P-glycoprotein-9 and anthelmintic resistance status in selected UK strains of the ovine gastrointestinal nematode *Teladorsagia circumcincta*

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BSc (Hons), MSc (Distinction)

Submitted in fulfilment of the requirements for the

Degree of Doctor of Philosophy

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Abstract

Throughout the world, control of parasitic nematodes in livestock has been compromised by the emergence and spread of anthelmintic resistance. *Teladorsagia circumcincta* is the most important gastrointestinal nematode parasite of small ruminants in temperate regions and the major resistant species in the United Kingdom (UK). In most cases the genetic factors which underpin resistance to broad-spectrum anthelmintics are still poorly understood.

Recent work conducted independently in New Zealand (NZ) and Scotland has implicated the involvement of a particular P-glycoprotein (Pgp) gene, *Tci-pgp-9*, in multiple-anthelmintic resistance in *T. circumcincta*. The focus of this study is to further characterise *Tci-pgp-9* and its possible role in ivermectin (and multi-drug) resistance using two UK field isolates of *T. circumcincta*, one which is anthelmintic susceptible (MTci2) and another that is multiple-anthelmintic resistant (MTci5).

The generation of full-length cDNA sequence data from these isolates allowed genetic comparisons which identified the presence of nine non-synonymous SNPs in the *Tci-pgp-9* coding sequence of the MTci5 isolate. The 3.8 kb, *Tci-pgp-9* transcript from the MTci2 and MTci5 isolates shared 95.5 % identity at the nucleotide level and 99.5 % identity at the protein level. Twelve sequence variants were identified in the first internucleotide binding domain, designated *Tci-pgp-9-IBDA*, some of which shared a high level of identity with sequence variants identified in near-isogenic NZ strains. Multiple allelic variants were present in the majority of individuals, but a reduction in the number of allelic variants present in individuals of MTci5 relative to the MTci2 isolate was evident. A further reduction in the number of alleles present in individuals was also observed in individuals derived from an IVM treated population of MTci5, suggesting that IVM treatment applied purifying selection pressure. Quantitative real time PCR analysis showed a 3.7-fold increase in *Tci-pgp-9* gene copy number in the MTci5 isolate relative to the MTci2 isolate, which was consistent with a 3.4-fold increase observed in the NZ study. None of the common haplotypes identified were unique to any given isolate, and the relationship between haplotype and copy number was not straightforward.

This study provides evidence that *Tci-pgp-9* is under anthelmintic selection, but the precise role of this specific P-glycoprotein gene, and its alleles, in the phenotypic expression of anthelmintic resistance in *T. circumcincta* remains to be determined.

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Acknowledgements

I am very grateful to the many people who have encouraged and supported my project. In particular, I would like to thank Dr Stewart Bisset of AgResearch Hopkirk Institute New Zealand, whose research inspired this PhD project and for his role in supervising this work. His hospitality and advice during my time in New Zealand was very much appreciated. I wish to thank Prof Nick Jonsson for his advice and assistance with statistical analyses and his general support during the writing process which really helped push me over the 'finish-line'. Thank you also to my supervisors Drs Philip Skuce and Fiona Kenyon, of Moredun Research Institute, for all your help and guidance provided throughout the project. I am very grateful for the funding provided by a new collaboration between the Moredun Research Institute and AgResearch Hopkirk Research Institute which gave me the opportunity to carry out this project in both Scotland and New Zealand. Financial assistance, in the form of travel grants, was provided by the British Society for Parasitology and Louisiana State University Travel Award Fund, and ensured that I could present my findings at conferences.

Within Moredun, thanks are due to Dr Dave Bartley, Alison Morrison, Dr Heather McDougall and Dr Kim Willoughby for helping with numerous experiments, teaching me new techniques and being a sounding board for my ideas. Within AgResearch, thanks are due to Charlotte Bouchet, Dr Jacqui Knight and Dr Ian Sutherland, for continuous encouragement and hospitality throughout my time in New Zealand. I gratefully acknowledge the input of everyone.

Finally, I would like to thank my family for encouraging my interest in science and their never-ending support over my many years as a student. Special thanks go to Dr Barbie Govan who has put up with me over the last two years! Her belief in me and general positivity has helped me immensely.

"Research is what I'm doing when I don't know what I'm doing"

Wernher von Braun

"It always seems impossible until it's done"

Nelson Mandela

Author's Declaration

The work presented in this thesis is my own work, unless otherwise acknowledged, and has not been submitted for any other degree.

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January 2014

Abbreviation List

Abbreviation	
AADs	Amino-Acetonitrile Derivatives
ABC	ATP-Binding Cassette
AMOVA	Molecular Analysis of Variance
ATG	Initiation Codon
ATP	Adenosine Tri-Phosphate
bp	Base Pairs
BZ	Benzimidazole
cDNA	Complementary DNA
CET	Controlled Efficacy Test
Cq	Quantification Cycle
C_T	Threshold Cycle Value
DEFRA	Department for Environment, Food and Rural Affairs
d.f.	Degrees of Freedom
DMSO	DiMethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Trisphosphate
dsDNA	Double Stranded Deoxyribonucleic Acid
DTT	Dithiothreitol
ED_{50}	Effective Dose (50%)
EDTA	Ethylenediaminetetraacetic acid
EHA	Egg Hatch Assay
FECRT	Faecal Egg Count Reduction Test
FITC	Fluorescein Isothiocyanate
GABA	Gamma-Aminobutyric Acid
gDNA	Genomic DNA
Hco-MTPL-1	*Haemonchus contortus* Monepantel-1
H-gal-GP	*Haemonchus* Galactose-containing Glycoprotein
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
IVM	Ivermectin
L_1	First Stage of the Larval Lifecycle
L_2	Pre-infective Second Stage of the Larval Lifestyle
L_3	Infective Third Stage of the Larval Lifecycle
L_4	Mature Fourth Stage of the Larval Lifecycle
L_5	Sexually Mature Adult Stage of the Larval Lifecycle
LB	Lysogeny Broth
LD_{50}	Lethal Dose (50%)
LDA	Larval Development Assay
LEV	Levamisole
LFIA/LFI	Larval Feeding Inhibition Assay
LMIA/LMI	Larval Migration Inhibition Assay
LN_2	Liquid Nitrogen
$MgCL_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
MIQE	Minimum Information for Publication of Quantitative Real Time PCR Experiments
ML	Macrocyclic Lactones
MOX	Moxidectin
mRNA	Messenger RNA
MTci5	Moredun *Teladorsagia circumcincta* Isolate 5
MTci5PT	MTci5 Post-Treatment
Na_2CO_3	Sodium Carbonate
NaCl	Sodium Chloride
NBDs	Nucleotide Binding Domains

NZ	New Zealand
NZv	New Zealand Allelic Variant
OXF	Oxfendazole
PCR	Polymerase Chain Reaction
PGE	Parasitic Gastroenteritis
Pgp	P-Glycoprotein
PPi	Inorganic Pyrophosphate
RACE	Rapid Amplification of cDNA Ends strategy
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
SL	Spliced Leader Sequence
SNPs	Single Nucleotide Polymorphisms
SOC	Super Optimal Broth with Catabolite Repression
spp	Species
TAE	Tris-Acetate-EDTA
Tci-pgp-9	*Teladorsagia circumcincta* P-glycoprotein-9
TGA	Termination Codon
TST	Targeted SelectiveTreatments
UK	United Kingdom
UKv	United Kingdom Allelic Variant
unc	Unco-ordinated gene
UTR	Untranslated Region
WAAVP	World Association for the Advancement of Veterinary Parasitology
X-gal	5-bromo-4,4-chloro-3-indolyl-ß-$_D$-galactopyranoside

International Universal Base Coding

Code	Translation
A	Adenosine
C	Cytidine
G	Guanine
T	Thymidine
B	C, G or T
D	A, G, or T
H	A, C, or T
R	A or G (puRine)
Y	C or T (pYrimidine)
K	G or T (Keto)
M	A or T (aMino)
S	G or C (Strong -3H bonds)
W	A or T (Weak -2H bonds)
N	Any Base
V	A, C, or G

Chapter 1

General Introduction and Background

1.1 Impact of Nematode Parasites on UK Livestock

Agriculture contributes approximately £4.7 billion per annum to the economy of the United Kingdom (UK), the largest producer of sheep and goat meat in Europe, with an estimated worth of £1.02 billion (DEFRA, 2012). The economic cost of gastrointestinal parasites in the UK, through animal production losses, costs associated with preventative measures and the treatment of affected animals, was estimated in 2005 to be in the order of £84 million per annum (range £48-120 million) (Nieuwhof & Bishop, 2005), and is likely to be much higher now. In cool temperate areas, grazing ruminants are infected by a variety of parasitic nematodes. The common gastrointestinal parasites that infect UK sheep flocks are *Teladorsagia circumcincta, Haemonchus contortus*, *Trichostrongylus spp.*, and *Nematodirus spp*. The dominant species in UK flocks is usually considered to be *T. circumcincta* (Stear *et al.*, 2009), although all species can cause ill-thrift and production losses when present in large numbers. The disease caused by such infections in sheep is called parasitic gastroenteritis (PGE) (Armour, 1986; Taylor *et al.*, 2007; McNeilly *et al.*, 2009). Nematode control currently relies heavily upon the intensive use of broad-spectrum anthelmintics, which has, in turn, led to the emergence of anthelmintic resistance.

1.2 Teladorsagia circumcincta

Teladorsagia circumcincta, commonly known as the “brown stomach worm”, belongs to the Order Strongylida, and is a member of the Rhabditina (Clade V) family in the Phylum Nematoda. This parasitic nematode is most commonly found in cool temperate regions of the world, where its definitive host is sheep. This section will describe the life-cycle of this important parasite, the disease caused and the preventative measures used to manage infections.

1.2.1 Teladorsagia circumcincta Life-cycle

The life-cycle of *T. circumcincta*, shown in Figure 1.1, begins when eggs, produced by adult females present on the mucosal surface of the abomasum, are excreted onto pasture in the faeces. Here, first stage larvae (L_1) emerge from the eggs and ingest faecal bacteria. The larvae undergo two further moults as they pass through the pre-infective second stage (L_2) to the infective third (L_3) stage of their life-cycle. The cuticle from the L_2 stage is retained as a larval sheath and offers L_3 protection from desiccation whilst on the pasture (O'Connor *et al.*, 2006). The progression to third stage larvae occurs within 2 weeks under optimal conditions of 18-26 °C and 60 % humidity (Gruner & Suryahadi, 1993; Urquhart *et al.*, 1996; O'Connor *et al.*, 2006). The infective L_3 then migrate out of the faeces onto the pasture where they are ingested by grazing sheep. The larvae shed their cuticle when they encounter high CO_2 concentrations in the rumen, then enter the highly acidic environment of the abomasum, after which, the larvae migrate to the gastric glands within the first 24 hours and mature through fourth (L_4) and fifth life-cycle stages, finally becoming sexually mature adults (L_5), around 10 days post-ingestion. The larvae may enter a hypobiotic state where they arrest their development *in vivo* until environmental conditions become favourable.

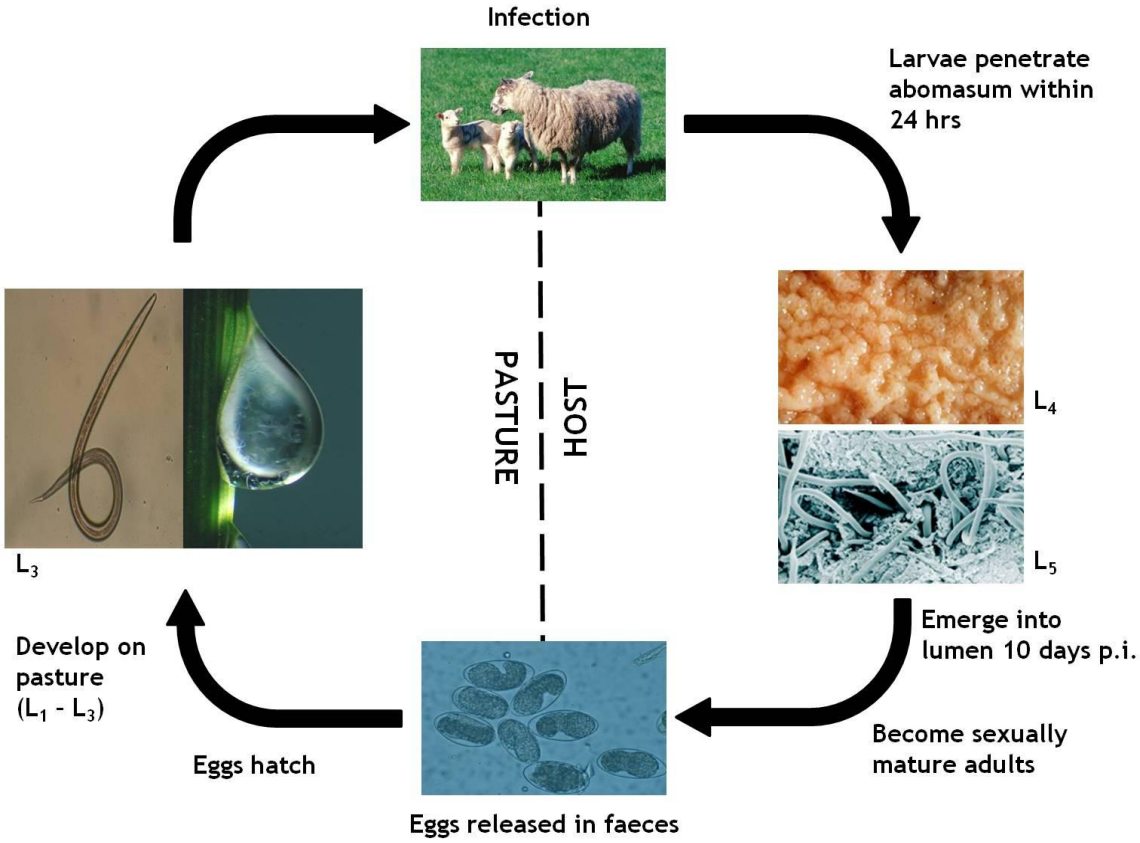


Figure 1.1 Life-cycle of *T. circumcincta*

Schematic showing the life-cycle of *T. circumcincta*. The first three larval stages are found on pasture with the remaining development occurring in the host. Images courtesy of Dave McBean and Fiona Kenyon (Moredun Research Institute).

1.2.2 Clinical Disease Caused by T. circumcincta

The damage in the abomasum is primarily caused when larvae arise from the gastric glands, resulting in increased pH levels followed by a strong inflammatory response in the host. This leads to a gastropathy that leads to protein loss, which is exacerbated by the adult worms which continue to reside at the mucosal surface of the abomasum (Simpson, 2000), resulting in reduced nutrient acquisition (McKellar, 1993). The effects of PGE ranges from reduction in appetite and liveweight gain, to diarrhoea, dehydration and, in severe cases, death (McNeilly *et al.*, 2009). Two types of PGE caused by *T. circumcincta* infection have been described; type I - an acute disease (Sutherland & Scott, 2010), and type II which is generally a sub-clinical disease (Urquhart *et al.*, 1996; Abbot *et al.*, 2004), which has been shown to suppress the appetite of the host (Greer *et al.*, 2008). Type I disease is caused by the ingestion and

maturation of large numbers of L_3 from pasture by lambs in their first grazing season, whereas, the less common, type II disease is caused by the delayed maturation of larvae that were ingested in the previous grazing season. The type II disease usually occurs the following late winter/early spring after the larvae have either, emerged from a period of arrested development in the host (Gibbs, 1986; Urquhart *et al.*, 1996), or alternatively, over-wintered in the host as arrested L_4 (Sargison *et al.*, 2007). Outbreaks of the type II disease have become more common and severe in South East Scotland in recent years (Sargison *et al.*, 2007; Kenyon *et al.*, 2009a).

1.3 Nematode Control Strategies

1.3.1 Broad-Spectrum Anthelmintics and Mode of Action

The control of parasitic nematodes currently relies heavily upon the strategic use of anthelmintic drugs combined with pasture management. Traditionally there were three main classes of broad spectrum anthelmintic available: benzimidazole (BZ), imidazothiazoles (e.g. levamisole (LEV) and related compounds), and macrocyclic lactones (MLs) (e.g. ivermectin (IVM) and moxidection (MOX). This was extended in recent years to five by the addition of two novel classes of anthelmintics: the amino-acetonitrile derivatives (AADs) and spiroindoles used as part of a dual-active, derquantel-abamectin.

The first broad spectrum anthelmintic was released in the 1960s. Thiabendazole, a member of the BZ class, was introduced in 1961 (Brown *et al.*, 1961) and, after just three years of intensive use, the first cases of resistance in sheep nematodes were reported (Drudge *et al.*, 1964). The BZs act by binding to ß-tubulin, preventing the formation of microtubules resulting in cell lysis, due to the inability to transport secretory granules or secrete enzymes (Lacey, 1988; Prichard, 1990), which subsequently inhibits larval motility and feeding and interferes with other vital cellular processes (McKellar & Jackson, 2004; Mitreva et al., 2007; von Samson-Himmelstjerna et al., 2007). The imidazothiazole class was introduced in 1970 with LEV proving to be the most commonly used member in this class. Levamisole acts on neuromuscular junctions as an agonist for nicotinic receptors (McKellar and Jackson, 2004), causing muscle paralysis which

results in the eventual death of the parasite. Resistance to levamisole was first observed in laboratory selected worms 6 years later (Le Jambre, 1976) and was reported in the field 9 years after its introduction (Sangster *et al.*, 1979; Le Jambre, 1979; Prichard *et al.*, 1980).

The MLs, originally isolated from *Streptomyces avermilitis* (Putter *et al.*, 1981; Campbell *et al.*, 1983) were introduced next. The release of IVM revolutionised the livestock anthelmintic market and boasted almost 100 % anti-parasitic efficacy against endo- and ecto-parasites (Geary, 2005). The MLs, such as IVM and moxidectin (MOX), bind to unique invertebrate-specific glutamate-gated chloride channels, causing irreversible activation which leads to muscle paralysis and the inhibition of motility, reduced pharyngeal pumping and inhibition of oviposition due to effect on uterus muscles (Geary *et al.*, 1993). Ivermectin is also believed to be an agonist of γ-aminobutyric acid (GABA) and disrupts GABA-mediated neurotransmission (Ros-Moreno *et al.*, 1999).

Recently, a fourth class of anthelmintic, the amino-acetonitrile derivatives (AADs), was introduced. Monepantel was the first member of this class to be released, initially marketed as Zolvix® (Novartis Animal Health) in Australia and New Zealand in 2009, and later released in Europe in 2010. Its use as a single quarantine drench or in conjunction with other anthelmintic classes has been advised to sustain its efficacy levels (Jackson *et al.*, 2009). Monepantel causes paralysis of the larva by targeting Hco-MTPL-1 receptors that are a unique, nematode-specific clade of acetylcholine receptor subunits (Kaminsky *et al.*, 2008).

The fifth class of anthelmintic to be released was the spiroindoles. Derquantel, a member of this class, is only used in combination with abamectin as part of a dual-active drench, largely due to the fact that derquantel itself has very poor efficacy against *T. circumcincta*. Abamectin, like IVM, interacts with the glutamate-gated receptors in the pharynx, inhibiting nerve communication by increasing membrane permeability to chloride ions. The resulting irreversible neuromuscular blockage and leads to the paralysis, starvation and eventual death of the worm. Spiroindoles are nicotinic cholinergic antagonists which block cation channels in nematode muscle cell membranes, resulting in flaccid paralysis of the nematode (Little *et al.*, 2011). By combining two active

compounds, the aim is to slow the development of drug resistance, as resistance to one active ingredient is covered by the other (Leathwick & Hosking, 2009).

1.3.2 Alternative Nematode Control Strategies

Intensive chemoprophylaxis is not a sustainable method of controlling parasitic nematodes as the prevalence of drug resistance increases (Jackson & Miller, 2006). Current worming strategies on farms involve rotation between the classes of anthelmintic, thereby reducing the generation of worm resistance to a single class of anthelmintic. Alternative strategies for controlling parasitic nematodes whilst limiting the development of resistance to anthelmintics have been considered.

1.3.2.1 Grazing Management

Grazing management has been proposed as a method to assist with nematode control. An evasive strategy, first proposed by Michel (1985), involves moving livestock to another pasture before the larvae resulting from their contamination are likely to appear. The livestock are not returned to the contaminated pasture until the number of infective larvae has declined. Co-grazing sheep and cattle has also been suggested as a method for sustaining parasite control as they tend to harbour different nematode species (Abbot *et al.*, 2004).

1.3.2.2 Selective Breeding

Over-dispersion typically occurs within the hosts of gastrointestinal nematodes, where the majority parasites are harboured by a relatively small number of host animals and the majority of animals harbour relatively few parasites (Stear *et al.*, 2006). Selective breeding for resilience or resistance to parasite infection has been proposed as a medium-long-term solution to the problem of anthelmintic resistance in sheep parasites. Animals that harbour fewer parasites, relative to susceptible animals, and that mount effective immune responses to those parasites are considered resistant to parasitic infection, whilst animals that still perform well and are able to maintain live-weight gain under parasite challenge are considered as being resilient to the parasite infections (Bisset *et al.*, 2001).

1.3.2.3 Refugia-based Approaches

Adopting *refugia*-based approaches along with selective breeding for desirable nematode resistant and resilient traits has been proposed to prolong the efficacy of the current anthelmintics (Kenyon *et al.*, 2009b). This approach maintains a population of worms that are not exposed to anthelmintic, thereby preserving the genes for susceptibility within the untreated parasite population. The anthelmintic resistant genotypes are diluted within the untreated parasite population. One practical approach to slow the development of resistance is the targeted selective treatments (TST), first described by Bisset & Morris (1996). In this study, it was used as a strategy to identify resilient animals, but the authors also recognised that it could be useful for reducing selection pressure for anthelmintic resistance by only targeting anthelmintics to those animals that are likely to benefit from treatment rather than treating the whole flock. The majority of hosts have a low worm burden, with approximately 80 % of the worms found in only 20-30 % of hosts (Sréter *et al.*, 1994). Targeting treatment to those animals that are disease susceptible (non-resilient and/or non-resistant) or those that produce most pasture contamination, ensures a sufficient number of animals are left untreated, thus maintaining the worms *in refugia* (Greer *et al.*, 2009; Kenyon *et al.*, 2009b). Replicated field trials showed that lambs in a

TST group received approximately 50 % fewer anthelmintic treatments throughout the trial, and grew as well as lambs that had suppressive monthly anthelmintic treatment (Kenyon *et al.*, 2009b; Kenyon *et al.*, 2013).

1.3.2.4 Vaccines

The development of vaccines against sheep parasites has been challenging, but has seen success with the development of a vaccine against *H. contortus* using a "hidden" antigen approach (Smith & Zarlenga, 2006). Substantial protection against natural haemonchosis in grazing sheep was observed following immunisation with a combination of the H-gal-GP and H11 glycoprotein complexes (Smith *et al.*, 2001a). Antibodies specific for *H. contortus* gut membrane proteins, specifically, H-gal-GP and H11, are ingested during the parasite's blood-meal, disrupting the digestion in the parasite, leading to starvation, loss of fecundity and weakness (Smith & Zarlenga, 2006). Unfortunately, this vaccine did not provide any cross-protection against *T. circumcincta* (Smith *et al.*, 2001b; Knox & Smith, 2001), most likely due to insufficient titres of host antibody being ingested for the approach to be highly effective.

