotes were found in the sample. This isolate was derived from a goat population and goats are known to propagate resistance at a faster pace due to the high metabolic rate and concomitant reduction in drug bioavailability.

It was unfortunate that the frequency of the F200Y mutation could not be analysed in the *ScKiTc* isolate, because it would be interesting to see if this SNP was occurring at a low frequency in the population, i.e. pre-existing. The failure of the Pyrosequencing assay to successfully genotype even one out of 90 worms suggests that sequence variation at the primer sites might be a possible explanation. However, the *ScKiTc* isolate is unlikely to be very differentiated from the mainland populations considering that the SSCP primers annealed well and that the banding patterns representing each allele class were very similar to those observed in the other isolates, indicating good homogeneity in sequence. Furthermore, the isotype I β-tubulin gene is, according to these results and that of Silvestre & Humbert (2002), very highly

conserved between UK (*MTci5*) and French populations, thus, genetic drift seems unlikely in *ScKiTc*. Moreover, previous studies have shown little genetic differentiation between the *ScKiTc* isolate and other UK and French populations (Grillo *et al.,* 2007; Braisher *et al.,* 2004). Instead, it is likely that poor template quality/ low DNA concentration is responsible for the failed Pyrosequencing assays.

In the case of *MTci2*, it is apparent from the results of the F200Y Pyrosequencing assay, that this isolate has been exposed to BZ selection at some point. It is possible that *MTci2* was isolated soon after this selection event, and the reintroduction of genetic diversity via gene flow (which has probably occurred amongst all mainland resistant isolates) was prevented. It is unlikely that the sample sizes of these populations were inadequate as usually a sample of 30 individuals is considered satisfactory for analysis of genetic diversity and a minimum of 40 worms were examined from each population here.

5.6.5.3 SSCP allele diversity amongst UK T. circumcincta isolates

The results of SSCP analysis across five other isolates were interesting. Firstly, the two mainland susceptible isolates (*MTci1* & *MTci2*) were similar in profile, with the DE alleles being the predominant class (75-95%). Very low frequencies (or an absence) of the class A and B alleles were also apparent in these isolates. Conversely, in the *MTci4* and *MTci5* isolates, class A alleles were far more common (~50%) and the DE alleles showed lower frequencies of 20-30%. The *MTci3* isolate showed similarities with both sets of isolates, showing around 30% A and 55% DE allele frequencies. In all five mainland isolates, the frequency of type B alleles was always low (< 5%), however these alleles were common in the *ScKiTc* isolate at a frequency of around 30%. Class DE alleles were also prevalent in *ScKiTc* with a frequency of 45%, but this was only half the frequency observed for DE alleles from the susceptible mainland counterparts. The *MTci4* isolate was of particular interest to include in this study due to the fact that it was propagated in goats. It was of interest whether, as Leignel *et al.,* (2002) described previously, there was divergence of this species as a consequence of the different host species. However, by this assay, the *MTci4* isolate seems similar to *MTci5* in the genetic constitution of the isotype I β-tubulin gene.

The *ScKiTc* isolate was also of interest due to the geographical isolation of this population. The harsh conditions of the environment for the sheep hosts on St Kilda result in frequent

population crashes and it might be predicted that the genetic diversity of the nematode population would also undergo bottlenecks. Moreover, the harsh environmental conditions and the poor rate of infection due to low host spatial density places these nematodes under a persistent set of natural selection pressures (Gulland *et al.,* 1993). These factors might be expected to reduce the effective population size of this species, however, population diversity is thought to assist these worms in their evolutionary survival strategy (Wimmer *et al.,* 2004). Furthermore, these results indicate that this isolate is still highly polymorphic at this locus and this suggests that there is no evidence of a significant loss of genetic diversity. This is consistent with the findings of Grillo *et al.* (2007). Indeed, analysis of the genotype frequencies of this isolate have shown that nine out of a possible ten SSCP genotype combinations were present, whereas only three and five genotypes were observed in the comparable susceptible mainland isolates, *MTci2* & *MTci1,* respectively. The most likely explanation is that the laboratory passage of these isolates for twenty years or so has reduced the level of genetic diversity, however, previous studies have rejected this possibility (Roos *et al.,* 1990; Otsen *et al.,* 2001). It is intriguing that the *ScKiTc* population was the only one to show a deviation from HWE, and this was highly significant. Since previous reports have shown little genetic differentiation of the *ScKiTc* isolate in comparison to mainland populations (Grillo *et al.,* 2007; Braisher *et al.,* 2004), this is very surprising indeed. However, as the findings of Silvestre & Humbert (2002) suggest, genetic drift may well explain the differences in allele frequencies of geographically isolated populations at the isotype I β-tubulin locus. Furthermore, perhaps a lower *Ne* (for the reasons explained above) has contributed to genetic drift. The lack of information regarding the anthelmintic selection that this population has received over the years accompanied by the poor identification of individual haplotypes by SSCP makes these findings inconclusive. Hence, further work is desirable to examine the diversity of alleles at the isotype I β-tubulin locus amongst mainland and geographically isolated *T. circumcincta* populations.