Attempts were made to immunise sheep against *T. circumcincta* using ConcanavilinA-binding extracts prepared from L_4 larvae (Halliday & Smith, 2011). Despite a similar extract prepared from *Ostertagia ostertagi* significantly protecting calves (Halliday & Smith, 2010), the *T. circumcincta* extracts did not contain significantly protective antigens (Halliday & Smith, 2011). Recently, Nisbet *et al.* (2013) successfully immunised sheep using a nematode sub-unit vaccine, containing a cocktail of eight recombinant proteins, against *T. circumcincta*. It is promising that induced levels of protection were higher than those observed in any other systems using a recombinant vaccine against a parasitic nematode in the definitive ruminant host (Nisbet *et al.*, 2013).

Although the vaccines described above have been shown to work in trials, the only commercially available nematode parasite vaccine is a live attenuated Bovilis® Huskvac preparation used to vaccinate calves against the lungworm, *Dictyocaulus viviparus*. Vaccines offer certain advantages over anthelmintics,

due to their lack of chemical residues they require shorter (or zero) withdrawal periods for food products, and it could be argued that vaccines are more environmentally-friendly as faecal residues are reduced or eliminated (Wall & Strong, 1987; Vercruysse *et al.*, 2004). The disadvantages include the requirement for regular vaccination, especially with hidden antigen vaccines, and the specificity for a single species when natural infections typically comprise multiple species.

1.4 Anthelmintics

1.4.1 Anthelmintic Resistance

The widespread prophylactic use of anthelmintics has led to the global emergence of resistance in ruminant nematodes. Anthelmintic resistance in *T. circumcincta* has been documented in most countries where the parasite is endemic (Kaplan, 2004). Anthelmintic resistance has been reported in four of the five major classes of anthelmintics, with no reported cases of resistance in the recently released spiroindoles class, as yet. The emergence of anthelmintic resistance developed soon after their commercialisation (Table 1.1).

As well as resistance to single classes of anthelmintic, resistance to multiple classes of anthelmintic have developed. The reported first case of BZ and IVM double resistance in *T. circumcincta* isolated from UK goats was in 1992 (Jackson *et al.*, 1992). Triple resistant isolates from sheep were also reported in Europe in 2001 (Sargison *et al.*, 2001). Resistance has also been reported to combination drenches (Pomroy, 2006; Leathwick et al, 2009). In the UK, drenches usually contain only a single class of anthelmintic, however in the Southern Hemisphere, many drenches contain several (up to 4) different actives in combination.

Table 2.1 Emergence of Anthelmintic Resistance

Examples of anthelmintics from each of the major classes are shown along with the year of their release and the year in which resistant nematodes were first reported in sheep. To date, no reports of anthelmintic resistance have been submitted for Derquantel. (Adapted from Kaplan (2004))

Drug	Year of Release	First Report of Resistance
Benzimidazole		
Thiabendazole	1961	Drudge *et al.*, 1964
Imidothiazoles		
Levamisole	1970	Sangster *et al.*, 1979; Le Jambre, 1979
Macrocyclic Lactones		
Ivermectin	1981	van Wyk & Malan, 1988
Moxidectin	1991	Leathwick, 1995
Amino-Acetonitrile Derivatives		
Monepantel	2009	Scott *et al.*, 2013
Spiroindoles		
Derquantel	2009	-

1.4.2 Mechanisms of Anthelmintic Resistance

Anthelmintic resistance is accepted as a pre-adaptive phenomenon (Jackson & Coop, 2000), where the gene or genes responsible for resistance may have already existed at low frequency within a species. A survival advantage is conferred to individuals carrying a resistance gene or genes when exposed to anthelmintics. The emergence of anthelmintic resistance occurs after continual selection with anthelmintic resulting in a high frequency of individuals carrying resistance allele(s) within the population. Anthelmintic resistance develops through a limited number of ways: (i) alterations of the drug receptor so that the target is no longer recognised by the anthelmintic, rendering it ineffective; (ii) inactivation or removal of the anthelmintic through increased drug metabolism; (iii) changes in drug distribution, such as the up regulation of cellular efflux mechanisms, prevent the anthelmintics reaching their target; (iv) amplification of the target gene(s) to overcome anthelmintic action or a reduction in drug receptor expression (Wolstenholme *et al.*, 2004; Prichard & Roulet, 2007). Mechanisms (ii) and (iii) effectively reduce the drug concentration at the receptor sites, whilst mechanisms (i) and (iv) affect the receptor response as a consequence of drug binding (Prichard & Roulet, 2007). Anthelmintic resistance is heritable, and due to the lack of appropriate selection pressure

that could be applied against the mutations responsible for the resistances, would seem to be irreversible (Barton, 1983; Jackson & Coop, 2000). No reversion was observed after a long-term absence of the selecting anthelmintic or class of anthelmintic (Dash, 1986; Martin *et al.*, 1988), for example, no reversion to sensitivity to BZs was observed in field populations of *H. contortus* and *T. colubriformis* after the removal of selection pressure (Roos *et al.*, 1995; Wolstenholme *et al.*, 2004).

Resistance to BZs has been attributed to the substitution of phenylalanine to tyrosine at either residue 167 or residue 200 of the ß-tubulin isotype 1 gene which disrupts BZ binding by removing high affinity receptor binding sites (Lacey & Gill, 1994). Substitutions at these residues do not occur simultaneously in a ß-tubulin allele, the occurrence of both substitutions simultaneously may be lethal in some way (Beech *et al.*, 2011). There appears to be no reduction in fitness associated with being genetically resistant, despite the conserved nature of ß-tubulin (Elard *et al.*, 1998). Without a fitness cost to being BZ-resistant, there is no selection pressure for the parasites to revert back to susceptibility (Leignel *et al.*, 2010). Multiple mechanisms may be responsible for the development of BZ resistance as evidence of selection at a Pgp locus during the selection for BZ resistance has been shown in *H. contortus* (Blackhall *et al.*, 2008).

The first report of IVM resistance was by van Wyk & Malan (1988) just eight years after its release. Similarly, MOX resistance was reported in sheep four years after its release on to the market (Leathwick, 1995; Watson *et al.*, 1996). Glutamate-gated and GABA-gated chlorine channels have been identified as the molecular targets for MLs. In *H. contortus*, it has been reported that there is no single allele associated with resistance, but changes in allele frequency have been observed (Blackhall *et al.*, 1998, Blackhall *et al.*, 2003). Njue *et al.*, (2004) reported that IVM resistance in a UK isolate of *Cooperia oncophora* was associated with an amino acid substitution, from leucine to phenylalanine, at codon 256 (L256F) of avr-14B which encodes a subunit of the glutamate-gate chlorine channel in this species. Further investigation into Belgian isolates of the cattle nematodes *O. ostertagi* and *C. oncophora*, did not possess the avr-14B L256F substitution (El-Abdellati *et al.*, 2011). The same subunit, avr-14B, was recently shown to have a relatively minor role in IVM resistance in *T. circumcincta* (Martínez-Valladares *et al.*, 2012). P-glycoproteins (Pgps) have also

been implicated in IVM-resistance. Studies that co-administered IVM or MOX with Pgp-inhibitors (such as verapamil) showed an increased efficacy of these drugs against MOX-resistant *H. contortus* (Xu *et al.*, 1998; Molento & Prichard, 1999) and will be discussed further below in Section 1.5.

Resistance to the AAD class of anthelmintic has been artificially selected in laboratory strains of *H. contortus* (Kaminsky *et al.*, 2008; Rufener *et al.*, 2009; Rufener *et al.*, 2010). Recently, resistance to monepantel was reported in a New Zealand goat herd where *T. circumcincta* and *T. colubriformis* populations were already multi-drug resistant (Scott *et al.*, 2013). In this case, resistance to monepantel had arisen on this property after switching from BZ, ML and LEV combination drenches, due to their lack of efficacy, to monepantel. The goats received 17 separate doses of monepantel within a two year period, after which there was no evidence of efficacy in the FECRT, indicating the development of resistance to the AAD class of anthelmintic. No cases of derquantel resistance have been reported, most likely because derquantel is combined with abamectin and resistance to one active ingredient is covered by the other (Leathwick & Hosking, 2009; Geurden, *et al.*, 2012).

1.4.3 Detection of Anthelmintic Resistance

Anthelmintic resistance is widespread and likely to be under-reported. The detection of anthelmintic resistance has relied upon laborious and cumbersome *in vivo* techniques such as the faecal egg count reduction test (FECRT) and the controlled efficacy test (CET); and *in vitro* methods including the egg hatch assay (EHA), the larval development assay (LDA) and to a lesser degree the larval migration inhibition assay (LMIA) and the larval feeding assay (LFIA). Of the tests mentioned above, the FECRT is the most widely used providing a reasonable indication of the reduction in faecal egg output as a result of anthelmintic treatment in most cases. It has been routinely used to monitor the emergence of anthelmintic resistance which is shown when the therapeutic effect of the drug is reduced by >5 % (Coles *et al.*, 1992). The lack of sensitivity means that the FECRT detects resistance once >25 % of the parasite population expresses resistance genes (Martin *et al.*, 1989; Sangster, 2001).

An understanding of the genetic basis of anthelmintic resistance will allow the development of molecular-based diagnostic tests with greater sensitivity than the FECRT. Qualitative changes (such as mutations, insertions and deletions), and quantitative changes (such as alteration of gene expression) have been targeted for the development of DNA based tests. The only example of a qualitative change being utilised in this way is the single nucleotide polymorphism that is responsible for the phenylalanine to tyrosine substitution at amino acid 200 of ß-tubulin isotype-1 which has been shown to reduce the affinity of BZs to ß-tubulin (Kwa *et al.*, 1994; Elard *et al.*, 1996). This mutation, along with SNPs that cause a similar substitution at residue 167 (F167Y), has permitted the development of a PCR diagnostic test to screen for allele(s) that are associated with BZ-resistance in the parasite population (Kwa *et al.*, 1994; Elard *et al.*, 1999). Considerably more work is required to identify and develop reliable genetic markers of anthelmintic resistance in the other broad-spectrum anthelmintic classes (von Samson-Himmelstjerna *et al.,* 2006; von Samson-Himmelstjerna *et al.,* 2007). While these tests are available, their use is not widespread, and are only routinely used in research laboratories.

1.5 P-glycoproteins

Anthelmintic resistance has been attributed to several genetic factors including qualitative and quantitative changes in glutamate-gated chloride channels, ß-tubulin and several Pgps (Xu *et al.*, 1998; Prichard & Roulet, 2007; James & Davey, 2009; Dupuy *et al.*, 2010). P-glycoproteins were first reported in mutant cell lines that displayed an altered permeation rate of drugs entering the cell via hydrophobic pathways (Juliano & Ling, 1976).

The Pgps were the first multi-drug resistance transporter molecules found to be involved in the efflux of ML and it was observed that Pgp-deficient mice accumulated MLs in the brain, resulting in neurotoxicity (Schinkel *et al.*, 1994; Lankas *et al.*, 1997). Recent studies have linked polymorphisms in Pgps with the multi-drug resistant phenotype in *T. circumcincta* (Bisset, 2007; Bartley *et al.*, 2009; Dicker *et al.*, 2011a; Dicker *et al.*, 2011b), *H. contortus* (Blackhall *et al.*, 1998; Williamson *et al.*, 2011), *Cooperia oncophora* (Demeler *et al.*, 2013), *Parascaris equorum* (Janssen *et al.*, 2013) and *Onchocerca volvulus* (Bourguinat

et al., 2008). Pgps are members of the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily and act as non-specific efflux pumps that export a range of xenobiotic agents. In the context of anthelmintic resistance, they could potentially function by pumping anthelmintics out of target cells, thereby reducing intracellular drug accumulation, leading to the parasite being exposed to sub-lethal doses (James *et al.*, 2009). The ABC superfamily is an evolutionarily ancient group of proteins that are conserved across the Animal Kingdom. Their importance in multi-drug resistance is illustrated by the fact that almost 5 % of the *Escherichia coli* genome is occupied by genes encoding 79 distinct ABC proteins (Zimniak *et al.*, 1999).

A typical Pgp molecule, as shown in Figure 1.2, comprises two nucleotide binding domains (NBDs) and two transmembrane domains, which form an aqueous chamber within the membrane (Schinkel *et al.*, 1995; Rosenberg *et al.*, 1997; Ambudkar *et al.*, 2003; Buss & Callaghan, 2008). Typical substrates for Pgp tend to be hydrophobic, with a molecular mass of 300-4000 daltons (Buss & Callaghan, 2008; Aller *et al.*, 2009). MLs are highly hydrophobic and are excellent substrates for Pgp transport in mammals (Prichard & Roulet, 2007) and, therefore, IVM resistance may develop as a result of increased drug efflux by Pgps. Zhao *et al.* (2004) showed that Pgps are expressed mainly in the pharynx, gut or excretory cells of *Caenorhabditis elegans* and suggested that Pgps may protect the individual from a range of xenobiotics. Increased expression of Pgps has also been associated with IVM resistance in *H. contortus* (Xu *et al.*, 1998), *C. oncophora* (Areskog *et al.*, 2013, De Graef *et al.*, 2013) and *C. elegans* (James & Davey, 2009) and was confirmed to be reversible by co-administering Pgp inhibitors such as the calcium channel blocker verapamil (Molento & Prichard, 1999). Lespine *et al.* (2008) proposed that the active life of the MLs could be enhanced by inhibiting the drug transporters and may be able to slow down the appearance of resistant parasites in hosts such as cattle in countries where resistance is not yet widespread. Recently it was demonstrated, for the first time under field conditions, that the co-administration of Pgp modulators and IVM improved the activity and extended the active life of IVM (Lifschitz *et al.*, 2010). The free-living non-parasitic nematode, *C. elegans*, and the parasitic nematode *H. contortus*, are closely related to *T. circumcincta*

(Geary & Thompson, 2001), therefore these studies support the theory that Pgps may be implicated in IVM resistance in *T. circumcincta*.

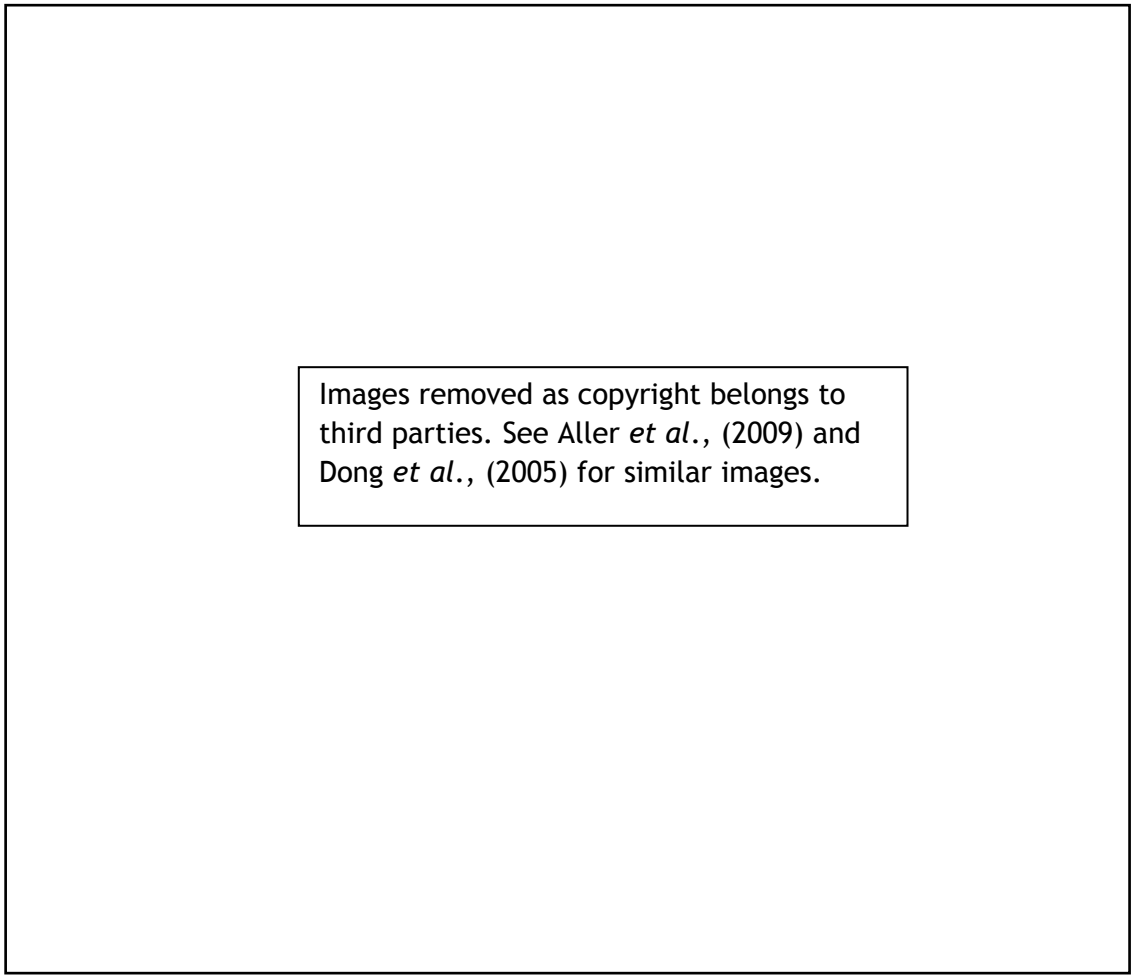


Figure 1.2 Model of substrate transport by Pgp

A, Schematic model of a Pgp protein with α-helices in the transmembrane domains and a mix of α-helices and ß-sheet in the nucleotide binding domains (NBD). *B*, Schematic model of the substrate expulsion; the substrate (magenta) partitions into the bilayer from the extracellular surface and enters the internal drug binding pocket through an open portal. ATP (yellow) binds to the NBDs and causes a conformational change, releasing the substrate into the extracellular space. *C*, The energy dependent cycle of substrate efflux is shown. The substrate (red) is actively pumped into the extracellular space as a result of ATP hydrolysis (Adapted from (Aller *et al*., 2009; Dong *et al*., 2005)).

1.5.1 Recent Studies on Tci-pgp-9

As sheep farming is a major part of the economies of Scotland and New Zealand (NZ), both countries possess research institutes which focus on livestock and animal health. Studies into Pgp expression and its association with anthelmintic resistance in *T. circumcincta* were being conducted independently, and collaboration between groups from these institutes was thus established. In Scotland, at the Moredun Research Institute, field isolates of anthelmintic susceptible and multiple anthelmintic resistant *T. circumcincta* were maintained and characterised (Bartley *et al.*, 2009; Dicker *et al.*, 2011a; Dicker *et al.*, 2011b). In the meantime in New Zealand, at the AgResearch Wallaceville Animal Research Centre, a set of laboratory derived near-isogenic strains of *T. circumcincta* had been developed by introgressing resistance genes from a multi-drug resistant field strain into an otherwise genetically drug-susceptible inbred *T. circumcincta* background (Bisset, 2007). Interestingly, both groups observed increases in expression and polymorphisms of Pgps in multiple anthelmintic resistant *T. circumcincta,* despite the respective resistant isolates being selected in very different ways.

The field isolates, maintained by annual passage through sheep hosts at Moredun Research Institute, represent two populations of worms of UK origin which are otherwise genetically unrelated. One isolate, MTci2 (**M**oredun ***T***. ***ci****rcumcincta* strain **2**), has been shown to retain susceptibility to the major classes of anthelmintics (Skuce *et al.*, 2010; D. Bartley, Pers. Comm.), and the other isolate, MTci5 (**M**oredun ***T***. ***ci****rcumcincta* strain **5**) was first described by Sargison *et al.* (2001) and was confirmed to be resistant to fenbendazole, LEV and IVM (Bartley *et al.*, 2004; Bartley *et al.*, 2005). Comparisons of the MTci2 and MTci5 isolates were conducted and one specific Pgp gene in *T. circumcincta*, classified "*Tci-pgp-9",* as it aligned with *Ce-pgp-9* of *C. elegans*, appeared to be associated with an IVM resistant phenotype (Dicker *et al.*, 2011b). Increased Pgp expression was observed at the mRNA level following comparisons of the MTci2 and MTci5 isolates. The constitutive expression of *Tci-pgp-9* NBD2 was measured using Real Time PCR in each life-cycle stages and *Tci-pgp-9* constitutive expression was greater in the MTci5 isolate at all life-cycle stage relative to the MTci2 isolate (Table 1.2). The inducible expression of *Tci-pgp-9* was measured after IVM exposure in the MTci5 isolate, and this was compared to the non-

exposed MTci5 isolate. Results revealed that *Tci-pgp-9* expression was increased in the IVM-exposed MTci5 population compared to a population that was at rest (no IVM-exposure) (Dicker *et al.*, 2011b).

Table 1.2 Increase in Expression of *Tci-pgp-9* in *T. circumcincta*

The table shows the increases in constitutive and inducible expression of *Tci-pgp-9* NBD2. Values represent fold increases observed between expression in the resistant and the susceptible isolates, over the different life-cycles of *T. circumcincta* (Adapted from Dicker *et al.*, 2011b).

Life-cycle Stage	Constitutive Expression	Inducible Expression
Eggs	55.27	1.10
L_1	5.06	1.71
L_3	17.49	1.54
L_4	14.04	No Data
Adults (L_5)	6.75	0.69

Investigations into the same *Tci-pgp-9* gene had earlier been conducted in the AgResearch Hopkirk Research Institute, New Zealand (Bisset, 2007). In that study, full-length genomic DNA sequences for *Tci-pgp-9* were generated from overlapping lambda clones obtained by screening a genomic library from an Australian strain of *T. circumcincta* whose anthelmintic resistance status was unknown. In addition, near-isogenic lines of multiple-anthelmintic resistant and susceptible *T. circumcincta* were developed for the study through a lengthy process of inbreeding and back-crossing experiments. First, a laboratory isolate of *T. circumcincta*, which was known to be susceptible to all anthelmintic classes, was inbred for two generations to reduce the genetic variation within this worm population. Simultaneously a multiple-anthelmintic resistant field isolate, maintained at AgResearch and used in several previous studies of anthelmintic resistance (Sutherland *et al.*, 1997; Sutherland *et al.*, 1999; Sutherland *et al.*, 2000, Sutherland *et al.*, 2002; Sutherland *et al.*, 2003), was screened with oxfendazole (OXF), LEV and IVM for five generations. Late fourth-

stage (virgin) female worms from the inbred susceptible population were crossed with male worms from the drug-screened multiple-anthelmintic resistant isolate by surgically implanting into a young goat. The resulting F_1 cross progeny were allowed to breed for two further generations with anthelmintic screening with OXF, LEV and IVM at the F_2 and F_3 generations. Adult males from the F_3 generation were then back-crossed with late fourth-stage females belonging to the initial inbred anthelmintic-susceptible isolate. Once more the F1 cross progeny were allowed to breed for two further generations with anthelmintic screening at each of the F_2 and F_3 generations. Adult males from the F_3 generation progeny were once again back-crossed with late fourth-stage females from the initial anthelmintic-susceptible isolate. The resultant progeny were bred for two further generations with anthelmintic screening at each of these generations to give a multiple-anthelmintic resistant strain of worms that were expected to be similar genetically ($7/8^{ths}$ similar) to the inbred anthelmintic-susceptible strain but carrying the anthelmintic resistance genes derived from the original parental anthelmintic resistant field isolate (Bisset, 2007). These "near-isogenic" worm populations were used to study the genetic basis of anthelmintic resistance in *T. circumcincta*, focusing on glutamate-gated chloride channel genes (*Tci-avr-14* and *Tci-GluCl-a*), the γ-aminobutyric acid receptor gene (*Tci-GABRG-2*), three different Pgp genes (*Tci-pgp-1*, *Tci-pgp-2* and *Tci-pgp-9*) and an axonemal dynein gene (*Tci-che-3*). An advantage of using near-isogenic strains for genetic comparisons is that only substantial differences should be visible at loci linked to anthelmintic resistance, either directly or closely linked to other loci that are implicated in anthelmintic resistance.

With regard to the *Tci-pgp-9* gene, amplification and alternative splicing was observed during comparisons of the isogenic strains. Four possible coding SNPs of interest were identified when two near-isogenic populations, one which was susceptible to anthelmintics and another that was resistant, were compared. It has been shown that a SNP has the capacity to change an amino acid in the drug target protein and so changes its affinity (James *et al.*, 2009). For example, BZ-resistance in most nematode species is associated with a common F200Y SNP in the ß-tubulin isotype I gene, which can be used as a molecular diagnostic for BZ-resistance. Therefore, it is conceivable that the coding SNPs identified in this study could potentially provide a genetic marker

for anthelmintic resistance in *T. circumcincta* (Bisset, 2007). The first of the proposed residue changes is asparagine (N) in susceptible isolates to serine (S) in resistant isolates at residue 79 (N79S), and results in the loss of an N-linked glycosylation site. N-glycosylation is believed to influence the configuration of the Pgp molecule (Schinkel *et al.*, 1993) and the presence of two or three N-glycosylation sites in this region is strongly conserved in mammalian Pgps. The second and third proposed residue changes are, threonine (T) to serine (S) at residue 86 (T86S) and asparagine (N) to aspartic Acid (D) (N1043D), respectively. The fourth amino acid substitution, located at amino acid 1097, is found in the second NBD located towards the 3' end of the gene and changes glutamic Acid (E) to glutamine (Q) (E1097Q). Bisset (2007) also showed that the abundance of the *Tci-pgp-9* gene was 3.4-fold higher in the near-isogenic anthelmintic resistant strain when compared to the anthelmintic susceptible strain. The increase is likely to represent gene duplication or increased copy number in the resistant strains when compared to its susceptible counterpart (Zhao *et al.*, 2004).

1.6 Thesis Aims

The present study was undertaken to further characterise the *Tci-pgp-9* gene in selected UK strains of *T. circumcincta*. It follows on from the work carried out by Stewart Bisset (2007) and Alison Dicker (2010), as outlined above.

The specific aims of the present study were:

(i) Determine the full-length cDNA sequence of *Tci-pgp-9* gene from the MTci2 and MTci5 isolates of *T. circumcincta*, and to conduct genetic comparisons of these isolates to identify polymorphisms. Compare the full-length *Tci-pgp-9* cDNA sequences derived from the UK isolates and the NZ near-isogenic strains of *T. circumcincta* to identify which, if any, polymorphisms are shared between these geographically diverse isolates.

(ii) Determine whether each isolate could be differentiated into sub-populations based on their anthelmintic resistance phenotype, using bioassays. Verify the utility of genetic comparisons between unrelated UK isolates by comparing the sub-populations collected from each isolate.

(iii) Identify which allelic variants of *Tci-pgp-9* are present in the UK isolates.

Chapter 2

General Materials and Methods

2.1 Introduction

General molecular biological and parasitological methods that were routinely used in multiple experiments are described in this chapter. Specific protocols that were followed for particular experiments will be described in the relevant chapter(s).

2.2 Teladorsagia circumcincta Isolates

Two UK field isolates of *Teladorsagia circumcincta*, namely MTci2 and MTci5, were the principal strains used in the present study. The first, MTci2, which is considered to be anthelmintic susceptible, was obtained from the Central Veterinary Laboratories (Weybridge, UK) in 2000. This strain was originally isolated from the field pre-1970, prior to routine use of LEV and IVM anthelmintics. It has been maintained since then by passage through parasite-naïve lambs without any exposure to anthelmintics. Although BZ anthelmintics may already have been in use at the time of MTci2's isolation, its susceptibility to this anthelmintic class has since been verified using egg hatch assays (Skuce *et al.*, 2010; D. Bartley, Pers. Comm.). In these assays, an ED_{50} of 0.09 µg/ml was observed (D. Bartley, Pers. Comm.), where resistance is indicated by an ED_{50} of >0.1 µg/ml as stipulated by Coles *et al.* (1992). Although the strain has not been exposed to anthelmintics or manipulated in any way since its isolation from the field, it may nevertheless contain low numbers of individuals that possess naturally occurring genetic mutations associated with anthelmintic resistance.

The second isolate, MTci5, originates from a Scottish lowland sheep farm and was first described by Sargison *et al.* (2001), where lambs displayed clinical evidence of PGE and lower liveweight gain than was expected, despite regular anthelmintic dosing. Prior to the isolation of the MTci5 strain, the parasites on the farm had been treated with IVM for the previous two years, before which BZ, LEV and ML anthelmintics had been rotated on an annual basis (Sargison *et al.*, 2001). Subsequent CET and FECRTs have confirmed the efficacies of

fenbendazole, LEV and IVM against this strain to be 59 %, 88 % and 60 %, respectively, indicating moderate levels of resistance to each of these drug classes (Bartley *et al.*, 2004; Bartley *et al.*, 2005). Furthermore, as MTci5 was isolated from a closed flock with no recent import of animals or, importantly, parasites, it was considered likely that the anthelmintic resistances observed had been selected on-farm (Bartley *et al.*, 2004). Like MTci2, MTci5 has since been maintained by annual passage through parasite naïve Suffolk-greyface cross lambs, and has not undergone any subsequent selection with anthelmintics.

A separate population of MTci5, representing progeny from adult survivors of IVM treatment, was generated specifically for the current study. An experimentally infected 'donor' lamb was treated with IVM at the manufacturer's recommended dose rate (0.2 mg/kg). Faecal samples were collected from day 21 post-treatment. Eggs were recovered and cultured to hatch to L_1, as described in Chapter 2.3.1. This IVM-screened population is, henceforth, referred to as "MTci5PT" (MTci5 Post-Treatment).

2.3 Collection of T. circumcincta Life-cycle Stages

Eggs, L_1 and L_3 are the easiest of the life-cycle stages to work with as they do not require the necropsy of donor animals. Faeces were collected from parasite-infected lambs and eggs and/or larvae were extracted from the faeces using the routine techniques listed below.

2.3.1 Egg Extraction & Collection of First Stage Larvae

Faeces were collected from previously parasite-naïve Suffolk-greyface cross lambs that had been dosed 21 days earlier with ~15,000 larvae of one or other of the above *T. circumcincta* isolates. The faeces were initially homogenised in tap water and then passed through sieves with decreasing mesh sizes (250 µm, 120 µm, 64 µm and 38 µm) to separate the nematode eggs from the particulate faecal matter. The eggs were washed with water and spun at 1000 x *g* for 2 minutes. The supernatant was removed and the pellet, containing the eggs and faecal debris, was resuspended in saturated salt solution. The tubes were inverted gently and then centrifuged at 1000 x *g* for a further 2 minutes. Nematode eggs float in saturated salt solution and, therefore, the top 1 cm of supernatant was transferred to a 38 µm sieve and the eggs washed thoroughly in tap water to remove residual salt solution. Egg hatching was encouraged by transfer to a petri-dish and incubation overnight at 25 °C. The L_1 were separated from unhatched eggs using the Baermann technique, where viable larvae migrate through 25 µm filters, for 2 hours at 25 °C.

2.3.2 Coproculture & Collection of Third Stage Larvae

Faeces from donor animals were collected and incubated at 20-27 °C for approximately 10 days, during which time the eggs hatched and larvae underwent two successive larval moults to progress to L_3. The L_3 were stimulated to migrate from the faecal material by submersion in water at room temperature for 4 hours. The supernatant was collected and passed over a 25 µm Baermann filter to ensure the collection of viable L_3 which were stored in water at 4 °C until required.

2.4 RNA Extraction Procedure

Pools of L_3 were collected by centrifugation at 1000 x *g* for 2 minutes. The supernatant was discarded before transferring the larvae, suspended in a minimal volume of water, to a cryovial. The cryovial was briefly immersed in liquid nitrogen (lN_2) before adding its contents to a pre-chilled (-80 °C) mortar and submerging them in lN_2. The larval pellet was thoroughly homogenised with a pestle, ensuring the pellet remained frozen by periodic addition of lN_2. The homogenate was collected in the centre of the mortar and 1-2 ml of Trizol® Reagent (Invitrogen) was immediately added. Trizol® reagent is used to isolate RNA by single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture (Chomczynski & Sacchi, 1987). The Trizol® Reagent solidified and the pellet was ground until it thawed to the liquid phase. Aliquots (1 ml) of the Trizol-homogenate were transferred to 1.5 ml Eppendorf tubes and incubated at room temperature for 5 minutes. To these tubes, 200 µl of chloroform was added and the contents were vigorously mixed for 15 seconds followed by 2 minutes incubation at room temperature. Phase separation was completed by centrifugation at 12000 rpm for 15 minutes at 4 °C. The upper aqueous phase, containing RNA, was transferred to a 1.5 ml Eppendorf tube to which 500 µl isopropanol was added and mixed by inversion. The tubes were incubated at room temperature for 10 minutes and then centrifuged at 12000 rpm for 10 minutes at 4 °C to pellet the RNA. The supernatant was discarded and the pellet washed with 1 ml of 75 % ethanol with vortexing. The RNA was collected in a pellet by centrifugation at 7500 rpm for 5 minutes at 4 °C. The ethanol supernatant was discarded and the pellet was air-dried to remove the residual ethanol. The pellet was resuspended in 50 µl Ultrapure™ DNAse/RNAse-Free Distilled Water (Gibco®) and the purity and concentration of RNA was determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo-Fisher Scientific) and stored at -80 °C.

2.5 Crude Genomic DNA Lysate Production

Crude genomic DNA (gDNA) lysates were generated for use as template in pyrosequencing assays (Chapter 3), allele-specific PCR (Chapter 5) and the relative quantification of *Tci-pgp*-9 using real time PCR (Chapter 6). Individual L_3 were transferred into each well of a 96-well plate (Axygen) containing 10 µl of a 3 % solution of recombinant PCR grade proteinase K (Roche) in PCRDirect lysis reagent (Tail) (Viagen Biotech). The L_3 were lysed by incubating at 55 °C for 16 hours, followed by incubation at 90 °C for 1 hour to denature the proteinase K and stored at -80 °C.

2.6 Agarose Gel Electrophoresis, DNA Gel Extraction and Quantification

Amplified products from successful PCR reactions were visualised using agarose gel electrophoresis and exposure of gels to ultra-violet light. One percent molecular grade agarose (Bioline) was added to 1X Tris-acetate-EDTA (TAE) buffer (pH 8.0), containing 40 m*M* Tris base, 20 m*M* acetic acid, and 1 m*M* Ethylenediaminetetraacetic acid (EDTA). The fluorescent nucleic acid gel stain, GelRed™ (Biotium) was added to the gel as a DNA intercalator to allow its visualisation using an AlphaImager®2200 (Alpha Innotech) ultraviolet transilluminator and imager. DNA was purified either directly from the PCR product using a Wizard® SV Gel and PCR Clean-Up System (Promega), or extracted from the agarose gel using a QIAquick® Gel Extraction Kit (QIAgen) - following the manufacturer's instructions each time. The concentration of purified DNA was determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo-Fisher Scientific) and stored at -80 °C.

2.7 DNA Sequencing Procedure

Two different cloning systems were used; chemically competent *E. coli* cells were used in work conducted at Moredun Research Institute (UK), and electro-competent cells were used in work conducted at Hopkirk Research Institute (NZ).

2.7.1 UK-based Cloning and Sequencing

Successfully amplified products were ligated into pGEM®-T Easy Vector (Promega) following the manufacturer's protocol. The vector was transformed into chemically competent *E. coli* JM109 High Efficiency Competent Cells (Promega) as per the manufacturer's protocol. To obtain the maximal transformation efficiency of *E. coli*, 950 µl of super optimal broth with catabolite repression (SOC) medium (Invitrogen) at room temperature was added (Hanahan, 1983). The transformed *E. coli* were incubated at 37 °C for 2 hours in a ThermoForma Orbital Shaker at 200 rpm. Transformed *E. coli* were spread on lysogeny broth (LB) -agar plates supplemented with 0.08 mg/ml X-Gal (5-bromo-4-4chloro-3-indolyl-ß-$_D$-galactopyranoside, Promega), 0.1 mg/ml Ampicillin and 0.5 n*M* IPTG, and incubated at 37 °C overnight. X-Gal and IPTG were used to blue-white screen for successful recombinants which possess a functional *lacZ* gene as part of the transformed pGEM®-T vector plasmid. PCR checks were conducted on selected colonies to confirm the presence of original PCR product in the plasmid. Successful recombinants were grown as overnight cultures in LB growth medium supplemented with 0.1 mg/ml Ampicillin at 37 °C. The plasmids were purified from the *E. coli* culture using a QIAprep Miniprep Spin Kit (QIAgen), following the manufacturer's instructions. The concentration of plasmid DNA was determined by spectrophotometry using a NanoDrop® ND-1000 Spectrophotometer (Thermo-Fisher Scientific). DNA sequencing was outsourced commercially to Eurofins/MWG/Operon, and the resulting sequences were analysed using Lasergene® 10 (DNASTAR Inc.) bioinformatics software.

2.7.2 NZ-based Cloning and Sequencing

Amplified PCR products that were selected for sequencing were cloned into pCR™4-TOPO® TA vector (Invitrogen) by adding 1 µl of PCR product to a ligation reaction of 0.6 µl PCR-grade water, 0.4 µl dilute salt solution (1:4 dilution of saline solution supplied with the kit) and 0.4 µl pCR™4-TOPO® TA vector (Invitrogen), followed by incubation at room temperature for 5 minutes. Electroporation cuvettes (0.1 cm) were chilled on ice and SOC medium was equilibrated to room temperature. Electro-competent Transform One-Shot® TOP10 cells (Invitrogen) were thawed on ice and diluted 1:4 with sterile PCR grade water. To each ligation reaction, 45 µl of electro-competent cells was added and then transferred immediately to the electroporation cuvette and stored on ice until electroporation. Electroporation was conducted using a Bio-Rad Gene Pulser system set to low range 200, high range 500, capacitance 25, and voltage 1.5 kV. Immediately after electroporation, 200 µl of SOC medium was added to the cuvette followed by incubation at 37 °C for 1 hour with occasional mixing. Transformed cells (50 µl) were spread on LB-agar plates supplemented with 0.05 mg/ml kanamycin and incubated overnight at 37 °C. Colony PCR checks were conducted to confirm the presence of inserted PCR products of the appropriate size in the plasmids. Selected recombinants were amplified as overnight cultures of LB growth medium supplemented with 0.05 mg/ml kanamycin at 37 °C. The plasmids were purified from the bacterial culture using a QIAprep Miniprep Spin Kit (QIAgen), following the manufacturer's instructions. The concentration of plasmid DNA was quantified by spectrophotometry using a NanoDrop® ND-1000 Spectrophotometer (Thermo-Fisher Scientific). DNA sequencing was sourced externally with Massey Genome Service (Massey University, Palmerston North, New Zealand) and sequences were analysed using Lasergene® 10 (DNASTAR Inc.) bioinformatics software.

Chapter 3

Genetic comparisons of *Tci-pgp-9* in UK isolates of *T. circumcincta*

3.1 Introduction

A complete genome is a valuable resource for defining genetic mechanisms involved in drug resistance as well as genes implicated in host-parasite interactions. A concerted effort has been made to generate the complete genomes of species found throughout the five clades of the Phylum Nematoda, however, few have been completed to date. Clade V nematodes, *Caenorhabditis elegans* (The *C. elegans* Sequencing Consortium, 1998) and *C. briggsae* (Stein *et al.*, 2003), and *Meloidogyne hapla* (Opperman *et al.*, 2008) from Clade IV, have completed genomes. Draft genomes have been submitted for members of the other clades, such as Clade III parasitic nematodes *Ascaris suum* (Jex *et al.*, 2011) and *Brugia malayi* (Ghedin *et al.*, 2007), and the Clade I roundworm, *Trichinella spiralis* (Mitreva *et al.*, 2011). Gastrointestinal nematode parasites of livestock belonging to Clade V, including *T. circumcincta, Ostertagia ostertagi, Cooperia onchophora,* and *Nematodirus battus* have been selected for genome sequencing by the Genome Institute at Washington University. Recently, drafts of the *Haemonchus contortus* genome and transcriptome have been published, which represent the first genome to be published for a strongylid nematode (Laing *et al.*, 2013; Schwartz *et al.*, 2013).

Genetic comparisons are difficult in the context of understanding anthelmintic resistance, mainly due to the absence of a completed *T. circumcincta* genome. There are only minimal datasets such as the nematode transcriptome resource - NEMBASE4 (Elsworth *et al.*, 2011), which forms a database of expressed sequence tags and partial sequences, many of which remain to be annotated. Focus has thus shifted to specific candidate genes that have been associated with a drug resistance phenotype. Helminth parasites are thought to avoid the action of anthelmintics in a number of ways: alteration of the drug target; decreasing drug metabolism; changing the distribution of the drug by decreasing membrane permeability; or by amplifying the target genes to overcome the drug action, or indeed a combination of any/all of the above mechanisms (Wolstenholme *et al.*, 2004).

In *T. circumcincta*, ß-tubulin, acetylcholine receptors and glutamate-gated chloride channels have been shown to be the main targets for BZ, LEV and IVM, respectively. Xenobiotic efflux pumps, specifically Pgps, have been suggested as the mechanism by which parasites may resist the detrimental effects of multiple anthelmintic classes (Kerboeuf *et al.*, 1999; Prichard, 1999). Recent studies focusing on Pgps from *T. circumcincta* yielded partial sequences of multiple Pgp genes (Dicker *et al.*, 2011b), as well as showing an increased expression of specific Pgp genes in a multiple-resistant isolate of *T. circumcincta*. Further supporting the theory that Pgps are implicated in anthelmintic resistance are the studies into Pgp expression in *H. contortus* (Xu *et al.*, 1998; Williamson *et al.*, 2011), *C. oncophora* (De Graef *et al.*, 2013) and *C. elegans* (James & Davey, 2009) and the observed enhanced efflux of the BZ derivative, triclabendazole, by Pgps in the trematode, *Fasciola hepatica* (Wilkinson *et al.*, 2012). Interestingly, a specific Pgp in *T. circumcincta* has been highlighted by two independent groups as having a putative role in IVM resistance in this parasite. An increase in *Tci-pgp-9* copy number was observed when comparing a resistant NZ isolate with its susceptible counterpart (Bisset, 2007) and increased expression of *Tci-pgp-9* was observed in an anthelmintic resistant UK isolate, MTci5, when compared to an unrelated anthelmintic susceptible UK isolate, MTci2 (Dicker, *et al.*, 2011b). As part of the Bisset (2007) study, the only available full-length coding sequence of *Tci-pgp-9*, to date, was initially predicted from gDNA sequence derived from two lambda clones obtained by screening a genomic library from an Australian strain of *T. circumcincta*, whose anthelmintic resistance status was unknown. The *Tci-pgp-9* cDNA sequence was later confirmed by cDNA sequence generated from the inbred susceptible and back-crossed resistant, near-isogenic strains of *T. circumcincta* from NZ (described in Chapter 1.4.2). Three non-synonymous SNPs/amino acid substitutions were identified from cDNA clones representing N-terminal and C-terminal transmembrane domains amplified from pools from the inbred susceptible and multiple resistant near-isogenic strains. A fourth non-synonymous SNP was identified in the near-isogenic strains after haplotype sequence analyses focused on the second internucleotide binding domain of *Tci-pgp-9* (Bisset, 2007).

Non-synonymous SNPs are changes in the nucleotide sequence that, when translated into amino acid sequence and ultimately protein, alter the encoded residue and thus may change the tertiary structure of the protein molecule in question. Depending on their location in the gene, non-synonymous SNPs may alter a drug binding site directly, reducing that drug's binding affinity. Synonymous SNPs are "silent" mutations in the coding sequence that do not alter the encoded residue. As a result codons used frequently may be changed to rarer codons, possibly influencing the timing of co-translational folding and insertion of the Pgp molecule into the appropriate membrane (Kimchi-Sarfaty *et al.*, 2007). Therefore, silent SNPs may play a role in altering the drug binding site of the Pgp molecule through changes in protein folding and tertiary structure, without any change in the actual amino acid sequence.

In the field, the detection of anthelmintic resistance relies heavily upon the FECRT. There is considerable variation when interpreting the outcomes of the FECRT with the possibility of a test returning a false-positive (Miller *et al.*, 2006). Generally, the FECRT has been considered a diagnostic technique with low sensitivity, unable to detect levels of resistance below ~25 % (Martin *et al.*, 1989). A molecular test would provide greater sensitivity, although the identification of polymorphisms that have a potential use as diagnostic markers of anthelmintic resistance in parasitic nematodes remains elusive, with one notable exception. Currently, a genetic test is available that identifies key mutations in the ß-tubulin isotype-1 gene that are strongly linked to BZ-resistance in veterinary nematodes (von Samson-Himmelstjerna *et al.*, 2007). The phenylalanine (F) to tyrosine (Y) substitution at residue 200 (F200Y) was initially proposed as a marker for BZ-resistance (Kwa *et al.*, 1994) and later, similar substitutions were observed at residue 167, F167Y (Silvestre & Cabaret, 2002) and glutamic acid (E) to alanine (A) substitution at residue 198, E198A (Ghisi *et al.*, 2007). Originally identified in *H. contortus*, these genetic markers for BZ-resistance have also been identified in other trichostrongylid species, including *T. circumcincta* (Elard *et al.*, 1996), *Trichostrongylus colubriformis* (Grant & Mascord, 1996) and *C. onchophora* (Njue & Prichard, 2003) and, most recently, *Nematodirus battus* (A. Morrison, Pers. Comm.). To date, no robust markers have been identified for the imidazothiazoles or ML classes of anthelmintic.

The aim of this Chapter was to identify any polymorphisms by comparing the anthelmintic susceptible (MTci2) and resistant (MTci5) isolates, and generate the full-length cDNA sequence of the *Tci-pgp-9* gene. The *Tci-pgp-9* sequences generated from UK isolates were compared to *Tci-pgp-9* cDNA sequences generated from NZ isolates.