5.6.6 Summary

This study has demonstrated that SSCP is a useful technique for the rapid determination of the diversity of alleles across a small region of a gene. This information can then be used to selectively clone and sequence novel alleles in the population, which is both cost- and time-effective. The particular region assayed in this study was not ideal, as it did not resolve

sufficiently to discriminate between individual alleles. It did, however, allow a classification of similar alleles, the sequencing of which supported the identification method. It also allowed more sequence variation to be identified, which is useful in analysis of the origins of BZ resistance alleles. The classification system was then used to rapidly survey five UK *T. circumcincta* isolates for information regarding the genetic diversity of the isotype I β-tubulin gene, and the quantity and type of resistance alleles found. This study has highlighted four key findings, the first being that the *MTci5* isolate shows a staggering level of genetic diversity in both susceptible and resistant alleles, the likes of which have never been reported from one population. The second finding was that multiple BZ resistance alleles were found in two other mainland Scottish isolates, from classes A, C and DE and these were probably brought together via gene flow. The third finding is that class A alleles were strongly associated with resistance and therefore, if this technique was refined to distinguish more clearly between alleles, this would provide a good method of estimating the potential of a population to develop resistance. Finally, these results strongly support those of Silvestre & Humbert (2002) and provide evidence for the F200Y isotype I β-tubulin mutation having arisen spontaneously on multiple occasions and the *MTci5* isolate represents a mix of these different resistance alleles which have been brought together by gene flow.

As mentioned previously, most studies have observed a loss of polymorphism in the isotype I β-tubulin gene following BZ selection (Roos *et al.,* 1990; Kwa *et al.,* 1993; Beech *et al.,* 1994; Otsen *et al.,* 2001). This loss of variation can be due to selection upon specific loci (e.g. F200Y mutation of isotype I β-tubulin gene) allowing only certain alleles to persist, combined with a lack of recombination in the region surrounding these loci. This is known as a 'hitchhiking effect'. In this situation, the other genes in the genome should not display a reduced level of polymorphism, unless they are linked to the isotype I β-tubulin gene. This is the reason for employing control genes in such analyses, since the reduction in polymorphism of the gene of interest, may be due to bottlenecks or founding events after drug selection. In the latter scenario, other loci will show a decreased level of polymorphism. Hence, most SSCP studies include a control gene, that is, a gene which is not expected to show any effects of drug selection. This study used a different approach to control for gross changes in population structure; the use of microsatellite markers. As the results of Chapter 3 suggested, there was no effect of drug selection across these five neutral markers, and this is perhaps better than analysing a single control gene by SSCP. Moreover, the same markers were used