3.2 Materials and Methods

3.2.1 Sequence Differences Identified by Chromatogram Comparisons

Comparisons of cDNA sequences from pools of larvae were conducted to gain insight into the different genotypes likely to be present in each of the MTci2 and MTci5 strains. Four regions of the *Tci-pgp-9* gene were focused upon, these related to four amino acid substitutions, N79S, T86S, N1043D and E1097Q, identified during comparisons of the New Zealand susceptible and resistant near-isogenic strains in the Bisset (2007) study. First-strand cDNA was synthesised by reverse transcription from the RNA extracted from pools of larvae (described in Section 2.4) using the following method. Initially, a mixture was prepared containing 5 µg of RNA, 1 µ*M* oligo$(dT)_{20}$ primer (Invitrogen), 0.2 µ*M* dNTP mix (0.2 µ*M* each) (Invitrogen), and made to 13 µl with Ultrapure™ DNAse/RNAse-Free Distilled Water (Gibco®), before being incubated at 65 °C for five minutes and then transferred to an ice bath for a further minute. To this mix, using the components of the SuperScript® III Reverse Transcriptase kit (Invitrogen), 4 µl of 5X First-Strand Buffer, 2 µ*M* DTT, 40 units of RNaseOUT™ Recombinant RNase Inhibitor (Invitrogen) and 200 units of SuperScript™ III reverse transcriptase were added. Reverse transcription was carried out by incubating the mixture at 50 °C for 45 minutes and 70 °C for 15 minutes, and the resulting first-strand cDNA was stored at -20 °C. Fragments of *Tci-pgp-9* cDNA, that included the proposed locations of the four SNPs (Bisset, 2007), were amplified and the PCR product was purified (described in Section 2.6) and sequenced. Sequence generated from the MTci2 larvae was compared with sequences generated from MTci5 larvae. Automated DNA sequencers generate a four-colour electropherogram, commonly known as a chromatogram, where each colour represents an individual nucleotide: adenine (A) - green, thymine (T) - red, cytosine (C) - blue and guanosine (G) - black. The chromatograms from different sequencing runs can be

directly compared using the simple, web-based Sequence Difference of Chromatograms (SeqDoC) program, available at: http://research.imb.uq.edu.au/seqdoc/ (BioMed Central Ltd.). The SeqDoC program produces a difference profile graph by subtracting the differences between a reference and test sequence, allowing the rapid identification of SNPs, deletions/insertions and point mutations (Crowe, 2005). The difference profile is calculated by normalising the amplitude of the chromatogram peaks, in both the reference and test sequences and automatically aligning them. The test trace values are subtracted from the reference trace, resulting in a difference trace with bidirectional peaks highlighting the base changes between the sequences. The SeqDoC program was used to detect variations present in the generated sequences which may have been missed by base-calling analysis in the DNASTAR Lasergene® 10 bioinformatics suite.

3.2.2 SNP Quantification Using Pyrosequencing

The frequency of suspected SNPs around the E1097Q amino acid substitution, highlighted in the SeqDoC analysis, were determined in the MTci2, MTci5 and MTci5PT populations of *T. circumcincta* using pyrosequencing. Pyrosequencing is a 'sequencing-by-synthesis' method which is based on the detection of released pyrophosphate (PPi) during nucleotide incorporation by DNA polymerase (Ronaghi, 2001). Adenosine-triphosphate (ATP) sulfurylase coverts the PPi, released during a nucleic acid polymerisation reaction, to ATP in the presence of adenosine phosphosulphate. The ATP produced provides energy to luciferase which oxidises luciferin to oxyluciferin, a reaction that releases light. Ronaghi (2001) states that the overall reaction, from polymerisation to light detection, takes place within 3-4 s at room temperature. The light generated is proportional to the quantity of ATP produced which is dependent on the amount of PPi released, which, in turn, is equimolar to the amount of nucleotide incorporated. Therefore, the light emitted is proportional to the amount of incorporated nucleotide. The nucleotides are added individually, with unincorporated nucleotides and ATP being degraded by an apyrase enzyme before the addition of the next nucleotide. Sequential addition of nucleotides

and the resultant light signals generated allowed the determination of the template sequence.

Eighty-four crude larval lysates (generated using the method described in Chapter 2.5) from individuals belonging to each of the MTci2, MTci5 and MTci5PT populations of *T. circumcincta*, respectively, were used as template gDNA. Due to low concentrations of gDNA extracted from individual L_3, an initial amplification step was required before the pyrosequencing PCR products could be generated. The sense primer, GAAGATCACAGGAGCCGTCAAG [FTcP13], was paired with the antisense primer, ATGGCGATCCGTTGTTTCTG [FTcP14] to produce a 1203 bp product that contained both introns and exons that flanked the E1097Q polymorphism identified in the SeqDoC analysis (Figure 3.3). The PCR mastermix for each reaction included 0.5 units of Platinum *Taq* Polymerase, 1 µl 10X *Taq* buffer, 2.5 m*M* $MgCl_2$, dNTPs (4 µ*M* each) (all Invitrogen), 1 µ*M* each primer and 1 µl template (crude lysate). End-point PCR was performed using the following program: 94 °C for 8 min to denature template and to activate the *Taq* enzyme, followed by 35 three-step (denaturing, annealing and extension steps) cycles of 94 °C for 10 sec, 50 °C for 20 sec and 68 °C for 40 sec, followed by a final elongation step of 68 °C for 7 minutes. Successful PCR products of 1203 bp were diluted 1:10 and used as the template in a nested-PCR using the sense primer, CGCTCGCGTCTCTTTATGATC [Pyro F2] with the biotinylated antisense primer, GCGGATGGATCTGTCGAAA [PyroBiotin 2], which were designed using the Pyrosequencing™ Assay Design Software Version 1.0 (Biotage). The biotinylated primer was included to facilitate conjugation of the nested-PCR product to streptavidin-coated sepharose beads later in the DNA purification process. The nested-PCR product was generated using NovaTaq™ Hot Start Mastermix kit (Novagen®) and each 50 µl reaction contained 25 µl of 2X NovaTaq™ Hot Start Mastermix (which included DNA polymerase and dNTPs), 0.4 m*M* Pyro F2 primer, 0.185 m*M* PyroBiotin 2 primer, 1.5 m*M* $MgCl_2$, and made to 50 µl with Ultrapure™ DNAse/RNAse-Free Distilled Water (Gibco®). The thermocycling program used was: 10 minutes initial denaturation at 94 °C, 40 three-step cycles of denaturing at 94 °C for 60 s, primer annealing at 52.5 °C for 30 s and an extension phase of 72 °C for 30 s. A final extension step at 72 °C for 5 minutes followed, with a final hold at 4 °C. The successful amplification of the nested-PCR products was confirmed by agarose gel electrophoresis, as described in Chapter 2.6, before

continuing with the pyrosequencing assay. PyroMark™ Binding Buffer (Qiagen) was used to conjugate the biotin-labelled PCR products to streptavidin beads and using the PyroMark™ Vacuum Prep Workstation (Qiagen) the products were washed using PyroMark™ Washing Buffer (Qiagen) to remove the remaining components of the PCR reaction. Added to each well of a PyroMark™ Q96 Plate Low (Qiagen) was 38.4 µl PyroMark™ Annealing Buffer (Qiagen), 1.6 µl PyroMark™ Sequencing Buffer (Qiagen) and 0.4 µ*M* sequencing primer: TGGCAATGATCTACGC [Pyro S2]. The sequencing primer was required for SNP identification mode (Biotage PyroMark™ ID) and was located immediately before the SNP analysis region. The purified single-stranded DNA was transferred to this plate after removal from the streptavidin beads by alkali denaturation with PyroMark™ Denaturing Solution (Qiagen). The PyroMark™ Q96 Plate Low 96-well plate was incubated at 80 °C for 2 minutes to allow annealing of the sequencing primer, then cooled to room temperature for 5 minutes before transferring to the Biotage PyroMark™ ID. SNP identification mode was used to test for polymorphisms present in the target sequence (Figure 3.1) and was carried out on each of the MTci2, MTci5 and MTci5PT isolates.

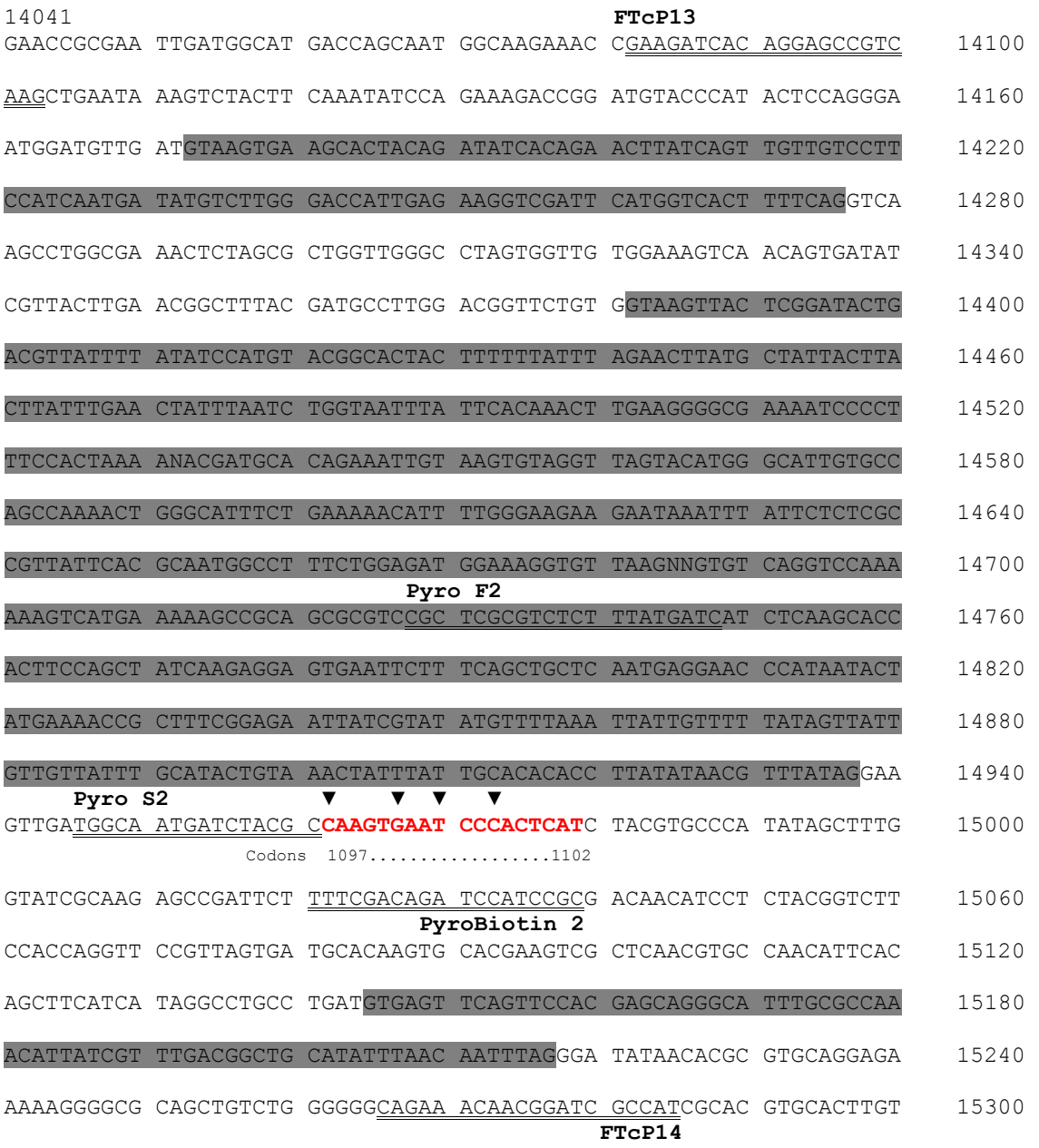


Figure 3.1 Pyrosequencing Target Sequence

A fragment of MTci2 gDNA sequence was amplified, using FTcP13 and FTcP14 primers (underlined). The fragment is located between 14082 bp and 15295 bp in the *Tci-pgp-9* gDNA sequence (Bisset, 2007). This fragment contained both introns (shaded) and exons. An additional PCR product was amplified using the nested primers Pyro F2 and PyroBiotin 2 (underlined). The nested-PCR product included the target sequence for the pyrosequencing assay (highlighted in red) immediately following the sequencing primer (Pyro S2). The four SNPs (arrows) tested were located in codons 1097, 1098, 1099 and 1100 of the *Tci-pgp-9* amino acid sequence.

3.2.3 Generation of Full-Length Tci-pgp-9 cDNA Sequence

The first step in identifying polymorphisms within the *Tci-pgp-9* gene was to generate and compare the full-length cDNA sequence from anthelmintic susceptible and resistant UK field isolates, MTci2 and MTci5, respectively. Initially, the regions flanking the initiation (ATG) and termination (TGA) codons - the 5' untranslated region (UTR) and the 3' UTR, were targeted for primer design. The spliced leader (SL) sequence, found in most nematodes (Bektesh, 1988; Blaxter & Liu, 1996; Redmond & Knox, 2001), was utilised to amplify the 5' UTR of *Tci-pgp-9*, upstream from the initiation codon. The SL1 sequence, first identified in *C. elegans* by Krause & Hirsch (1987), was used: 5' GGTTTAATTACCCAAGTTTGAG 3' [Cel SL1]. To enable DNA sequencing downstream of the termination codon, a 3' Rapid Amplification of cDNA Ends (RACE) strategy was adopted. This method utilises the naturally occurring polyadenylated tail found in mRNA as a generic priming site for an adapter primer. The adapter primer comprises a defined sequence at its 5' end and a $(dT)_{18}$ 3' tail: 5' <u>ACTTCGTTCTCCATTAGCGCG(</u>$T)_{18}$ 3' [3'RACE dT]. The adapter primer (underlined in the 3'RACE dT primer sequence above) is incorporated at the 3' end of the gene during first strand cDNA synthesis, and provides a binding site for an adapter-specific nested primer, 5' ACTTCGTTCTCCATTAGCGCG 3' [3'RACE]. First-strand cDNA synthesis was conducted using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's protocol, replacing the Oligo$(dT)_{20}$ primer with the 3'RACE dT primer in the reaction mix. The Cel SL1 and 3'RACE primers were paired with primers designed within conserved regions of the *Tci-pgp-9* cDNA sequences, kindly supplied by Stewart Bisset (AgResearch, Hopkirk Institute, NZ). Using this strategy, the 5' and 3' UTR sequences were obtained for the UK isolates, and allowed the design of primers within these regions to obtain the full-length *Tci-pgp-9* coding sequence.

Primer pairs were designed in the 5' and 3' UTR sequences to ensure amplification of full-length transcripts: 5' ATGGGCTTCCTAAAGAAGAACGGGA 3' [FTcP36] and 5' AASATCGTAGGGGTGAGCTCA 3' [FTcP35] for the MTci2 isolate, and for the MTci5 isolate, 5' GTTTGAGGTAATATGGGCTTCCT 3' [FTcP24] and 5' CGTATGGAASATCGTAGGGGTGA 3' [FTcP38]. The primer annealing positions are shown in Figure 3.2. The FTcP24/FTcP38 and FTcP36/FTcP35 primer pairings

were selected due to their superior amplification of the full-length *Tci-pgp-9* gene compared to other primer combinations that were tested.

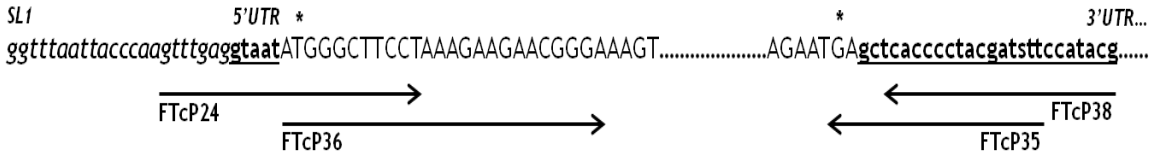


Figure 3.2 Full-length Transcript Primers

Schematic showing the positions of primers used to amplify the full-length *Tci-pgp-9* from MTci2 (primer pair FTcP36 + FTcP35) and MTci5 (primer pair FTcP24 + FTcP38), respectively. The initiation (ATG) and termination (TGA) codons are annotated with an asterisk and the 5' and 3' UTRs are underlined and in bold. The SL1 sequence is shown italicised and is found immediately upstream of the 5'UTR.

The coding sequence of the *Tci-pgp-9* gene forms a large transcript of ~3.8 kb. To ensure accurate sequence data had been generated, the full-length transcript was amplified in its entirety using Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen). This enzyme mixture was selected because it is composed of recombinant *Taq* DNA polymerase, *Pyrococcus species* GB-D polymerase and Platinum *Taq* antibody (Innis, 1988; Barnes, 1994). By adding *Pyrococcus* GB-D polymerase to regular *Taq* Polymerase, an approximately 6-fold increase in fidelity is observed. This is thanks to the proofreading ability of the *Pyrococcus* GB-D polymerase through the 3' to 5' exonuclease activity (Tindall, 1988).

The RACE-adapted first-strand cDNA, synthesised from RNA extracted from pools of L_3 for each of the MTci2 and MTci5 isolates (described in Section 2.4), was used as the template DNA in the following 50 µl mastermix: 5 µl of 10X High Fidelity PCR Buffer, 0.2 µ*M* of each deoxyribonucleotide triphosphate (dNTP), 2 m*M* $MgSO_4$, 1 unit of Platinum® *Taq* High Fidelity, 1 µl Template DNA, and made to 50 µl with Ultrapure™ DNAse/RNAse-Free Distilled Water (Gibco®). The thermocycling program followed for amplification of full-length transcripts was: 2 minutes initial denaturation at 94 °C, 35 three-step cycles of denaturing at 94 °C for 30 s, primer annealing at 55 °C for 30 s and an extension phase of 68 °C for 4 minutes. A final extension step at 68 °C for 5 minutes followed, and

a final hold at 4 °C. Successful PCR reactions were confirmed by agarose gel electrophoresis and the DNA was purified as described in Section 2.6. Full-length amplicons were ligated into pGEM®-T Easy Vector (Promega) as described in Chapter 2.7.1 and transfected into chemically competent JM109 *E. coli*. Clonal amplification of the plasmids was conducted, cDNA purified and DNA sequence obtained. Initially the PCR products were sequenced with SP6 and T7 primers which anneal to sites located in the plasmid vector, flanking the inserted sequence. After confirmation of successful ligation of the full-length *Tci-pgp-9* transcript into the plasmid, the purified cDNA was used for further rounds of sequencing.

The design of sequencing primers was based on the consensus sequence of multiple contiguous DNA sequencing alignments of the *Tci-pgp-9* gene, generated from pools of both the MTci2 and the MTci5 isolates. On average, the length of high quality sequence generated was ~500 bp, therefore, primers were designed successively at 500 bp intervals along the full-length *Tci-pgp-9* gene, in both the sense and antisense directions (Figure 3.3). The primer sequences used and the intended annealing orientations are shown in Table 3.1. Three clones possessing the full-length *Tci-pgp-9* products, from each of the MTci2 and MTci5 isolates, were sequenced in both the sense and antisense directions. Sequencing results were assembled and analysed using DNASTAR Lasergene® 10 bioinformatics software. Comparisons between sequencing results from each isolate were conducted using the multiple sequence alignment program ClustalW2, available online at the European Bioinformatics Institute (www.ebi.ac.uk/Tools/msa/clustalw2). The *Tci-pgp-9* sequences from the UK isolates were compared to the full-length cDNA sequence generated from the NZ stains, due to the unavailability of a UK reference sequence. Polymorphisms present in these geographically diverse isolates were compared to identify any shared SNPs.

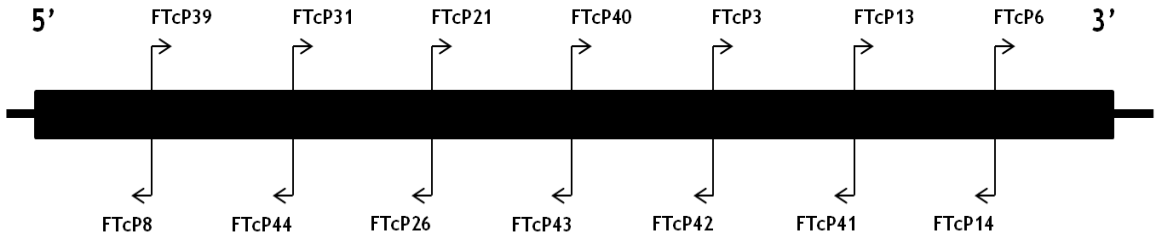


Figure 3.3 Full-Length *Tci-pgp-9* Sequencing Primers

Schematic showing the approximate locations of primers used to sequence *Tci-pgp-9*. Primers were located at ~500 bp intervals, SP6 and T7 primers were also used at the 5' and 3' ends of the sequence.

Table 3.1 Full-Length *Tci-pgp-9* Sequencing Primers

Primers positioned at ~500 bp intervals in both the sense (S) and antisense (A) directions, ensuring complete coverage of the *Tci-pgp-9* gene.

Primer Name	Primer Sequence	Sense (S) or Antisense (A)
FTcP39	5' GCCCTTATGATCCAATTCGTGGCGCA 3'	S
FTcP31	5' GTATTCTTCTCCGTGATGATGGG 3'	S
FTcP21	5' TTGGTGACCGAGGAACCCAGATG 3'	S
FTcP40	5' CGACTGAAGAAAGAACTCGAAGAAGAAGG 3'	S
FTcP3	5' GTCAAGTCGGCTCTCGA 3'	S
FTcP13	5' GAAGATCACAGGAGCCGTCAAG 3'	S
FTcP6	5' TGGGGGCAGAAACAACGGATCGCCATC 3'	S
FTcP8	5' GAAACCGCCGAAAAACTGC 3'	A
FTcP44	5' GCCATAGAACCCATCATCACGGAGAA 3'	A
FTcP26	5' TGCTGAACAATATGTTCACTTTCG 3'	A
FTcP43	5' CCTTCTTCTTCGAGTTCTTTCTTCAGTCG 3'	A
FTcP42	5' CGACACCACTGCAGACGGAGAC 3'	A
FTcP41	5' GAGCATATGGAAGATAAGACCGGCAGC 3'	A
FTcP14	5' ATGGCGATCCGTTGTTTCTG 3'	A

3.3 Results

3.3.1 Comparisons of Sequence Chromatograms

Sequencing chromatograms for *Tci-pgp-9* in the MTci2 and MTci5 isolates were compared and analysed using the SeqDoC program. The difference profiles were checked at the locations of the four amino acid substitutions identified in the NZ isolates of *T. circumcincta* at residues N79S, T86S, N1043D and E1097Q. The difference profiles of the N79S, T86S, and N1043D SNPs were flat, indicating no difference in the chromatograms from the MTci2 and MTci5 isolates. The final SNP found at E1097Q showed a double peak in the MTci2 chromatogram and a single peak in the MTci5 chromatogram, resulting in a peak on the difference profile (Figure 3.4). This result suggested that both cytosine and guanine had been identified at this position, although the base-calling software identified this base as cytosine which was the higher of the peaks at this position. When cytosine is incorporated at this position, the codon, CAA, is translated into glutamine (Q). Incorporating a guanine at this point changes the residue to glutamic acid (E) with a codon of GAA.