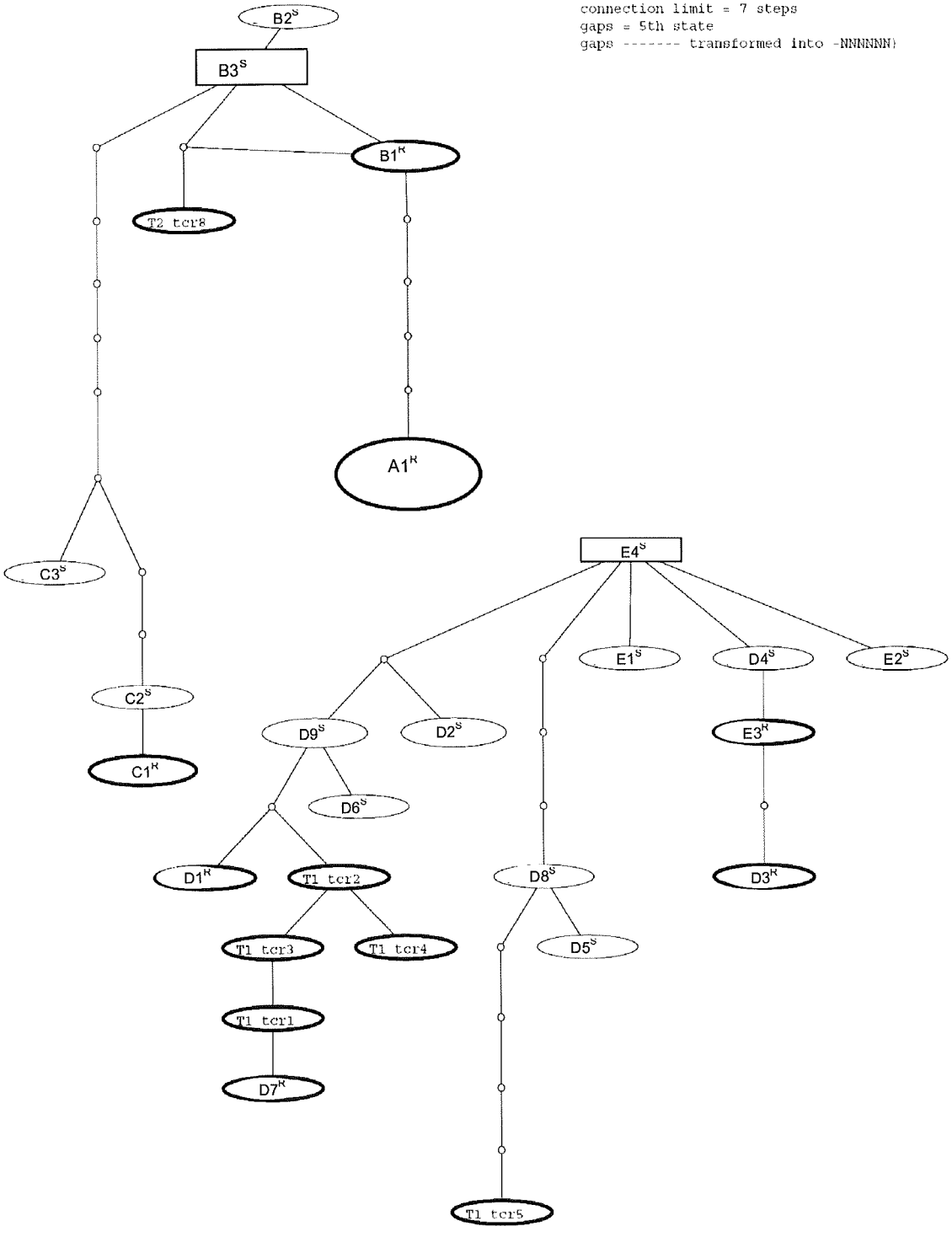
to compare two of the most distant populations, unselected *MTci5* and *MTci1*, and there was little variation between these populations (Grillo *et al.,* 2007). Similarly, Leignel & Humbert (2001) studied the genetic variation of the mtDNA ND4 gene between BZ resistant and susceptible *T. circumcincta* populations and found no evidence of selection upon this neutral locus.

5.6.7 Future work

A useful extension to the present study would include sequencing of alleles from the other isolates, for comparison with the *MTci5* dataset. This would also provide a more accurate indication of the diversity of the isotype I β-tubulin gene and in particular, the number of BZ resistance alleles. Based on what we now know are the most variable sites in this region, a new SSCP assay could be designed, which would reduce the fragment length and improve resolution, thus permit identification of individual alleles. It would be advised that this assay should include positions 60 through to 249 of the sequence (see Figure 5.20, Tables 5.1 to 5.3) to maintain the level of discrimination between all alleles sequenced so far. This would generate an 189bp fragment as opposed to a 278bp fragment. The majority of this fragment would represent intron sequence and would extend 66 bases into exon 2 (from codons 178 to 200). This would be a useful assay for the presence and diversity of resistance alleles amongst populations, and could be extended for use in nematodes of importance to cattle to avoid the spread of BZ resistance in the future.

It would also be advisable to examine other small regions of the isotype I β-tubulin gene to look for evidence regarding potential effects of IVM resistance, given the findings of Eng *et al.,* 2006).

Figure 5.61 Phylogenetic network estimation of isotype I *β*-tubulin alleles sequenced from the *MTci5* isolate (analysis performed by V. Hypsa, University of Glasgow).



6.0 Chapter 6 General Discussion

This study has examined the trichostrongylid nematode, *Teladorsagia circumcincta*, and has provided some valuable insights into different aspects of anthelmintic resistance in this species. In particular, this study deals with the origins and mechanisms of BZ resistance and the population genetic structure of a MDR isolate (see Figure 6.1). Since anthelmintic resistance has now reached critical levels in some countries and the discovery, testing and registration of novel anthelmintics is a slow and costly process, a number of approaches towards sustainable control are being evaluated. This study has attempted to contribute to the research on molecular and phenotypic characterisation of anthelmintic resistance, with the long term aims of preserving the efficacy of the anthelmintics currently available and identifying means of slowing the rate of resistance development against future products. However, effective conservation of efficacy requires a comprehensive understanding of a) the origins of resistance and the selection processes, b) the mechanisms underpinning resistance and c) the genetic consequences of anthelmintic treatments at the population level. Considering that we currently lack genetic markers associated with macrocyclic lactone and tetrahydropyrimidine/ imidazothiazole resistance, it was decided to study a well documented SNP, the F200Y isotype I β-tubulin mutation, as a genetic marker for BZ resistance. With this in mind, the aim of this thesis was to characterise a MDR field isolate of *T. circumcincta* (*MTci5*) and to gain some understanding of the effects of drug selection upon available markers. Underpinning all of the experiments in this study was a method whereby this isolate was pressurised separately with three broad-spectrum anthelmintics. Adopting the approach of examining a locus before and after selection is a more powerful way of analyzing the genetic consequences of selection than comparing two genetically unrelated populations. This is due to the high level of genetic diversity of nematodes, which can result from stochastic differences as well as the genetic changes associated with anthelmintic selection. Furthermore, all of the molecular techniques described in this thesis were performed on the same adult worms, thus a library of information has been created at the individual worm level from the parent *MTci5* isolate and the three drug-selected F1 generations derived from it. Moreover, the F1 generations have been archived for future anthelmintic selection experiments when novel markers for resistance become available.

6.1 Analysis of population genetic structure of MTci5 isolate

The population genetic structure of the *MTci5* isolate has been investigated using molecular assays. There are two theories regarding MDR status, the first being that individual worms are each resistant to all three broad spectrum anthelmintics, or alternatively, that MDR isolates comprise sub-populations which are each resistant to only one or two drugs (admixture). The findings presented in this thesis suggest that the *MTci5* isolate is a freely interbreeding population with triple resistance, with no evidence of genetic sub-structuring. This has important implications for the use of combination drenching and future approaches to control. Whilst combination drenching is more effective against MDR populations than the use of single drenches, there will be still be a proportion of survivors,* and thus, where MDR is diagnosed, combination drenching will, if used prophylactically, inevitably increase the gene pool for resistance. Under these circumstances, it would be advisable to employ combination drenches for quarantine and therapeutic purposes, instead of routine prophylactic treatments. The microsatellite analysis also showed that there is no evidence of linkage between anthelmintic resistance genes and these neutral markers. Hence, these markers were useful in estimation of any potential hitchhiking effects of selection and to act as negative control loci when examining the effects of selection at the isotype I β-tubulin locus. A future research priority will be to identify markers which show evidence of selection by different anthelmintic treatments, particularly IVM and LEV, since key resistance loci for these drugs are yet to be found. Such an approach will require extensive genome sequencing of *T. circumcincta*.

6.2 Mechanisms of BZ resistance

Resistance to anthelmintics, particularly to BZ, has traditionally been assumed to be a feature of modification of the target site (Sangster, 1996). Research on BZ resistance suggests that mutations in the β-tubulin genes are clearly key mechanisms, however, there have been suggestions that other mechanisms could be involved (Beugnet *et al.*, 1997; Pouliot *et al.*, 1997; Xu *et al.*, 1998; Berge *et al.*, 1998; Sangster *et al.*, 1999; Smith & Prichard, 2002; Kerboeuf *et al.*, 2003; Kotze *et al.*, 2006a,b; Blackhall *et al.*, in press). The results presented in this thesis clearly demonstrate the importance of the F200Y isotype I β-tubulin mutation to the BZ resistance phenotype of the *MTci5* isolate. For instance, there was clear selection of

* Between 6 and 8% of the infrapopulation survived combination drug treatments, as demonstrated by the controlled efficacy test conducted in *MTci5* by Bartley *et al.* (2005).