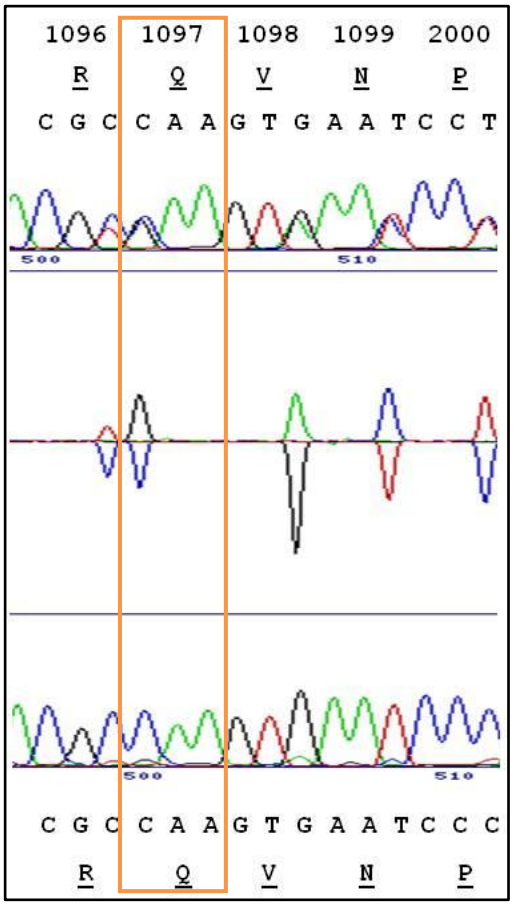


Figure 3.4 SeqDoC Analysis of E1097Q SNP in UK Isolates of *T. circumcincta*

The difference profile generated by comparison of the MTci2 and MTci5 *Tci-pgp-9* sequences. The codon of interest, highlighted by orange box, was CAA encoding glutamine (Q) and GAA encoding glutamic acid (E). At residue 1097, Q was associated with a particular set of haplotypes which appeared to be strongly selected for by drench screening in the NZ-R near-isogenic strain. In the MTci2 isolate, the first base of the codon appears to be either cytosine or guanine whereas in the MTci5 isolate it is predominantly cytosine at this position. This is shown by the double peak in the MTci2 profile (top profile) and the single peak in the MTci5 profile (bottom profile) and highlighted as a bidirectional peak in the difference profile (central profile).

3.3.2 Quantifying SNP Frequency

Previously, Bisset (2007) showed that a Q at residue 1097 was associated with a particular set of haplotypes that appeared to be strongly selected for by drench screening in the NZ-R near-isogenic strain. Therefore, the E1097Q SNP was targeted during the pyrosequencing assay. Evidence from the SeqDoC analysis (Figure 3.4) indicated that polymorphism at this codon was present within pools of each UK isolate. The initial step was to amplify a 1203 bp fragment of *Tci-pgp-9* gDNA, which used the template gDNA for a nested-PCR reaction. The nested-PCR product (312 bp) was then used as the template for the pyrosequencing assays (shown in Figure 3.1). The potential polymorphisms shown in the SeqDoC analysis (Figure 3.4) were targeted and included: a transversion mutation of C→G in the first base of codon 1097, and three transition mutations in the third base of codons 1098 (G→A), 1099 (T→C) and 1100 (C→T). The transversion mutation in codon 1097 translates to a change in the amino acid encoded, either glutamic acid (CAA) or glutamate (GAA). The transition mutations result in silent mutations and do not change the residue encoded by the codon.

The pyrosequencing assay generated 18 bp of *de novo* DNA sequence for 84 individual larvae from the MTci2, MTci5 and MTci5PT isolates of *T. circumcincta*. No further evidence of an E1097Q SNP was obtained from the results of the pyrosequencing assay, although it has since become apparent that this may have been due to an issue with the design of primers used for this work (see Discussion).

3.3.3 Generation of Full-Length Tci-pgp-9 cDNA Sequence

The full-length *Tci-pgp-9* cDNA sequence was successfully amplified from pools of L_3 using primer pairs FTcP36 + FTcP35 for the MTci2 isolate and FTcP24 + FTcP38 for the MTci5 isolate (products are shown in Figure 3.5). The product was cloned into pGEM®-T Easy Vector (Promega), in triplicate, and DNA sequencing of the *Tci-pgp-9* gene was conducted on purified plasmid cDNA for both the MTci2 and MTci5 isolates. Each clone was sequenced in the sense and antisense directions, thus ensuring the consensus for each isolate was derived from a minimal coverage of six sequences. The DNA sequencing data were assembled to form a contiguous sequence for each UK field isolate and comparisons of the *Tci-pgp-9* gene sequence from each isolate were conducted. Common motifs shared by members of the ABC-transporter superfamily (Table 3.2) were identified by comparing with ABC-transporter sequence motifs characterised in *Brugia malayi* (Ardelli *et al.*, 2010) and annotated in the contiguous sequences (Figure 3.6), thus verifying that the sequenced transcript was that of a P-glycoprotein.

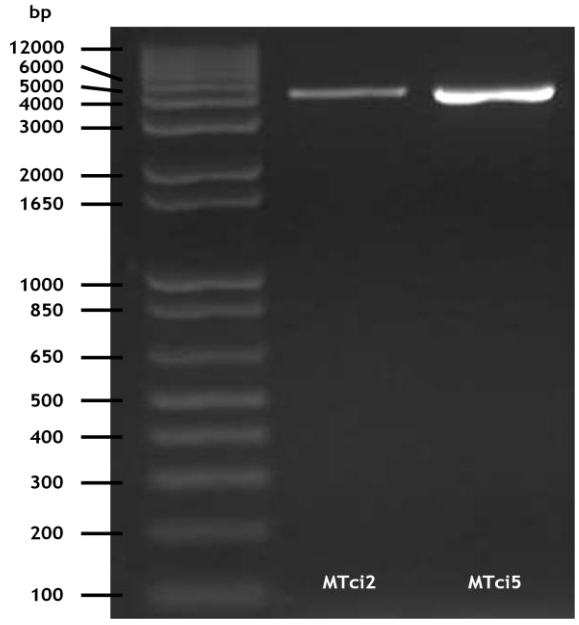


Figure 3.5 Full-length *Tci-pgp-9* cDNA PCR Products

Full-length *Tci-pgp-9* PCR products, approximately 3.8 kb, are shown on a 1 % agarose gel. The products are amplified from pools of L_3 from the MTci2 and MTci5 isolates of *T. circumcincta*.


(1)
MTci2 MGFLKKNGKVADSKGQEDSQIEEEKKEVVPKASIGQLFRYTTTFDKVLLLIGSVVAIGTGIGLPMMSIIM 70
MTci5 ....E..... 70
NZ-S ....E..... 70
NZ-R ....E..... 70
(A) (B)
MTci2 GNISQNFMSITGNTTSIQQFEHDVIQNCLKYVYLGCGVFTAATIQAMCFLTVCENLVNQLRRQFFKSILR 140
MTci5 140
NZ-S .....N.....T..... 140
NZ-R 140
MTci2 QDITWFDKNNSGTLATKLFDNLERVKEGTGDKLGLMIQFVAQFFGGFIVAFTYDWKLTLIMMSLAPFMII 210
MTci5 210
NZ-S 210
NZ-R 210
MTci2 CGAFIAKLMASAATREAKKYAVAGGIAEEVLTSMRTVIAFNGQPYECERYEKALEDGKSTGIKKSLYIGI 280
MTci5 280
NZ-S 280
NZ-R 280
MTci2 GLGITFLIMFSSYCLAFWVGTDFVFKNQMQGGTVMTVFFSVMMGSMALGQAGPQFAVLGTAMGAAGSLYQ 350
MTci5 350
NZ-S 350
NZ-R 350
Walker A
MTci2 IIDREPEIDSYSSEGVRPSNLKGKITVSNLKFTYPTRPDVPILKGVSFEAKPGETIALVGSSGCGKSTII 420
MTci5 420
NZ-S 420
NZ-R 420
NBD1
Q-Loop/Lid
MTci2 QLLLRYYNPADGKITIDGVEIDKINIEFLRNYVGVVSQEPMLFNTTIEQNIRYGREKVTDAEITAALRKA 490
MTci5 490
NZ-S 490
NZ-R 490
Signature Walker B D-Loop
MTci2 NAYNFVQSFPDGIYTNVGDRGTQMSGGQKQRIAIARALVRDPKILLLDEATSALDAESEHIVQQALENAS 560
MTci5 560
NZ-S 560
NZ-R 560
MTci2 KGRTTIVIAHRLSTIRNADKIIAMKNGEVVEVGNHDELIARKGLYHELVNAQVFADVDDTVGDAAVRRRT 630
MTci5 630
NZ-S 630
NZ-R 630
(234) (5)
MTci2 MSSSRSRSPSLASPEYKRLRSQLSVTEDTGVATAQNDPVKAEKDLERLKKELEEEGAAKANLFGILRHAR 700
MTci5 .....TAT.....S... 700
NZ-S .....S... 700
NZ-R .....TAT.....S... 700
MTci2 PEWPFIMFAVFSSVVQGCVFPAFSLFFSQIINVFSKQPGDPTLKQEGHFWALMFLVLGAVQATTMIIQCF 770
MTci5 770
NZ-S 770
NZ-R 770

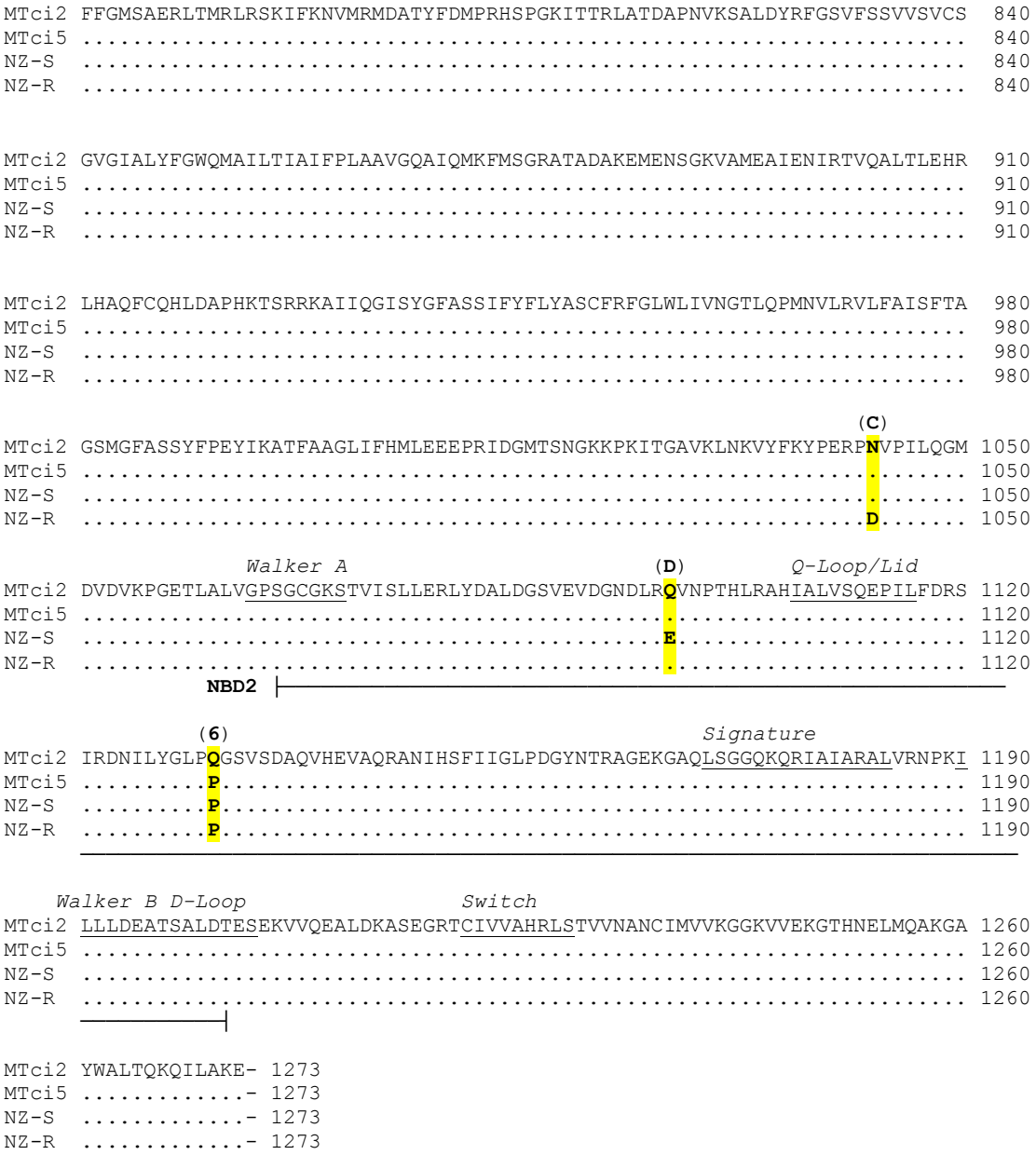


Figure 3.6 Amino Acid Sequence of *Tci-pgp-9*

Amino acid sequences from UK isolates, MTci2 and MTci5, aligned with sequence information derived from two near-isogenic strains *T. circumcincta* from NZ (Bisset, Pers. Comm.). The first of these was an inbred strain that was known to be susceptible to all available anthelmintics (NZ-S) and the other was a near-isogenic strain that was anthelmintic resistant (NZ-R). Sequence motifs are underlined and annotated above the sequence. The positions of nucleotide binding domains 1 and 2 (NBD1 and NBD2) are underlined and annotated below the sequences. Highlighted in yellow are six residue substitutions identified in the UK isolates (numbered 1-6), and four previously identified residue substitutions in NZ isogenic strains (annotated A-D).

Table 3.2 Summary of Signature Motifs in *Tci-pgp-9* Amino Acid Sequence

ABC-transporter sequence motifs were characterised in *Brugia malayi* (Ardelli *et al.*, 2010) and these were used to identify similar motifs in the *Tci-pgp-9* amino acid sequence. The motif amino acid sequences for each nucleotide binding domain are listed.

Motif	Motif Amino Acid Sequence	
	NBD1	**NBD2**
Walker A	GSSGCGKS	GPSGCGKS
Q-Loop/Lid	VGVVSQEPMLFNT	IALVSQEPIL
Signature	MSGGQKQRIAIARAL	LSGGQKQRIAIARAL
Walker B	ILLLDEAT	ILLLDEAT
D-Loop	SALDAES	SALDTES
Switch		CIVVAHRLS

At the nucleotide level, the MTci2 and MTci5 isolates share 95.45 % identity (identical at 3648/3822 bases) in their respective *Tci-pgp-9* cDNA sequences, whilst the protein sequences shared 99.53 % identity (1267/1273 residues). The full-length *Tci-pgp-9* cDNA and amino acid sequences for each isolate are listed in Appendix 1. Nine point mutations in total were identified between the UK isolates, resulting in six residue changes observed at V28E, A662T, T663A, A664T, R697S, and Q1131P, respectively (Figure 3.6). The remaining 165 silent, synonymous polymorphisms did not result in changes in encoded residue. As members of the ABC-transporter superfamily, Pgps share common motifs: the Walker A, Q-loop/lid, Signature, Walker B, D-loop and Switch sequences (Ardelli *et al.*, 2010) (Table 3.2). The presence of these motifs verified that the sequencing results were that of an ABC-transporter (annotated in Figure 3.6). The non-synonymous SNPs identified were not located in any of the conserved sequence motifs, although the Q1131P SNP was found in NBD2. Of the 165 synonymous SNPs, 12 were located in each of the sequence motifs in NBD1 and two were found in the Walker A motif in NBD2, the remaining 151 synonymous SNPs were located sporadically through the *Tci-pgp-9* cDNA sequence.

Comparisons were conducted between the *Tci-pgp-9* cDNA sequences from UK isolates of *T. circumcincta* and the only other full-length *Tci-pgp-9* cDNA sequence available, one that was originally predicted from overlapping clones

isolated from a *T. circumcincta* genomic DNA library generated from an Australian strain whose anthelmintic resistance status unknown (Bisset, 2007). The *Tci-pgp-9* sequence was subsequently updated using new information from several full-length cDNA clones derived from a set of near-isogenic inbred anthelmintic susceptible and multiple-resistant strains of New Zealand origin (S. Bisset, Pers. Comm.). The amino acid sequences of the UK and NZ inbred susceptible strains share 99.60 % identity (identical at 1268/1273 residues) and the resistant strains shared 99.76 % identity (identical at 1270/1273 residues). The MTci2 and MTci5 isolates both displayed a similar genotype to the anthelmintic resistant strain from NZ at three of the four non-synonymous SNPs originally identified in the Bisset (2007) study, at residues 79, 86 and 1097 (annotated A, B and D, respectively, in Figure 3.6). At the fourth non-synonymous SNP, residue 1043 (annotated C in Figure 3.6), the UK MTci2 and MTci5 isolates shared the susceptible genotype displayed in the NZ near-isogenic strains. There was no evidence from the above sequence information to suggest that the four non-synonymous SNPs identified in the NZ strains were present in the UK isolates of *T. circumcincta*.

SeqDoC analysis focussing on a particularly polymorphic region between residues 662-697 confirmed that four out of nine base changes resulted in non-synonymous SNPs and displayed peaks on the difference profile, these were the residue changes at A662T, T663A, A664T and R697S (Figure 3.7), the remainder were synonymous mutations. The genotype of residues 662, 663, 664, and 697 (numbered 2-5 in Figure 3.6) partitions between the UK and NZ strains based on their anthelmintic resistance phenotype. The MTci2 isolate shares identity with the NZ-S strain with “ATA” at residues 662-664 and is also associated with R at position 697. Similarly, the MTci5 isolate and the NZ-R strain have identical residues with “TAT” at 662-664, associated with S at position 697.

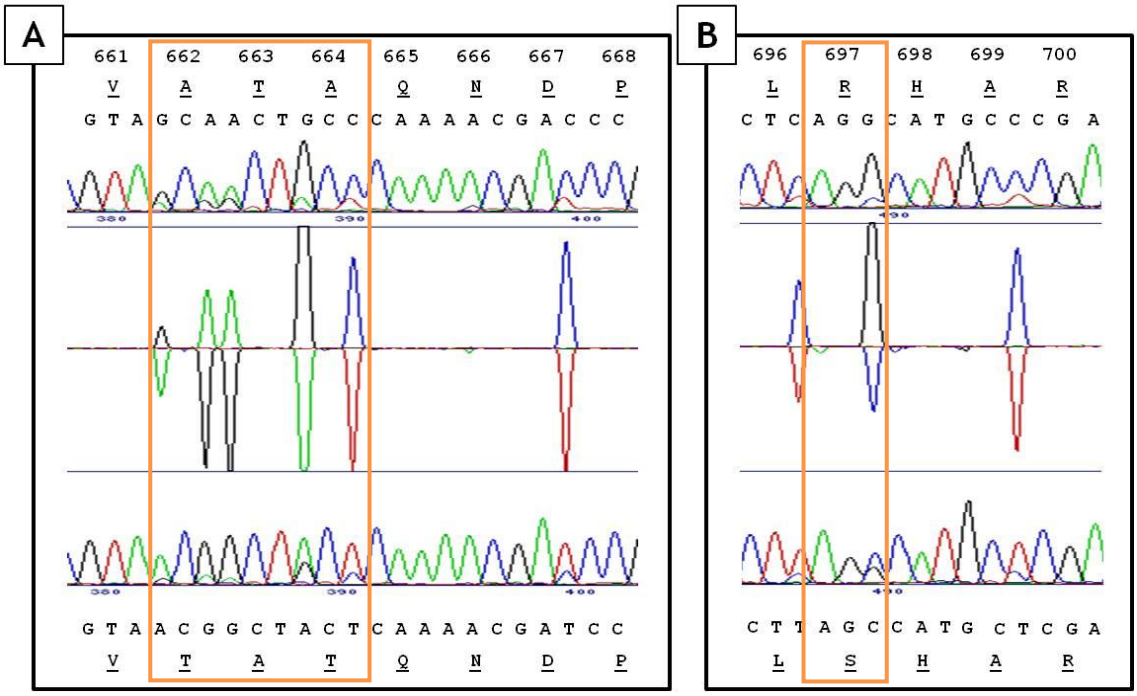


Figure 3.7 SeqDoC Analyses of Polymorphisms Present in *Tci-pgp-9*

Selected outputs from SeqDoC analysis of the full-length *Tci-pgp-9* sequences. The top profile is the reference sequence, in this case this was the *Tci-pgp-9* cDNA sequence from MTci2. The bottom profile is the test sequence of *Tci-pgp-9* cDNA from MTci5. The difference profile is displayed between the reference and test sequences. Peaks on the centraldifference profile indicate the position of SNPs when the MTci2 and MTci5 were compared. Panel *A* shows the observed SNPs at residues A662T, T663A, A664T in the full-length *Tci-pgp-9* sequence, highlighted by orange box. Panel *B* shows the SNP observed at R697S, in orange box. Additional peaks indicate the presence of synonymous SNPs which do not alter the residue encoded by the codon. Examples of synonymous SNPs are evident in the transition (C→T) mutations in the third base of codons 667 (Panel *A*), 696 and 699 (both Panel *B*).

3.4 Discussion

For the first time, the full-length cDNA transcripts of *Tci-pgp-9* have been amplified and sequenced from UK isolates of *T. circumcincta*. The sequences contain motifs that are common to all ABC-transporters (Ardelli *et al.*, 2010), verifying the amplified PCR products as Pgps. The *Tci-pgp-9* genes of the MTci2 and MTci5 isolates share very high homology, 95.45 % at the nucleotide level and 99.53 % amino acid identity, suggesting that conservation of this gene may offer a survival advantage to the individual. Polymorphisms were found throughout the *Tci-pgp-9* gene when comparisons were made between the MTci2 and MTci5 isolates. Nine mutations were non-synonymous SNPs, altering six residues (Figure 3.5), and 165 synonymous SNPs were identified, where nucleotide changes at the DNA level do not translate into amino acid changes. Since the Bisset (2007) study, the full-length cDNA sequences of *Tci-pgp-9* from the inbred anthelmintic susceptible and the near-isogenic anthelmintic resistant NZ strains have been confirmed (Bisset, Pers. Comm.). Interestingly, comparisons of the full-length cDNA sequences from the MTci2 and MTci5 isolates with the NZ strains of *T. circumcincta* revealed that four substitutions at amino acids 662-664 and 697 were shared. The anthelmintic susceptible strains, MTci2 and NZ-S, both displayed ATA and R at the 662-664 and 697 residues, whilst at the same positions in the resistant strains, MTci5 and NZ-R, the larvae exhibit TAT and S at the respective residues. The significance of these geographically distinct isolates sharing non-synonymous SNPs at these positions in *Tci-pgp-9* remains to be determined. Clearly, further confirmation of these SNPs in additional isolates of *T. circumcincta* is required before they could be considered as robust genetic markers of global IVM-resistance in this species. Certain amino acid substitutions, such as the F200Y SNP in ß-tubulin, have been shown to confer resistance to BZ anthelmintics. The search for a similar genetic marker of IVM-resistance in *T. circumcincta,* and other important nematode species, is ongoing.

The non-synonymous SNPs identified in the UK isolates differed from those initially identified in the NZ isolates (Bisset, 2007). Comparisons of the amino acid sequence of the NZ anthelmintic susceptible and resistant, near-isogenic strains revealed two point mutations, N79S and S86T, located in the first extracellular loop of the *Tci-pgp-9* molecule, with the first mutation resulting in the loss of an N-glycosylation site. Another two mutations (N1043D and E1097Q)

were found in the intercellular region near the second ATP-binding domain. These four residue substitutions do not appear to be conserved in the UK isolates of *T. circumcincta*.

The spread of point mutations observed throughout the *Tci-pgp-9* gene may influence the tertiary structure of the gene, altering drug binding which can be affected by multiple independent parts of the molecule (Gottesman & Pastan, 1993). All six residue changes are found in the cytoplasmic regions of the molecule and are unlikely to interact with the drug molecules directly; instead they may alter protein folding. Additionally, the silent SNPs may cause changes in substrate specificity, possibly by changing the timing of co-translational folding and thus the conformation of the Pgp molecule (Buss & Callaghan, 2008; Kimchi-Sarfaty *et al*., 2007). Changes in the codon sequence, and therefore codon usage, have been linked to the secondary structure of protein that they encode. Some residues show preference to be buried in the centre of the protein molecule and others prefer to be exposed on the surfaces of the folded protein (Saunders & Deane, 2010). Synonymous codons are not used with equal frequency (Tao & Dafu, 1998) resulting in the incorporation of "rarer" codons, the availability of which may impede translation speed. Structural information that is linked to translation speed (Saunders & Deane, 2010), may influence translation rate and subsequently protein folding (Kimchi-Sarfaty *et al*., 2007). Therefore, it is possible that both coding and non-coding SNPs have the ability to influence the rate of drug efflux in multi-drug resistant isolate of *T. circumcincta*.

The occurrence of alternative-splicing was raised by Bisset (2007), reporting an alternatively-spliced isoform of *Tci-pgp-9*, which resulted in the deletion of 45 residues in the first three transmembrane domains in the multiple resistant worms. Interestingly, an alternatively spliced Pgp was reported to occur in substantial quantities in two independently derived multidrug resistant Chinese hamster cell lines (Devine *et al*., 1991), which contained a deletion of a similar size and location to the alternatively-spliced *Tci-pgp-9* identified in the Bisset (2007) study. The deletion was absent from a parallel drug sensitive Chinese hamster cell line (Devine *et al*., 1991). In the present study, no evidence of an alternatively spliced *Tci-pgp-9* gene was observed in the UK isolates of *T. circumcincta*.

The SeqDoC analysis highlighted the nine point mutations that were responsible for the non-synonymous SNPs at residues 662, 663, 664 and 697. The analysis was also used to focus on the E1097Q identified in comparisons of the NZ inbred anthelmintic susceptible strain and the near-isogenic resistant strains of *T. circumcincta*. Comparisons of *Tci-pgp-9* from the MTci2 and MTci5 isolates at residue 1097 suggested that the pool of MTci2 larvae possessed both G and C at the first base of codon 1097, whereas the pool of MTci5 larvae possessed only C at this base. The G/C transversion is responsible for changing the residue that is encoded from E (codon GAA) to Q (codon CAA). The base-calling software displayed Q at residue 1097 because a higher proportion of the pooled MTci2 larvae displayed the CAA codon. SeqDoC analyses inferred that CAA codon was present in approximately 55 % of pooled larvae. The possibility of multiple genotypes present at this position in the MTci2 isolate (Figure 3.4) was investigated using a pyrosequencing assay, to quantify the frequency of this SNP within each isolate.

The pyrosequencing assay was conducted on 84 larvae from each of the MTci2 and MTci5 isolates and the results did not support those shown in the SeqDoC analysis. A single genotype was observed, with all individual larvae from both isolates displaying CAA at codon 1097. However, following detailed re-evaluation of the pyrosequencing primers used, it has since become apparent that the particular primers used in the pyrosequencing experiment may have effectively screened out all variants carrying a G in codon 1097. The FTcP13 and FTcP14 primers used in the initial amplification of the target region (Figure 3.1) were not sufficiently degenerate and therefore may have only amplified a proportion of the sequence variants that were present. The pyrosequencing primer design software was used to create primers to amplify the specific target region (Pyro F2 and PyroBiotin 2), as well as the sequencing primer (Pyro S2) located immediately before the target sequence. The Pyro F2 was positioned in intron sequence where it is difficult to design truly degenerate primers, whilst retaining the specificity of the primer to its target sequence. It is likely that this primer may be specific for a single haplotype, in this case one with CAA at codon 1097, thus the alternative GAA codon may would have been missed. Indeed this was shown to be the case. In the Bisset (2007) study, haplotype analysis of IBD71 (the second nucleotide binding domain in *Tci-pgp-9*) showed that the PyroF2

primer design is based on “Haplotype 4B” sequence which has Q at residue 1097. There was a SNP, which varies according to haplotype, in the second last base at the 3’ end of the PyroF2 primer; T at this position in Haplotype 4B, and C in all other haplotypes. Therefore, the nested-PCR has only amplified Haplotype 4B variants and so only the CAA codon at residue 1097 has been detected.

The sequencing primer PyroS2 was positioned immediately before the target sequence and ends on arginine at codon 1096. The third base of the arginine codon can accommodate any nucleotide and, therefore, sequencing primer finishing on the C of codon 1096 and has inadvertently screened out all variants that possess A, G or T at this position. This lack of degeneracy in the sequencing primer may explain why no GAA codons at residue 1097 were observed in the pyrosequencing assay. Increasing the degeneracy of the primers used in the pyrosequencing assay would ensure that all sequence variants are included in the analysis, but the reduction in specificity that comes with degenerate primers would have to be taken into consideration when interpreting the data. This is particularly pertinent when investigating a large and polymorphic gene family like the Pgps. Similarly, with PyroF2 primer design, only Haplotype 4B has been screened as the 3’ end of the PyroS2 primer matches none of the other haplotypes. Therefore, it was almost inevitable that only the CAA codon would be observed at residue 1097 in *Tci-pgp-9*.

The amplification and DNA sequencing of full-length cDNA sequence of *Tci-pgp-9* involved pairing the SL1 and 3’RACE primers. The spliced leader sequence is trans-spliced on to the 5’ end of primary transcripts during the maturation of the pre-mRNA (Blaxter & Liu, 1996), and conveniently provides a conserved sequence template for design of a sense primer(s). The spliced leader was first described in C. elegans (Krause & Hirsch, 1987) and since then numerous other SLs have been identified in this model nematode. The most abundant spliced leader sequence was SL1, which is found trans-spliced onto more than 80 % of C. elegans mRNA, and has also been identified in H. contortus (Redmond & Knox, 2001) and other strongyle species (Bektesh, 1988). To design the antisense primer, to pair with the SL1, a 3’ RACE strategy was adopted. During first-strand cDNA synthesis, the adapter primer sequence is incorporated into the naturally occurring polyadenylated tail found in mRNA at the 3’ end of the gene. This provided a target for the antisense adapter-specific nested

primer, 3'RACE, which ensures the termination codon as well as 3' UTR are included in the sequence generated. This approach ensured full-length *Tci-pgp-9* cDNA products were amplified (Figure 3.5).

In conclusion, full-length cDNA transcripts of *Tci-pgp-9* have been amplified and sequenced from UK isolates of *T. circumcincta*. Four substitutions at amino acids 662-664 and 697 were identified in comparisons of the *Tci-pgp-9* full-length cDNA sequence from anthelmintic susceptible and anthelmintic resistant isolates. The residue substitutions observed are conserved in each of the anthelmintic resistant isolates from both the UK and NZ. Before being considered as potential markers of IVM-resistance in this species, the presence of these point mutations in the *Tci-pgp-9* gene should be confirmed in additional *T. circumcincta* isolates. Future work could focus upon pyrosequencing targeted at the SNPs responsible for the substitutions at residues 662-664 and 697 to clarify the frequency of these SNPs within each isolate of *T. circumcincta*.

Chapter 4

Identification of sub-populations of *T. circumcincta* using *in vitro* bioassays

4.1 Introduction

Many different tests have been developed over the last 30 years to detect and quantify levels of anthelmintic resistance in parasitic nematodes of livestock. *In vivo* tests such as the Controlled Efficacy Test (CET) and Faecal Egg Count Reduction Test (FECRT), as well as *in vitro* tests such as the Egg Hatch Assay (EHA), Larval Development Assay (LDA), Larval Motility Assay, Larval Migration Inhibition Assay (LMIA), and the Larval Feeding Inhibition Assay (LFIA) (described in Chapter 1.3.3). There are numerous advantages of using *in vitro* techniques over those *in vivo*, for example; the reduced use of experimental host animals, and the cost savings associated with this reduction (outright purchase of sheep, husbandry expenses, anthelmintic purchase), assured exposure of the parasite to the anthelmintic in question, and removal of the inter-host variation in drug metabolism, which can complicate the determination of effective drug concentrations. Equally, the latter point can also be considered as a disadvantage of using *in vitro* techniques as the drug metabolism dynamics of the host are not necessarily reproduced or accounted for in these assays. Another disadvantage of *in vitro* methods is the requirement that larvae are exposed to far higher drug concentrations than would be encountered in the host animal.

The present study focuses on the effect of IVM treatment on *T. circumcincta*. Exposure to IVM causes hyperpolarisation of glutamate-gated chloride channels, and this acts on the parasite's neuromuscular system and ultimately, results in a flaccid paralysis particularly of the somatic and pharyngeal musculature (Blackhall *et al.*, 1998; Yates *et al.*, 2003), thereby inhibiting motility and feeding. The LMIA and LFIA are thus likely to be most suitable bioassays for measuring the paralytic effect of IVM. The EHA, for example, is only useful for measuring BZ efficacy as the other anthelmintic drugs tend to lack ovicidal activity. The LDA has been used successfully to detect BZ and LEV resistance in *T. circumcincta* but it is generally considered less useful

for detecting IVM resistance in this species as discriminating LD_{50} values overlapped when IVM-susceptible and IVM-resistant isolates were compared (Amarante *et al.*, 1997).

The LMIA has previously been optimised for cattle nematodes *Ostertagia ostertagi* and *Cooperia oncophora* (Demeler *et al.*, 2010), but remains to be fully optimised for use with sheep parasitic nematodes. Demeler *et al.* (2010) showed that re-suspension of IVM in 0.5 % DMSO was optimal for the LMIA with *O. ostertagi* and *C. oncophora* at IVM concentrations between 0.1 n*M* and 10 µ*M*. Bioassays requiring higher IVM concentrations would require a higher concentration of DMSO for resuspension; the toxicity of DMSO for *T. circumcincta* has yet to be reported.

The LFIA relies on measuring the disruption in feeding caused by macrocyclic lactones such as IVM. As well as inhibiting larval motility, IVM also causes flaccid paralysis of the pharynx (Gill *et al.*, 1995; Martin, 1996) thereby inhibiting worm feeding activity by reducing pharyngeal pumping. The concept used in the LFIA was first described by Geary *et al.* (1993) who used it to monitor the inhibition of ingestion, caused by IVM, of fluorescently-labelled *E. coli* by adult *H. contortus* worms. Later, it was applied to *H. contortus* and *T. circumcincta* first-stage (L_1) larvae (Jackson & Coop, 2000) and was further developed by Álvarez-Sánchez *et al.* (2005) to calculate the IC_{50} values (concentration causing inhibition of ingestion in 50 % of L_1 tested) for macrocyclic lactones and imidazothiazoles. In the present study, LFIAs utilising fluorescein isothiocyanate (FITC)-labelled *E. coli* were used to identify larvae with the ability to ingest bacteria using fluorescence microscopy of their intestinal content. Similar to the LMIA, the concentrations of IVM that caused 10 % (LFI_{10}) and 90 % (LFI_{90}) feeding inhibition, respectively, were determined. Between 10- and 100-fold lower anthelmintic concentrations are required to affect larval feeding than to inhibit larval motility (Gill *et al.*, 1995).

Studies into anthelmintic resistance often include comparisons of unrelated isolates of the same species, which are likely to possess different genetic backgrounds (Sangster *et al.*, 1999). This can make identifying genetic factors related to or responsible for an anthelmintic resistance phenotype difficult, due to natural variation or different selection pressures encountered

by parasites in different geographic locations, management systems or host breeds (Bisset, 2007).

FECRT studies conducted on the MTci5 isolate showed that IVM efficacy was 60 % (Bartley *et al.*, 2004; Bartley *et al.*, 2005), indicating that some susceptible individuals still reside in this population. The aim of the work described in this chapter was to obtain individual larvae that represent the most and least IVM resistant individuals, according to the LMIA and the LFIA, from within a single isolate of *T. circumcincta*. These larvae could then be subjected to molecular genetic analysis. Before the assay could be used to determine discriminating concentrations of IVM, it was first necessary to establish the optimal concentration of DMSO required because this was unknown in relation to *T. circumcincta*.

4.2 Materials & Methods

4.2.1 T. circumcincta Larvae

Eggs were recovered from faeces from donor animals that had been infected with larvae from the MTci2 and MTci5 isolates of *T. circumcincta*, larvae were coprocultured to L_3 as described in Chapter 2.3.2, for use in the LMIA. Exsheathment was induced by incubation in 1 % sodium hypochloride for 1-2 minutes at room temperature after which the larvae were washed with water, to remove the sodium hypochloride, and then resuspended in water. First-stage larvae of each of the MTci2 and MTci5 isolates were collected as described in Chapter 2.3.1 for use in the LFIA.

4.2.2 Larval Migration Inhibition Assay

Migration chambers were assembled in a similar fashion to those used previously by Demeler *et al*. (2010). A precision woven nylon mesh with a 25 µm mesh size (HPC Gears) was attached to one end of a 2 cm section of clear plexiglass tube. The 25 µm pore size was selected so that larvae would have to actively migrate through the pore rather than passing through under gravity. Six migration chambers were attached to a glass rod, aligning the chambers to the wells of a 24-well plate (Nunclon®, Thermo-Fisher Scientific) as shown in Panel *A* of Figure 4.1. The rods were positioned in a row of the 24-well plate and the incubated larvae, which had been incubated in various concentrations of DMSO prior to being transferred into the migration chambers, were added into the migration chamber which was immersed in identical concentrations of DMSO (Figure 4.1, Panel *B*) and incubated for a further 24 hours at 25 °C. The migrating larvae were found in the bottom of the well and larvae present on the outside of the chambers were washed into the well with water. The non-migrating larvae were washed from the inside of the migration chamber with water into empty wells in the row below (Figure 4.1, Panel *C*).

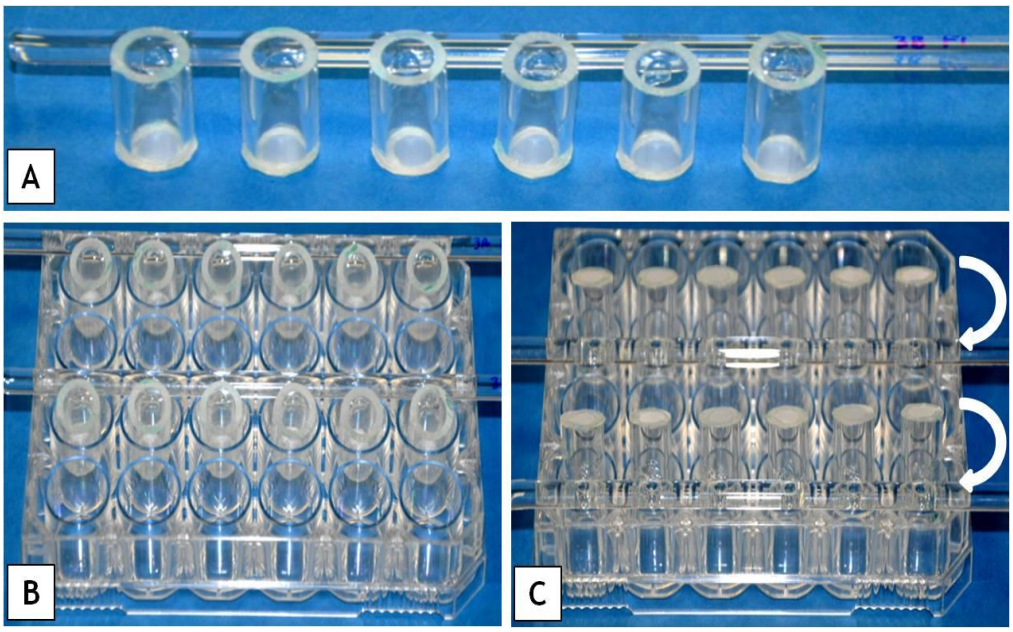


Figure 4.1 Migration Chamber Assembly

LMIA set up in a 24-well plate. Migration chambers are formed by fixing 25 µm mesh to one end of a 2 cm section of clear Plexiglas tube (Panel *A*). The chambers are aligned and secured to a glass rod and placed in rows A and C of a 24-well plate (Panel *B*). Migrating larvae collect in the well, whilst non-migrating larvae are washed from inside the chamber to rows B and D of the 24-well plate, indicated by the white arrows in Panel *C*.

The larvae were stained with helminthological iodine and counted under a stereomicroscope. The percentage larval migration inhibition (LMI) was calculated using Equation 1 and the mean LMI for the replicates was calculated:

$$LMI = 100 - \left(\left(\frac{N_{migrators}}{N_{total}}\right) \times 100\right) \qquad \textbf{EQUATION 1}$$

4.2.3 Effect of DMSO Concentration on LMIA

Higher concentrations of IVM require higher concentrations of DMSO for resuspension. Demeler et al. (2010) showed that resuspension of IVM in 0.5 % DMSO was optimal for the LMIA for use with the cattle nematodes *O. ostertagi* and *C. onchophora*, with relatively low concentrations of IVM. In the present study, the effects of varying the DMSO concentration was evaluated using both sheathed and exsheathed *T. circumcincta* L_3 from both the MTci2 and MTci5 isolates. Approximately 100 sheathed and 100 exsheathed larvae were added to

each well of a 24-well plate in duplicate and exposed to a range of DMSO concentrations (range: 0 - 5 %), and incubated for 24 hours at 25 °C. The larvae were transferred to migration chambers immersed in identical concentrations of DMSO and were allowed to migrate for a further 24 hours at 25 °C. The larvae were stained with helminthological iodine and counted under a stereomicroscope.

4.2.4 Effect of IVM Concentration on LMIA

The main aim of conducting the LMIA was to partition each isolate into sub-groups of larvae that represented the most and least IVM resistant individuals. The LMIA protocol optimised by Demeler *et al*. (2010) using cattle nematodes, *C. onchophora* and *O. ostertagi*, was closely followed in the present study, with alterations as described below. Stock solutions of ivermectin 1b (Sigma) were made with 100 % DMSO with final dilutions made with water, ensuring the final concentration of 2 % DMSO was maintained across all IVM concentrations. Aliquots of ~100 exsheathed larvae were incubated in IVM concentrations ranging from 0.057 to 514 µ*M* resuspended in DMSO, in the dark due to the photosensitivity of IVM, for 24 hours at 25 °C. The larvae were transferred to migration chambers immersed in the same IVM concentration, then allowed to migrate at 25 °C for a further 24 hours in the dark. The negative control throughout the LMIA was 2 % DMSO (no IVM) and the positive control was 100 % DMSO. Larvae that retained the ability to migrate through the mesh were washed from the outside of the migration chamber and individuals that were unable to migrate were washed from the inside of the migration chamber into fresh wells. Staining the larvae with helminthological iodine allowed quantification of the migrating and non-migrating larvae under a stereomicroscope.

4.2.5 Larval Feeding Inhibition Assay

The method used for the LFIA was a modification of the procedure developed by Geary *et al*. (1993). Viable L_1 were collected as described in Chapter 2.3.1 and aliquots of 150 L_1 were added to 0.5 ml Eppendorfs containing a range of IVM concentrations. Ivermectin 1b (Sigma) was resuspended in 2 % DMSO and each concentration (range: 0.1 – 70 µ*M*) was repeated in triplicate for both the MTci2 and MTci5 isolates. The larvae were incubated for 2 hours at 25 °C in the dark, with the addition of 7 µl fluorescein isothiocyanate (FITC)-labelled *E. coli* before overnight incubation, similarly at 25 °C in the dark. Labelling the *E. coli* with FITC was achieved by mixing 100 µl of concentrated (2250 µg/ml) lyophilised *E. coli* (Sigma) with 1 ml of bicarbonate buffer (2.66 g/l $NaHCO_3$, 1.96 g/l Na_2CO_3 and 1.5 g/l NaCl, at pH 7.4) containing 1 mg FITC (Sigma), and incubating at 20 °C for two hours. After the incubation period, the labelled *E. coli* were washed three times in phosphate buffered saline (PBS) and resuspended in 1 ml of PBS. Using an inverted fluorescence microscope (Olympus CK40), ingested FITC-labelled *E. coli*, present in the intestine of the larva, were visualised (Figure 4.2), taking care to ensure that fluorescence associated with the labelled *E. coli* was distinguished from the autofluorescence of the larvae (Geary *et al*., 1993).

The percentage of larval feeding inhibition (LFI) was calculated using Equation 2 and the mean LFI for the replicates was calculated:

$$LFI = 100 - \left(\left(\frac{N_{feeders}}{N_{total}}\right) \times 100\right) \qquad \textbf{EQUATION 2}$$

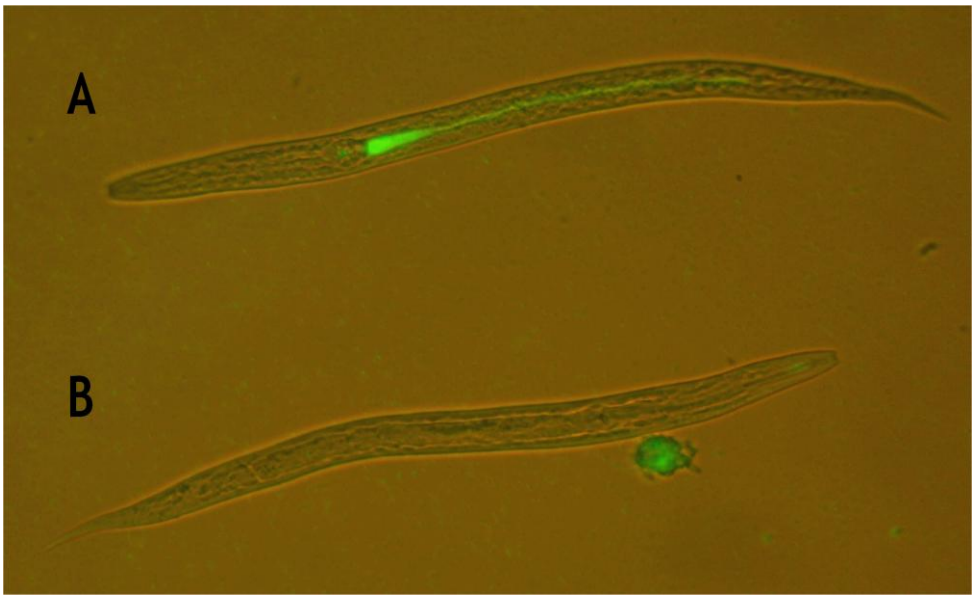


Figure 4.2 First stage larvae of *T. circumcincta* showing inhibited feeding

Fluorescence microscopy showing an individual larva that retains the ability to ingest FITC-labelled *E. coli* (**A**), alongside an individual that did not ingest FITC-labelled *E. coli* (**B**). The fluorescently-labelled *E. coli* are visible in the intestine of larva (**A**) but absent in the intestine of larva (**B**).