this mutation by BZ treatment in the absence of selection of five independent microsatellite markers, or of any particular SSCP allele class of the isotype I β-tubulin locus. However, there are two major results presented in this thesis, which suggest that the BZ resistance phenotype may not be entirely explained by this single mutation. Firstly, the survival of the $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes following BZ selection: if one assumes the $P200^{Tyr}$ resistance allele is completely recessive, as has been suggested in the literature (Elard *et al.,* 1998; Elard & Humbert, 1999), then this would clearly imply that at least one other resistance-conferring locus was present. Alternatively, if the $P200^{Tyr}$ allele was not fully recessive in the *MTci5* genetic background, then that would explain the survival of the $P200^{Phe/Tyr}$ genotypes, without recourse to additional resistance loci. However, it would not explain the survival of $P200^{Phe/Phe}$ genotypes, although it has to be accepted that there were only a very small proportion of these. In summary, the data on the survival of $P200^{Phe/Tyr}$ and $P200^{Phe/Phe}$ genotypes in the *MTci5* isolate following BZ exposure does not fit a simple model of the resistance phenotype being entirely due to the F200Y mutation, which is also fully recessive. The second finding which suggests that there may be additional BZ resistance mechanisms in the *MTci5* isolate, was the increase in susceptibility induced by the presence of verapamil hydrochloride (VPL) and piperonyl butoxide (PB). These are the inhibitors of the P-glycoprotein (P-gp) efflux mechanism and the cytochromes P450 (CYP) metabolic pathways, respectively.

The involvement of such additional mechanisms is a worrying prospect, particularly if these mechanisms have wide substrate-specificity as implicated previously. One potential solution would be to use inhibitors such as VPL or PB in combination with anthelmintic treatments. However, whilst there is a strong synergistic effect of VPL and PB inhibitors with BZ anthelmintics *in vitro*, their use is not easily translated to an *in vivo* situation, whereby host drug detoxification mechanisms will also be compromised. Such *in vivo* experiments have been conducted and verapamil has a dose-dependent toxic effect in mammals (from studies in jirds and sheep) regardless of the presence of an anthelmintic (Xu *et al.,* 1998; Molento & Prichard, 1999). A combination of CYP inhibitors and anthelmintics has been suggested previously to extend the life of an anthelmintic *in vivo,* as host-mediated detoxification of anthelmintics limits drug bioavailability. For instance, PB was used in combination with fenbendazole in sheep and goats and pre-administration of the inhibitor was shown to increase the bioavailability of the drug. Co-administration of these drugs in sheep infected with BZ-

resistant *T. circumcincta* increased the efficacy of the anthelmintic from 7.9% to 97.8% (Benchaoui & McKellar, 1996). This effect of PB *in vivo* could be caused by one of two processes: firstly, increased bioavailability of the drug through interruption of host CYP activity allowing prolonged exposure of parasites to the anthelmintic and, secondly, compromised resistance of the parasites through interruption of their CYP activity. Whilst this research is hugely encouraging, it is questionable whether these drugs will be licensed for use as a combination therapy in the field due to the dangers of host toxicity, particularly since variation between hosts will mean that some animals are more sensitive than others to these treatments. For example, the co-administration of verapamil with IVM in mice results in neurotoxic effects of the anthelmintic (Schinkel *et al.,* 1994). It is also possible that CYP and P-gp are not the only resistance mechanisms involved, and as yet, we do not have suitable markers for analysis of these loci. Consequently, there is a need to employ genome-wide approaches to investigate the presence of additional resistance-conferring loci and to assess their relative contribution to the resistance phenotype (Gilleard, 2006)

Future studies of the *MTci5* isolate and other MDR populations should investigate the frequency of the F200Y isotype I β-tubulin mutation over several rounds of BZ selection, to determine whether this allele goes to fixation. In a phenotypic sense, there is no evidence of reversion to susceptibility to BZ. For example, the *MTci3* population was isolated from Firth Mains farm in 1983, when BZ resistance was first diagnosed (Scott *et al.,* 1990). Consequently, BZ was withdrawn from use and in the intervening 24 years, there has been no evidence of reversion to susceptibility despite the continual passage of this isolate without selection (F. Jackson, personal communication). However, given the evidence from this study that the F200Y isotype I β-tubulin mutation may not be the only contributing BZ resistance mechanism, it would be interesting to correlate changes in resistance phenotype with the F200Y genotype. Furthermore, assessing the fitness of these parasite generations throughout the selection process would be interesting. For instance, Leignel & Cabaret (2001) observed that BZ resistant *T. circumcincta* were larger than susceptible worms (which the authors suggest may have implications for higher fecundity in resistant worms). However, studies by Hoekstra *et al.* (1997b) suggested that high LEV resistance had a severe fitness cost. Tests of physical size, fecundity, and viability of offspring, cross-resistance and tolerance of other natural xenobiotics, e.g. condensed tannins would be worthwhile in examination of the effects of continued selection.

Further research is also required on the isotype II β-tubulin locus. Having characterised a portion of this gene, subsequent steps should be taken to sequence the full length gene. This information could then be used to compare the sequences of BZ-selected worms versus BZ-susceptible worms. The latter comparison could be done relatively quickly and inexpensively by adopting the SSCP approach and then sequencing all novel haplotypes, as described in Chapter 5. It is possible that the frequency of the F200Y isotype II β-tubulin mutation, or indeed other polymorphisms are also correlated with BZ resistance (as described by Beech *et al.,* 1994). Unfortunately, time constraints prevented genotyping of the F200Y isotype II β-tubulin locus of those individuals which survived *in vitro* and *in vivo* BZ selection, yet displayed a homozygous susceptible ($P200^{Phe/Phe}$) genotype in the isotype I β-tubulin gene. It would also be interesting to find out whether the isotype II β-tubulin gene is subject to stage-specific expression as suggested by Clark *et al.* (2005) from studies in cyathostomes. An investigation of the potential role of other β-tubulin genes would also be worthwhile, considering that two additional loci, designated isotype 3 and isotype 4 have been identified in the *H. contortus* genome (G. Saunders, M. Berriman, C. Britton & J.S. Gilleard, personal communication).

Furthermore, whilst there has been no indication from the literature that quantitative changes in the β-tubulin genes of nematodes have an impact upon the expression of BZ resistance, this still has to be viewed as a potential mechanism. This is more likely in the case of drug handling systems but, over-expression of β-tubulin could lead to sequestration of BZ, for example. This area of research has been constrained by technology, however, in recent years, advances in genomics and proteomics will make valid quantitative comparisons between candidate genes and gene products between susceptible and resistant populations a viable prospect.

6.3 Origins and diversity of isotype I β-tubulin alleles

Chapter 5 addressed the diversity of the isotype I β-tubulin locus in the *MTci5* isolate and to a lesser degree, in some other UK *T. circumcincta* populations. The findings showed a very high level of genetic diversity at this locus. There were at least seven different haplotypes

carrying the P200^{Tyr} mutation in the *MTci5* isolate and the presence of multiple resistance alleles was also detected in two further Scottish isolates (*MTci3* & *MTci4*). This has not been described previously for other BZ resistant trichostrongylid populations. In the only previously published study involving sequencing of a large number of isotype I β-tubulin alleles, a maximum of two P200^{Tyr}-containing haplotypes were detected (Silvestre & Humbert, 2002). The only major difference between the Scottish and French parasite populations examined was that the French farms were closed to animal movement, whereas the Scottish farms were not. Hence, it seems likely that the different resistant haplotypes in the *MTci5* population have arisen in multiple locations and have been brought together by animal movement, thus, parasite gene flow. Hence, these findings are consistent with the theory of multiple, independent and spontaneous mutations at the P200 locus of the isotype I β-tubulin gene. It is possible that the recent development of triple resistance in UK sheep farms may be a direct result of extensive animal movement combined with poor knowledge transfer regarding adequate quarantine measures for introduced stock.

The characterisation of two distinct phylogenetic clades, of the isotype I β-tubulin gene has been described previously (Leignel *et al.*, 2002; Silvestre & Humbert, 2002). These have been termed *Type I* and *Type II* and both of these allelic 'types' can carry the P200^{Tyr} mutation. These 'types' were also observed in the *MTci5* isolate, with the A/B/C alleles sharing high homology with *Type II* and the D/E alleles sharing high homology with *Type I* alleles. There could be several explanations for this phenomenon. One possible explanation could be the presence of two genetically distinct sub-populations within the *MTci5* isolate, i.e. the presence of a cryptic species. However, this does not appear to be the case, for a number of reasons. For instance, the microsatellite analysis revealed no genetic sub-structuring of *MTci5*. More importantly, there were many *Type I /Type II (DE /ABC)* heterozygotes in the *MTci5* isolate and in the other UK isolates examined, as revealed by SSCP genotyping. Furthermore, the A, B, C, D & E alleles were in Hardy-Weinberg Equilibrium in the *MTci5* population. This proves that there are not separate populations of worms (each containing one of the two isotype I β-tubulin 'types') but instead, these are different alleles within one single interbreeding population. The most plausible explanation for the presence of these two divergent clades in *T. circumcincta* populations is that they reflect a historical admixture event. For example, two geographically disparate and consequently genetically divergent populations of *T. circumcincta* were brought together a long time ago. The highly conserved

nature of the isotype I β-tubulin gene (and perhaps some other genes) has maintained the integrity of these allele *'types'*. This admixture event would have to pre-date the separation of the UK and French parasite populations, since the same two allelic *'types'* are present in both populations. It would be interesting to analyse other geographical populations of *T. circumcincta,* for example, from North America and Australia, to assess whether these populations also display this phenomenon.