The LFIA was repeated on a biological replicate of viable L_1 collected on a separate day and the IVM concentrations which inhibited larval feeding by 10 % and 90 % (LFI_{10} and LFI_{90}, respectively) were identified. In order to select enough larvae from each sub-population for subsequent molecular analyses, the LFIA was scaled up by exposing ~600 L_1, from each of the MTci2 and MTci5 isolates, to the LFI_{10} and LFI_{90} doses of IVM. Larvae from each of the MTci2 and MTci5 isolates that did not feed at the LFI_{10} IVM concentration were identified, and individually transferred to separate wells in a 96-well plate (Axygen) each containing 10 µl of a 3 % solution of recombinant PCR grade proteinase K (Roche) in PCRDirect lysis reagent (Tail) (Viagen Biotech). This procedure was repeated for the larvae that had retained the ability to feed at the LFI_{90} IVM concentration.

4.2.6 Data Analysis

The percentage inhibition of larval migration (LMI) was determined for each concentration of DMSO using Equation 1 above. The LMI at the selected DMSO concentration of 2 %, and the percentage inhibition of feeding (LFI), were determined for each concentration of IVM using the equations 1 and 2 above. Probit analysis was then used to identify the IVM concentrations required for 10 %, 50 %, 90 %, 95 % and 99 % inhibition of either larval migration or larval feeding. Binary logistic regression analysis was conducted using inhibition of migration or feeding as the outcome variable (inhibited, not inhibited) and using strain (MTci2, MTci5), presence of sheath (sheathed, exsheathed) and concentration of DMSO or IVM as the explanatory variables. Probit and logistic regression analyses were conducted (using Minitab15 Statistical Software).

4.3 Results

4.3.1 Effect of DMSO Concentration on Larval Migration

Increasing DMSO concentration increased the inhibition of larval migration (Figure 4.3). Logistic regression analysis (Table 4.1) showed that isolate, presence of sheath and the concentration of DMSO were all highly significant factors (p <0.001). The MTci5 isolate had a positive coefficient (p <0.001) and the greatest odds ratio of the variables tested, indicating increased inhibition of migration in the MTci5 isolate compared with the MTci2 isolate. The absence of the larval sheath had a similar effect on the larval migration rates to that caused by the increasing the DMSO concentration, with both factors having similar odds-ratios.

Two percent DMSO was selected as the optimal concentration for resuspending IVM as the larval migration inhibition was similarly low in both the MTci2 and MTci5 isolates at 3.47 % (range: 0.64 – 6.01 %). For consistency with previous work and because there were clear differences in the effect of the sheath, exsheathed larvae were chosen as they were required for the molecular tests that would be undertaken subsequently.

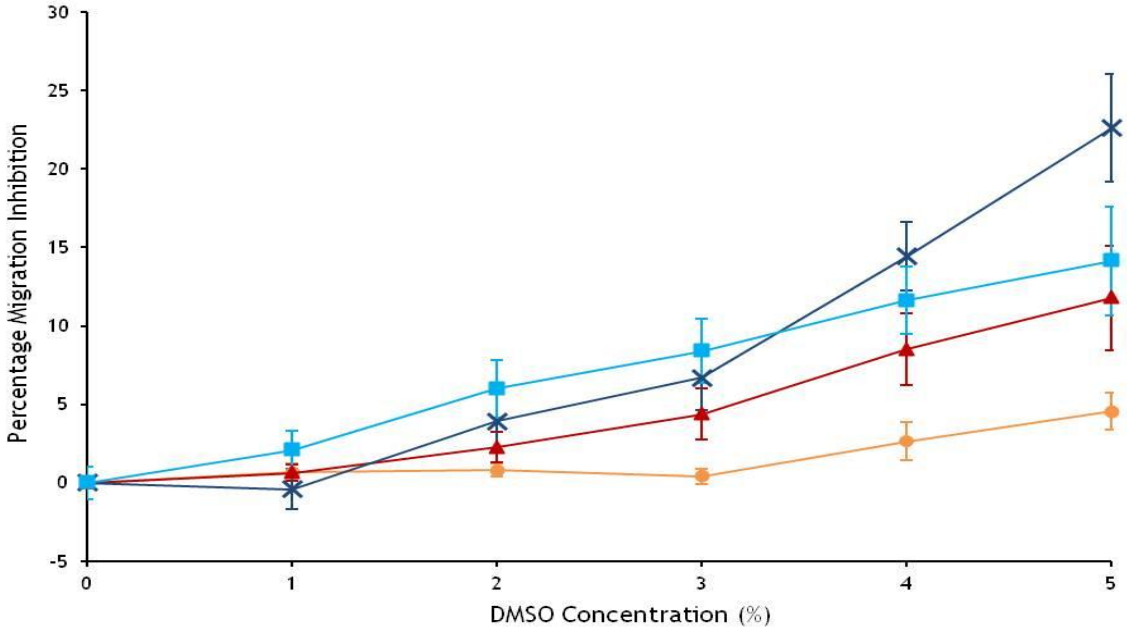


Figure 4.3 Effect of DMSO concentration on inhibition of L_3 migration

Larvae were exposed to a range of DMSO concentrations and their ability to migrate through a 25 µm mesh was determined. The percentage migration inhibition was calculated for each isolate of *T. circumcincta*: MTci2 sheathed (▲) and exsheathed (●) and MTci5 sheathed (×) and exsheathed (■).

Table 4.1 Logistic regression table for the effect of DMSO concentration on inhibition of L_3 migration

The variables tested included whether the larval sheath was present or not, increasing concentration of DMSO and the isolate of *T. circumcincta* (MTci2 or MTci5).

Predictor	Coefficient	SE Coefficient	Z-value	P-value	Odds Ratio	95 % C.I.	
						Lower	Upper
Constant	-4.5678	0.0682	-66.95	<0.001			
MTci5	1.1340	0.0476	23.96	<0.001	3.13	2.85	3.43
Exsheathed	0.4092	0.0432	9.48	<0.001	1.51	1.38	1.64
[DMSO]	0.3975	0.0134	29.61	<0.001	1.49	1.45	1.53

4.3.2 Effect of IVM on Larval Migration

In order to identify IVM concentrations that would partition the isolates into subgroups that were 'most resistant' and 'least resistant', exsheathed infective larvae (L_3) were exposed to increasing concentrations of IVM (range: 0.057 – 514 μM) resuspended in 2 % DMSO, and their ability to migrate through 25 μm mesh was measured. The LMI was calculated and plotted for each isolate at each individual IVM concentration (Figure 4.4).

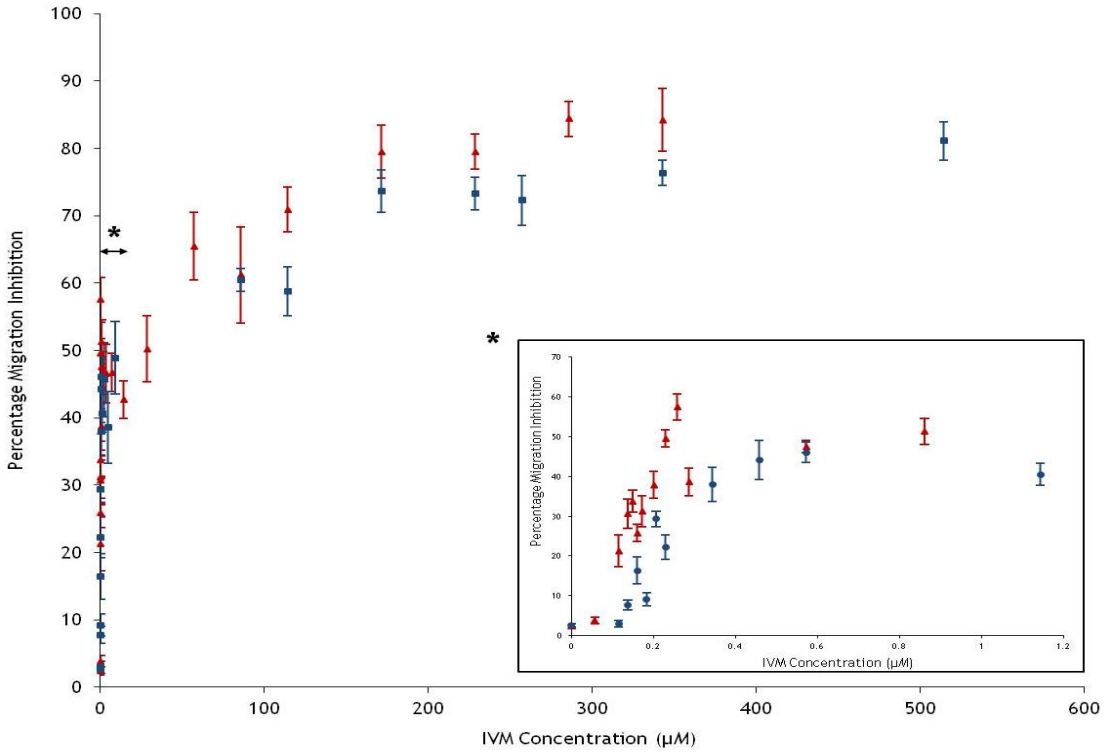


Figure 4.4 Third-stage Larval Migration Inhibition

Each isolate, MTci2 (▲) and MTci5 (■), was exposed to increasing concentrations of IVM and the ability of L_3 to migrate through 25 μm mesh was observed and the percentage migration inhibition was calculated. Inset panel shows an enlarged area of the graph marked (*).

Logistic regression analysis (Table 4.2) showed highly significant effects of increasing the concentration of IVM and isolate (p <0.001). The MTci5 isolate was protected against the inhibitory effect of IVM (Odds ratio = 0.83, 95 % C.I. = 0.79 – 0.86).

A Probit analysis was used in an attempt to estimate LMI_{10} and LMI_{90} for each of the isolates (Appendix 2). High levels of inhibition at relatively low concentrations of IVM meant that the Probit model was a poor fit for the data and it was not possible to estimate a value for LMI_{10}. The concentration of IVM responsible for LMI_{90} was estimated to be 287 µ*M* and 378 µ*M* for the MTci2 and MTci5 isolates, respectively. Using 2 % DMSO to resuspend IVM restricted the maximal concentration of IVM to 257.1 µ*M* as the solution reached saturation (Figure 4.5). The percentage of migration inhibition before the IVM solution became saturated was 81.8 % and 78.5 % for the MTci2 and MTci5 isolates, respectively. Hence, the predicted LMI_{90} was unattainable using the LMIA.

Table 4.2 Logistic Regression Table for LMIA

The potential explanatory variables tested were the concentration of IVM and the MTci2 and MTci5 isolates of *T. circumcincta*.

Predictor	Coefficient	SE Coefficient	Z-value	P-value	Odds Ratio	95 % C.I.	
						Lower	Upper
Constant	-0.568729	0.0131278	-43.32	<0.001			
MTci5	-0.188193	0.0216825	-8.68	<0.001	0.83	0.79	0.86
Log[IVM]	0.0074410	0.0001061	70.11	<0.001	1.01	1.01	1.01

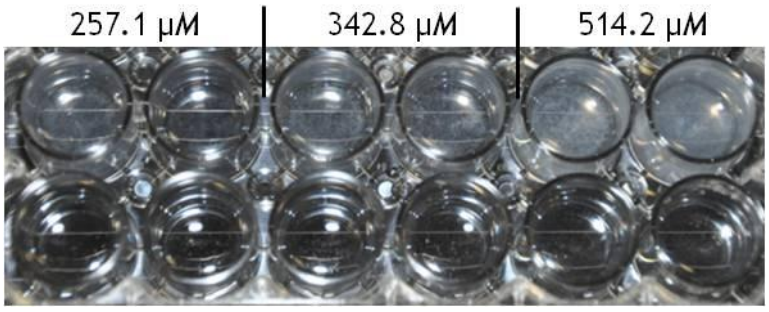


Figure 4.5 Saturated IVM Solution

Ivermectin 1b (IVM) was resuspended in 2 % DMSO and after incubation for 24 hours at 25 °C, the excess IVM solute recrystallised. The presence of IVM precipitate indicated that the solution was saturated and the exact molarity of IVM dissolved in solution could not be determined.

4.3.3 Effect of IVM Concentration on Larval Feeding

The percentage of larval feeding inhibition (LFI) at each concentration of IVM is shown in Figure 4.6 for a range of IVM concentrations. Logistic regression analysis (Table 4.3) showed that increasing concentration of IVM caused a highly significant (p <0.001) increase in inhibition of feeding, and confirmed that the MTci5 was significantly (p <0.001) less susceptible to the effects of IVM compared to the MTci2 isolate (Odds ratio = 0.65, 95% C.I. = 0.61 - 0.69).

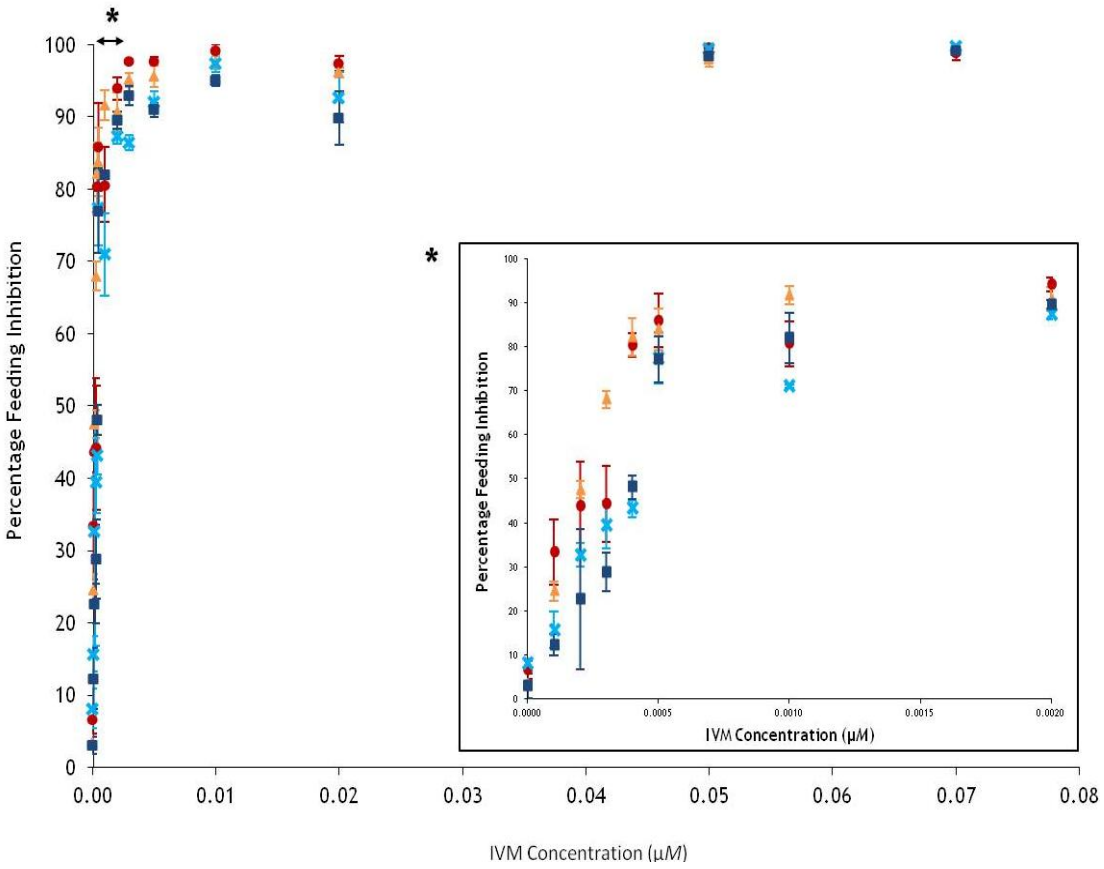


Figure 4.6 First-stage Larval Feeding Inhibition

Each isolate, MTci2 (biological replicates 1 (▲) and 2 (●)) and MTci5 (biological replicates 1 (×) and 2 (■)), was exposed to a range of IVM concentrations and the percentage larval feeding inhibition was calculated.

Table 4.3 Logistic Regression Table for LFIA

The variables tested in the logistic regression analysis were the concentration of IVM and the MTci2 and MTci5 isolates of *T. circumcincta*.

Predictor	Coeff.	SE Coeff.	Z-value	P-value	Odds Ratio	95 % C.I.	
						Lower	Upper
Constant	0.266	0.0232	11.47	<0.001			
MTci5	-0.432	0.0308	-13.99	<0.001	0.65	0.69	0.61
Log[IVM]	222.7	6.0305	36.93	<0.001	$5.19_{x10}{}^{96}$	$3.82_{x10}{}^{91}$	$7.06_{x10}{}^{101}$

A Probit analysis was conducted in an attempt to determine the LFI_{10} and LFI_{90} values (see Appendix 3). The Probit analysis shows a low Chi-square value of 0.027, showing the model was a good fit for the data overall despite very high levels of non-feeding at IVM concentrations close to zero. The estimated LFI_{90} was 0.013 m*M* and 0.016 m*M* for the MTci2 and MTci5 isolates, respectively, therefore the IVM concentration that caused 90 % feeding inhibition was 10 µ*M*. The model was a poor fit when focussing on the concentration of IVM responsible for LFI_{10}, with negative IVM concentrations of IVM predicted for both isolates. Therefore, it was decided to simply select concentrations of IVM on the basis of visual appraisal from the line graph (Figure 4.6). The concentration of IVM selected to collect the 10 % of non-feeding L_1 from each isolate was 0.1 µ*M*. Larvae from each isolate that represent the 'least' resistant individuals were selected and recorded as $MTci2_{NF(0.1\mu M)}$ and $MTci5_{NF(0.1\mu M)}$. Larvae representing the 'most resistant' individuals were designated $MTci2_{F(10\mu M)}$ and $MTci5_{F(10\mu M)}$.

4.4 Discussion

The aim of the work described in this Chapter was to use two *in vitro* bioassays as a tool to select IVM-susceptible and IVM-resistant larvae from within two genetically distinct isolates with differing expected levels of IVM resistance. However, this aim could only partially be met due to limitations in the bioassays and it is clear that considerable work is still required to optimise both of these for use with IVM and *T. circumcincta*. Nonetheless, it was possible to select sub-populations of larvae from each isolate that could reasonably be considered to represent highly resistant and less resistant phenotypes from within each of the isolates.

Although neither assay has been optimised, they both show some promise. Most importantly, there was a strong effect of increasing IVM concentration on inhibition of migration and feeding. However, these effects were seen at very low concentrations of IVM and over most of the range of concentrations tested there was little if any significant increase in inhibition of migration or feeding. Both bioassays also showed a clear effect of isolate in the expected direction, in that MTci5 larvae were significantly less likely to be inhibited by IVM in the LMIA and the LFIA. In the case of the LMIA, the protective effect was relatively small, likelihood of inhibition of migration being about 90 % of that in MTci2, whereas in the LFIA, the likelihood of being inhibited from feeding in MTci5 larvae was about half of that in MTci2 larvae. Consequently, the assays show considerable promise for further development in the future.

One of the biggest limitations of the bioassays was their relatively poor performance at the lower and upper end of the test concentrations. This resulted in a poor fit of the data to the Probit analysis model and an inability to determine meaningful values for LMI_{10} and LFI_{10}. There were also problems at the upper end of the concentration scale, in that 100 % inhibition was very rarely achieved, leading to LFI_{90} and LMI_{90} estimations that exceeded achievable concentrations. Rarely achieving 100 % migration inhibition may be a result of dead larvae falling through the pores of the sieve, or being pulled through by attachment to active larvae, rather than actively migrating through them. Although it was decided that the LMIA was not suitable for the purposes of the present study and those following it, it was possible to collect some larvae of

relatively higher and lower resistance to IVM as determined by the LFIA from each of the isolates MTci2 and MTci5. These larvae were used in the experiments described in subsequent chapters.

The LMIA with varying concentrations of DMSO showed that the absence of a sheath was protective against the effects of DMSO on migration. This was unexpected because larvae retaining the sheath would be expected to receive a degree of protection in the form of a physical barrier against its immediate environment. It is reasonable to assume the exsheathed individuals would be vulnerable to drug molecules as they lack this protective layer. Exsheathed larvae appeared more active following the stimulation to shed their sheaths and this may result in increased larval migration through the Baermann apparatus. Another possible explanation could be that exsheathed larvae tend to fall through the mesh more easily than those with an intact sheath. Any movement of the liquid in the migration chambers (caused by vibrations or water currents due to evaporation for example) may cause the larvae to lift briefly off the mesh surface and vertically fall through it.

This study used a modification of the method developed by Demeler *et al*. (2010), by using 2 % DMSO to resuspend IVM instead of 0.5 %. This facilitated the resuspension of higher concentrations of IVM, the concentration range 0.057 to 514 µ*M* was used in the present study, whereas in the previous study the IVM concentration range was limited to 0.001 - 10 µ*M* (Demeler *et al*., 2010), most likely due to the use of 0.5 % DMSO. The maximal concentration of IVM in the present study was approximately 200 µ*M*, at which point the recrystallisation of IVM was observed after 24 hours incubation without agitation. At this concentration, the larval migration inhibition was 81.8 % for the MTci2 isolate and 78.5 % for the MTci5 isolate. To attain 90 % larval migration inhibition the required IVM concentrations were estimated by Probit analysis to be 287 m*M* and 378 m*M* for the MTci2 and MTci5 isolates, respectively, which was higher than the maximal concentration of IVM that could be resuspended in 2 % DMSO. As the Probit analysis model was not a good fit, to determine the IVM concentration which causes LMI_{90}, would require further optimisation of the LMIA for *T. circumcincta*. Although higher concentrations of DMSO could possibly facilitate the resuspension of higher IVM concentrations, the toxicity of DMSO would have to be considered when interpreting the rate of inhibition as a result of IVM.

Therefore, an alternative bioassay was sought to collect sub-populations of the *T. circumcincta* isolates.

The larval feeding inhibition assay provided an alternative option to the LMIA bioassay for assorting the isolate into two sub-populations based on their ability to feed in different concentrations of IVM. The concentrations of IVM used (0.1 to 70 µ*M*) were lower than those of the LMIA, avoiding issues with resuspension in 2 % DMSO. The requirement for lower concentrations of IVM is supported by a finding reported by Gill *et al*. (1995), which showed that between 10- and 100-fold lower anthelmintic concentrations are required to affect larval feeding, than to inhibit larval motility. In retrospect, a lower concentration of DMSO could have been used as a similar study conducted by Geary (1993) into pharyngeal paralysis by IVM in *H. contortus* used 0.1 % DMSO to resuspend IVM. The effects of DMSO were avoided completely in the Álvarez-Sánchez *et al*. (2005) study which exposed *H. contortus*, *T. circumcincta* and *Trichostrongylus colubriformis* to serial dilutions of IVM in the form of Oramec oral solution (Merial). Focusing on much lower concentrations of IVM than in the present study could minimise potential confounding of results due to inhibitory effects of DMSO. In the present study, no mortality was observed in the L_1 that were exposed to IVM, the IVM instead paralysed the pharyngeal muscles, inhibiting ingestion of fluorescently-labelled *E. coli*. Thus, it was possible to collect two sub-populations based on their ability to ingest fluorescently labelled *E. coli*. One sub-population was collected from the 10 % of L_1 that displayed an inhibited feeding phenotype at 0.1 µ*M* IVM, these represented the 'least resistant' individuals from each isolate. A second sub-population was collected from the 10 % of L_1 that retained the ability to feed at 10 µ*M* IVM, representing the 'most resistant' individuals from each isolate. A proportion of larvae did not feed in the absence of IVM, 5.1 % and in MTci2 and 5.6 % in MTci5, possibly due to the toxicity of DMSO. Therefore, the susceptibility of individuals that did not feed at low IVM concentrations cannot be assumed, whereas retaining the ability to feed at high concentrations of IVM, which does indicate resistance. Had time allowed, the susceptibility to IVM could have been tested by passaging non-feeding LFIA survivors through a sheep to increase numbers and subsequently undertaking a controlled efficacy trial on the $MTci2_{(NF0.1\mu M)}$ and $MTci5_{(NF0.1\mu M)}$ sub-populations. As these sub-populations were derived from the same initial

population they were considered to have similar genetic backgrounds apart from a limited number of genes associated with IVM resistance, allowing meaningful genetic comparisons between these two sub-populations.