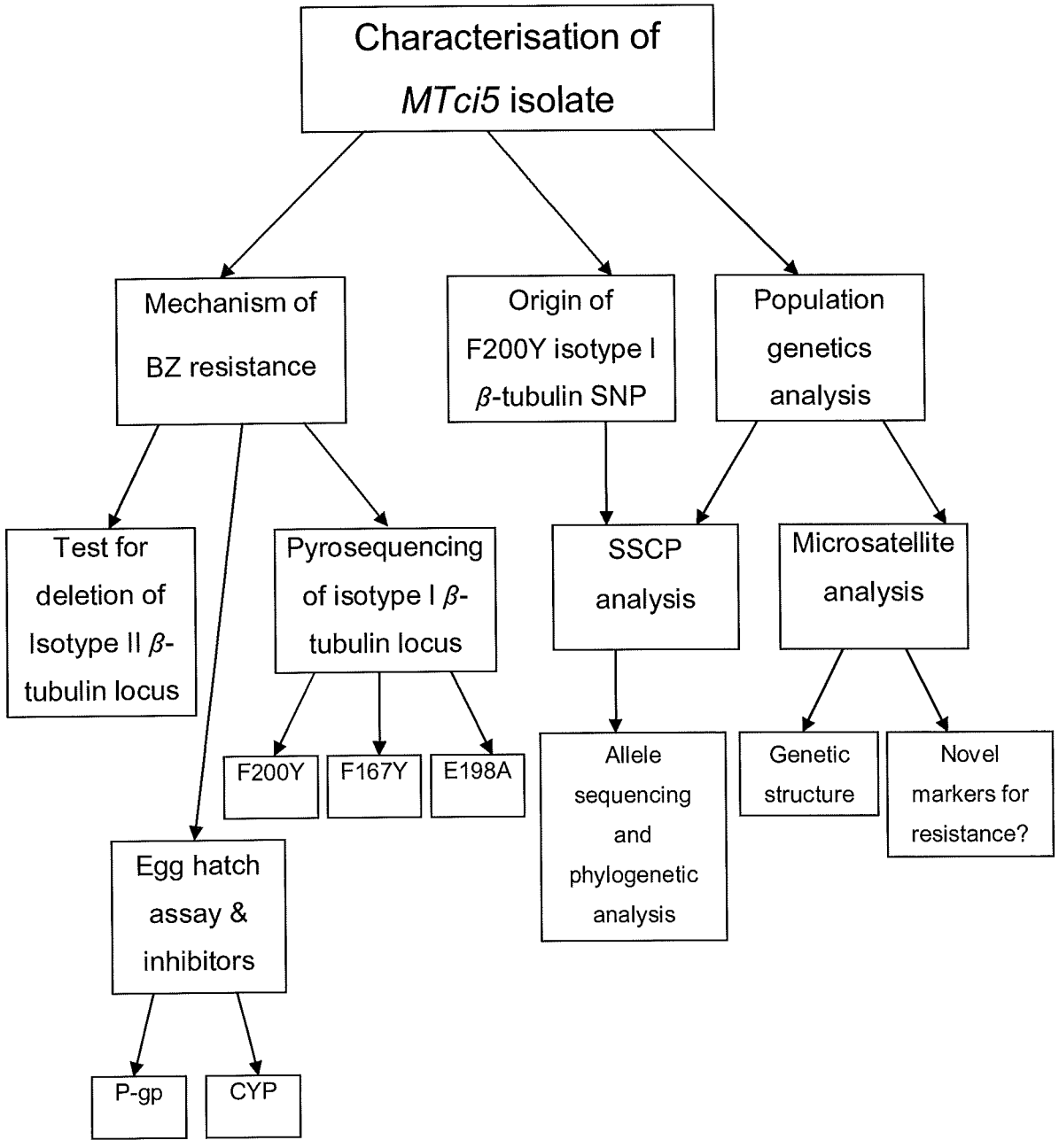
6.4 Linkage between resistance mechanisms

This study also aimed to investigate the possibility of linkage between resistance mechanisms. Previous studies have implicated the isotype I β-tubulin locus in IVM resistance of *Onchocerca volvulus* and *Haemonchus contortus* (Eng & Prichard, 2005; Eng *et al.,* 2006). This is an important issue, particularly since Eng *et al.* (2006) proposed that IVM selection favours the propagation of $P200^{Phe/Tyr}$ genotypes. If this is a universal phenomenon, and there is an IVM-resistance advantage of having this genotype, then this may explain why IVM resistance appears to have developed comparatively quickly in recent years. Again, there are important implications for the use of combination drenches. Currently, BZ and IVM combinations are not recommended for use against trichostrongylid nematodes due to widespread resistance to both of these anthelmintics, however, lessons should be learned for future anthelmintic control of nematodes of importance to cattle and humans, for instance. There was no evidence from this study to support an effect of LEV selection upon the isotype I β-tubulin locus. However, there was no consistent evidence of an effect of IVM or LEV selection upon the genotype frequency of the F200Y isotype I β-tubulin mutation in the *MTci5* isolate or of any particular SSCP haplotype at that locus.

6.5 Summary

In summary, this work has demonstrated the extraordinary genetic diversity of these parasitic nematodes and has shown that whilst the F200Y isotype I β-tubulin mutation is an important determinant of BZ resistance, the mechanisms underlying BZ resistance are much more complicated than first thought. These results also demonstrate that triple resistance occurs at the individual worm level, and therefore, future management of the MDR situation should take account of this. Further research is required to continue the quest for molecular markers for BZ, IVM and LEV resistance. Research into alternative methods of control should also continue. It is generally agreed that the best way to preserve the efficacy of the anthelmintics available, is to reduce our dependency upon them. There is much evidence, from the field, from laboratory experiments and from statistical modelling to suggest that the frequency and duration of treatment and the intensity of application are the most important factors in the selection for resistance. Hence, prophylactic use of anthelmintics should be replaced with targeted and selective treatment of individuals which are most susceptible to nematodoses or those that, due to over-dispersion, play a major role in the transmission of infection. However, practical affordable means of identifying the most susceptible stock in order to target them for anthelmintic treatments presents a considerable challenge. Whilst useful markers may well emerge from current research in this area, the growing problem of MDR needs urgent action in order to halt the relentless progression of anthelmintic resistance. Furthermore, research into immunologically-based alternative control strategies such as vaccines, genetic selection, optimized nutrition and 'neutraceuticals' may provide means of reducing our reliance upon chemoprophylaxis. However, none of these offers an immediate solution, nor are they likely to be effective in isolation. Effective biosecurity, to control the movement of resistant nematode populations, and a better use of grazing management, to minimise host-parasite contact, are currently the only options for many European farmers to reduce their anthelmintic dependency.

Figure 6.1 Flow diagram detailing the characterisation studies of the *MTci5* isolate.



Appendix

SOC media (Stored at room temperature)

1ml of filter sterilised 20% glucose solution was added to 99ml of following medium and autoclaved.

20g Tryptone (Sigma)

5g Yeast Extract (Sigma)

0.5g Sodium Chloride (Sigma)

10ml 1M Magnesium chloride (Sigma)

10ml 1M Magnesium sulphate (Sigma)

Made up to 1 litre with distilled water (Fisher Scientific)

20% Polyacrylamide gels for SSCP (prepared when required)

80ml acrylamide: bisacrylamide (37.5:1 mix) (Severn Biotech)

40ml 5x Tris Boreate EDTA (TBE buffer, see below)

76ml distilled water (Fisher Scientific)

1324µl 10% Ammonium Persulphate (APS) solution (Sigma)

175µl N,N,N',N'-Tetramethylethylenediamine (TEMED, Sigma)

SSCP loading buffer recipe (Stored at 4°C)

95% Formamide (Sigma)

10mM Sodium Hydroxide (Sigma)

0.25% Bromophenol blue (Sigma)

0.25% Xylene cyanol (Sigma)

Helminthological iodine (Stored at room temperature)

250g potassium iodide

50g iodine

Made up to 500ml with distilled water (Fisher Scientific)

10 x TAE buffer (Stored at room temperature)

0.4M TRIS (Sigma)

0.2M glacial acetic acid (Sigma)

0.01M EDTA (pH 8) (Sigma)

10 x TBE buffer (Stored at room temperature)

0.89M TRIS (Sigma)

0.02M EDTA (Sigma)

0.89M boric acid (Sigma)

TE buffer (Stored at room temperature)

10mM TRIS (pH7) (Sigma)

1mM EDTA (Sigma)

11.1 x buffer (Stored at -20 °C)

2M Tris.HCl pH8.8

1M NH_2SO_4

1M $MgCl_2$

0.53% 2-mercaptoethanol

10mM EDTA pH8

100mM each dNTP and 10mg/ml BSA

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