Of the two bioassays considered appropriate for studying the effects of IVM and collecting sub-populations of larvae, the LFIA was shown to be the more suitable. Due to difficulties encountered attempting to resuspend IVM in DMSO, the LMIA was discounted as a viable option, at least in this species, as concentrations of IVM did not reach concentrations high enough to inhibit more than ~85 % of larval migration. Although some degree of optimisation of the LMIA was carried out, the LMIA remains to be fully optimised for use with parasitic nematodes that infect sheep. Similarly, although the LFIA has been used in the present study as a tool for selecting individuals with different IVM-resistance phenotypes, plans have been made to scale-up the collection of different sub-populations using a specially adapted large particle fluorescence-activated cell sorter or FACS (F. Kenyon and D. McBean, Moredun Research Institute, Pers. Comm.). Larvae displaying different feeding phenotypes could be automatically counted and partitioned into separate sub-populations, reducing the time and effort taken to characterise a given isolate with respect to IVM sensitivity.

In conclusion, the LFIA was not fully optimised but instead used as a tool for selecting the ‘less resistant’ and ‘more resistant’ individuals from within the MTci2 and MTci5 isolates, for use in subsequent genetic comparisons.

Chapter 5

Analysis of *Tci-pgp-9-IBDA* Allelic Variants in *T. circumcincta*

5.1 Introduction

P-glycoproteins have been implicated as a mechanism for ML-resistance (Xu *et al.*, 1998; Molento & Prichard, 1999), although they are not the specific target sites for IVM. Instead the Pgp xenobiotic efflux pump affects the drug distribution of IVM, restricting access to its acknowledged target sites such as the glutamate-gated chloride channels and γ-aminobutyric acid chloride channels (Geary *et al.*, 1993; Ros-Moreno *et al.*, 1999). Increasing the number of Pgp molecules present at the cell surface will result in a greater capacity for drug efflux, thereby avoiding the toxic effects of a given drug. Gene amplification, gene expression and gene splicing are all potential mechanisms of increasing the number of Pgp proteins present in the cell membrane. As anthelmintic treatment provides a selective pressure, individuals that are susceptible to the drug action are removed from the population, along with their 'susceptible genotype', whereas survivors complete their life-cycle, passing on the 'resistant genotype' to their progeny, thus increasing the frequency of allele(s) that offer a survival advantage (Blackhall *et al.*, 1998). A reduction in genetic variation in a population can arise from non-random mating as a consequence of inbreeding or continual drug-selection (Bourguinat *et al.*, 2011) leading to an accumulation of the resistant genotype to a level which causes the loss of drug efficacy in the target population (Prichard, 1990).

A recent study examined the presence of *Tci-pgp-9* allelic variants in an inbred anthelmintic susceptible strain and a near-isogenic multiple anthelmintic resistant strain of *T. circumcincta* isolated in New Zealand (Bisset, 2007). The region targeted for genotyping using allele-specific primers was the N-terminal internucleotide-binding domain of Pgp-9, *Tci-pgp-9-IBDA*, which corresponds to the IBD77 region reported in the *H. contortus* Pgp-1 gene (*Hco-pgp-1*) by Sangster *et al.* (1999) [Accession Number: AF055175]. The *Tci-pgp-9-IBDA* target gDNA region was ~700 bp, which included exons that represent a 165 bp region between bases 1361-1526 in the *Tci-pgp-9* cDNA sequence (Appendix 1).

Polymorphism in the intronic sequence of *Tci-pgp-9-IBDA* allowed the identification and characterisation of nine allelic variants in the New Zealand strains of *T. circumcincta* (Bisset, 2007). P-glycoproteins appear to have arisen by a gene duplication event, fusing two related half Pgp molecules each consisting of a NBD and one transmembrane domain (van Veen *et al*., 2001). Possession of multiple allelic variants supports the theory that the *Tci-pgp-9* gene has been duplicated, and its maintenance suggests that this duplication is not detrimental to the survival of the individual.

There is scant information in the literature on the levels of variability that constitute an allele in parasitic nematodes. Each allele presents a unique combination of polymorphisms, and as such, a single nucleotide change would, technically, represent a new allele. Sangster *et al*. (1999) amplified Pgp-IBD fragments from *H. contortus*, and grouped alleles that shared 82-99 % identity at the nucleotide level. In a recent study by Bisset (2007), allelic variants were classified by tolerating "one-off mutations" and basing groupings on blocks of nucleotide substitutions, insertions or deletions within introns, which were repeatedly associated with particular allelic variants. A strong association between the multiple-anthelmintic resistant phenotype and a genotype with an increased occurrence of several allelic variants (NZ variants 2, 3, 6, 9 & 10) was observed, suggesting that such variants had been subjected to the strong positive selection pressures of anthelmintic screening (Bisset, 2007).

In this Chapter, individual larvae selected from the MTci2, MTci5, MTci5PT isolates and the two sub-populations (least and most resistant) from each of the MTci2 and MTci5 isolates, were used to identify which *Tci-pgp-9-IBDA* allelic variants were carried by UK strains of *T. circumcincta*. The process of identifying allelic variants developed by Bisset (2007) was closely followed.

5.2 Materials & Methods

5.2.1 Generation of Generic Tci-pgp-9-IBDA Fragments

Crude larval lysates were prepared from 84 individual L_3 randomly selected from the MTci2, MTci5, and MTci5PT isolates, using the methodology described in Section 2.5. These were subsequently used as the gDNA template for PCR amplification. Crude larval lysates of the sub-populations of L_1, 47 of each sub-population collected in Chapter 4, were also used as template gDNA for allelic variant analysis. The first step was to PCR amplify a fragment of the *Tci-pgp-9-IBDA* using the primer pair IBD77GF3B [5' GGNGTNGARATHGAYAARATHAAYATHGARTT 3'] (sense) and IBD77GR2 [5' TGNCCNCCAGACATYTGNGTNCC 3'] (antisense). Degenerate primers were used to increase the likelihood of amplifying potential sequence variants present in the isolates. A Platinum *Taq* mastermix was produced where each 10 µl reaction volume contained: 0.5 units Platinum *Taq* Polymerase, 1 µl 10X *Taq* buffer, 2.5 m*M* $MgCl_2$, dNTPs (4 µ*M* each) (all Invitrogen), 1 µ*M* each primer and 1 µl template (crude lysate). End-point PCR was performed using the following program: 94 °C for 8 minutes to denature template and to activate the *Taq* enzyme, followed by 35 three-step (denaturing, annealing and extension steps) cycles of 94 °C for 10 s, 50 °C for 20 s and 68 °C for 40 s, followed by a final elongation step of 68 °C for 7 minutes.

Next, a nested-PCR strategy was followed as it generally gave stronger, cleaner PCR products from the minimal concentrations of gDNA present in the crude L_3 lysates. The PCR product, generated using the IBD77GF3B/IBD77GR2 primers in the previous step, was diluted 1:50 with Ultrapure™ DNAse/RNAse-Free Distilled Water (Gibco®) and used as the cDNA template for nested-PCR with the primer pair IBD77GF4 [5' GAGTAGTKTCACARGARCCNATG 3'] (sense) and IBD77GR3 [5' TCNCCNACRTTNGTRTADATNCC 3'] (antisense) in a Platinum *Taq* mastermix similar to that used in the initial PCR step. The thermocycling program was initiated with a denaturing step of 94 °C for 8 minutes, followed by 35 three-step cycles of 94 °C for 10 s, 54 °C for 20 s and 68 °C for 40 s, and a final extension step of 68 °C for 7 minutes. To confirm successful PCR amplification of the *Tci-pgp-9-IBDA* domain and to assess evidence of sequence variants present in each *T. circumcincta* larva, the products of the nested-PCR

were run through the QIAxcel Advance System (QIAgen) using the QIAxcel Screening Kit (QIAgen), and analysed using the QIAxcel ScreenGel 1.0.2 software (QIAgen).

The QIAxcel Advanced System provides an automated alternative to using traditional agarose gel electrophoresis. Using capillary electrophoresis enables high resolution and sensitive separation of DNA and the QIAxcel ScreenGel 1.0.2 software provides a virtual gel image of up to 96 samples per run. The migration speeds across the independently operated capillaries were normalised using the QX Alignment Marker containing 15 bp and 1 kb DNA markers. The size of the PCR products was determined by their relative migration distance when compared to known fragments in the QX DNA Size Marker 50 – 800 bp v2.0 (QIAgen).

Generic products of various lengths were amplified by the degenerate primer pair IBD77GF4/IBD77GR3 in the nested-PCR, as was observed in a previous study (Bisset, 2007). The generic *Tci-pgp-9-IBDA* PCR products from 37 individuals (10 from MTci2, 11 from MTci5, and 16 from MTci5PT) were selected to account for each of the different sized products and were ligated into pCR® 4-TOPO® Vector (Invitrogen), transfected into One Shot® TOP10 Electrocomp™ Cells (Invitrogen) and grown overnight on Kanamycin (50 mg/ml) supplemented LB-agar plates as outlined in Section 2.7.2. The plasmids were purified and the cDNA inserts were sequenced at Massey Genome Service at Massey University, Palmerston North (New Zealand). The sequencing data generated were analysed with Lasergene® 10 (DNASTAR Inc.) bioinformatics software and aligned with gDNA sequence of *Tci-pgp-9-IBDA* allelic variants identified in NZ near-isogenic strains of *T. circumcincta* (Bisset, 2007).

Each UK allelic variant was sequenced in both the sense and antisense direction, with the exception of UKv2. Using different denaturing solvents during the *de novo* sequencing, such as DMSO or BigDye® Terminator v3.1 (Applied Biosystems), did not prove fruitful and each time the generated sequence was truncated at the same point when sequenced in both the sense and antisense directions. The complementarities shared in different parts of the gene were visualised using mfold (Zuker, 2003), a predictive DNA structure program (available at http://www.bioinfo.rpi.edu/applications/mfold).

5.2.2 Allele-specific PCR

The DNA sequencing data generated from the initial *Tci-pgp-9-IBDA* PCR products were aligned with 9 allelic variants (numbered 1-3, 5-10) that had been previously identified in NZ isolates of the *T. circumcincta* (Bisset, 2007). Seven allelic variants from the UK isolates shared high levels of identity (93 – 99 %) with the *Tci-pgp-9-IBDA* alleles identified in the NZ isolates, therefore, primers designed by Bisset (2007) were used for genotyping (Table 5.1). Four alleles found in the UK isolates did not align with the previously identified alleles and these were numbered 11-14. Allele-specific primers were designed in unique, polymorphic regions within the introns of *Tci-pgp-9-IBDA* and were used to differentiate between variants. The melting temperatures of the primers were calculated and matched using OligoCalc software (http://www.basic.northwestern.edu/biotools/oligocalc.html). The OligoCalc software allowed additional checks for potential hairpin formation within the primer as well as 3' complementarity and potential self-annealing sites. The allele-specific antisense primers, target sites shown in Figure 5.2, were paired with a degenerate (non allele-specific) IBD77GF5 sense primer [5' GAGTAGTKTCACARGARCCNATGCT 3'] located near the 5' end of the *Tci-pgp-9-IBDA* fragment initially amplified with primer pair IBD77GF3B/IBD77GR2. The template cDNA used for the allele-specific PCR reactions was the 1:50 dilutions of the PCR products that were amplified using the IBD77GF3B/IBD77GR2 primers. A Platinum *Taq* mastermix was used where each 10 µl reaction volume contained: 0.5 units Platinum *Taq* Polymerase, 1 µl 10X *Taq* buffer, 1.5 m*M* $MgCl_2$, dNTPs (4 µ*M* each) (all Invitrogen), 1 µ*M* each primer and 1 µl cDNA template. A touchdown PCR strategy was adopted where the annealing temperature in the first cycle is set above the expected annealing temperature and reduced by 0.5 °C each subsequent cycle to 'touchdown' to the expected annealing temperature (Don *et al.*, 1991). This approach increases the specificity of primers by reducing non-specific binding at higher temperatures, and increases the efficiency towards the end of the cycling by reducing the annealing temperature. The touchdown PCR thermocycling program followed here was: 94 °C for 8 minutes, then 12 three-step cycles with decreasing annealing temperature by 0.5 °C per cycle, starting with a denaturing step at 94 °C for 10 s, an annealing step at 62 °C for 20 s and an extension step 68 °C for

30 s, each cycle; this was followed by 24 three-step cycles of 94 °C for 10 s, 56 °C for 20 s and 68 °C for 30 s with a final extension step of 68 °C for 5 minutes. The PCR products were analysed using the QIAxcel Advanced System and the QIAxcel DNA Screening Kit (both QIAgen).

Table 5.1 Allele-Specific Primers

Allele-specific antisense PCR primers were combined with the sense primer, IBD77GF5 [5' GAGTAGTKTCACARGARCCNATGCT 3'], and used in *Tci-pgp-9-IBDA* genotyping reactions. Primer names starting with "IBD77RAS" were originally designed by Bisset (2007) and those starting with "FTcP" were designed in the present study, based on sequencing results from UK isolates.

Allele Variant	Primer Name	Primer Sequence (5' – 3')	Product Size (bp)
1	IBD77RAS7	GTAGATTCCCTGAAATAAGCTCAC	449
2	IBD77RAS11	CTTTTCCAGCGACGACCCGC	286
3	IBD77RAS12	GCGCCATTCCACCACTTTCTTAG	291
5	IBD77RAS4	CGTACTGTGGCGATCTCG	458
6	IBD77RAS5B	AGCTGAAAGGGCAGAGTCAGAG	610
7	FTcP 45	ACCCGTGTGATAACATTGGAGAG	361
8	IBD77RAS8A	TCCTGCCCTCTCCCTCTCAAC	323
9	IBD77RAS9A	GTGTGATAACGTCGGGGAAGATC	356
10	IBD77RAS10C	GAGTAGTCCTACAACACCGCT	341
11	FTcP 46	CACCTTTAAGATCGACAATACGAGC	246
12	FTcP 47	CCGTGTAAGGGTGGGGATGG	289
13	FTcP 48	AGAGAAACCAGCAGTAGTGAATGCAACG	422
14	FTcP 49	AAAATTATAGGCGTTCGCTTTGCGC	191
14	FTcP 50	CTTTCCAACGCTGCAACACTGACGA	305

5.2.3 Data Analysis

Phylogenetic analyses were conducted using the TreeDyn program which is part of the Phylogeny.fr platform (Dereeper *et al.*, 2008) available online at http://www.phylogeny.fr/ (Accessed 25th November 2013). A phylogenetic tree was constructed based on bootstrap (x100) alignment data to show the relationships between the allelic variants of *Tci-pgp-9-IBDA*, found in the UK and NZ strains of *T. circumcincta*. Allelic variants identified from the UK strains were numbered based on their relationships with allele variants previously identified in NZ strains (Bisset, 2007). Those that differed by ≥10 % were recorded as "new" variants and numbered accordingly.

Presence or absence of each allelic variant in DNA from each of the individual larvae tested was recorded as a binary outcome (0,1) in an Excel spreadsheet. Genetic analyses (haplotype generation, allele frequency estimations, genetic distance and molecular analysis of variance) were conducted using GenAlEx Version 6.501 (Peakhall & Smouse, 2012). Because the maximum number of allelic variants amplified by the primers exceeded two and ranged up to 10 in some individuals, it was assumed that there was gene amplification with 5 copies or more in some larvae. Hence, it was not possible to determine whether an individual that was observed to be homozygous had one or more copies of the gene. As a result, although it was possible to generate a form of haplotype without reference to copy number, the data were not suitable for standard genetic analyses for diploid, co-dominant, single locus analysis. Instead, to gain some insights into the relatedness of populations and sources of variance, the data were treated as multilocus binary data, in which each allelic variant was considered as a separate locus with two states (0, 1). Analysis of variance (ANOVA) for the number of allelic variants per individual larva, by population was conducted using Minitab15 statistical software.

5.3 Results

5.3.1 Sequencing Tci-pgp-9-IBDA Allelic Variants

The first step in identifying allelic variants, using a nested PCR strategy, was to amplify a specific region of *Tci-pgp-9-IBDA*. Amplification of a generic PCR product from individual L_3 lysates from each of the MTci2, MTci5, MTci5PT strains, and L_1 lysates from the sub-populations of MTci2 and MTci5 collected in Chapter 4, confirmed successful crude gDNA lysate. It was evident, from the QIAxcel virtual gel (Figure 5.1), that several allelic variants had been amplified, with a range of different sized PCR products observed. The presence of more than one product in the same lane indicated that individuals may possess more than one allelic variant. Selected PCR products of different sizes were cloned and sequenced from each of the isolates, and the results were aligned with nine *Tci-pgp-9-IBDA* allele sequences previously generated from NZ isolates of *T. circumcincta* (Bisset, 2007).

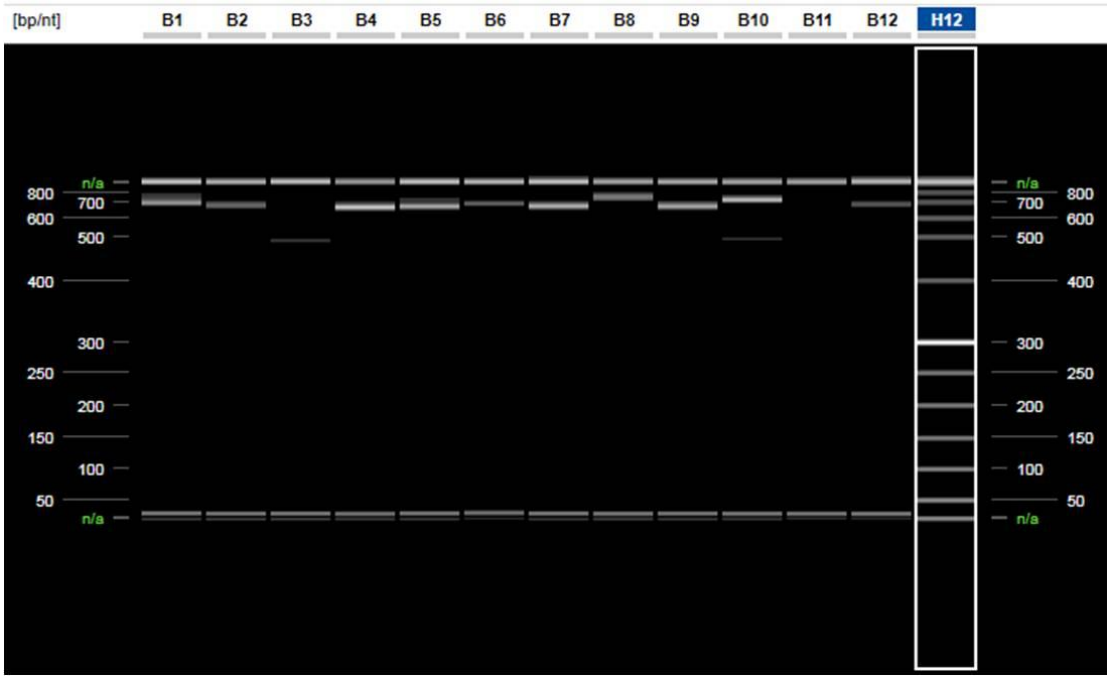
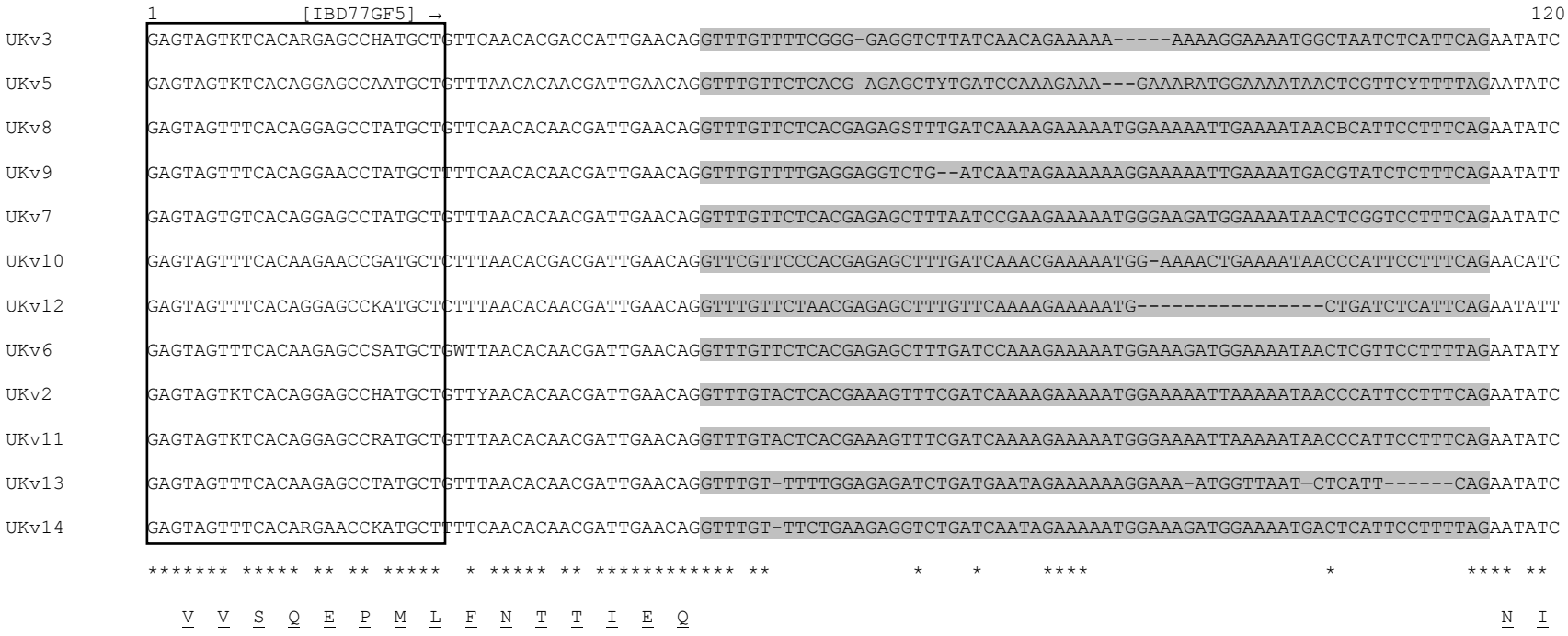


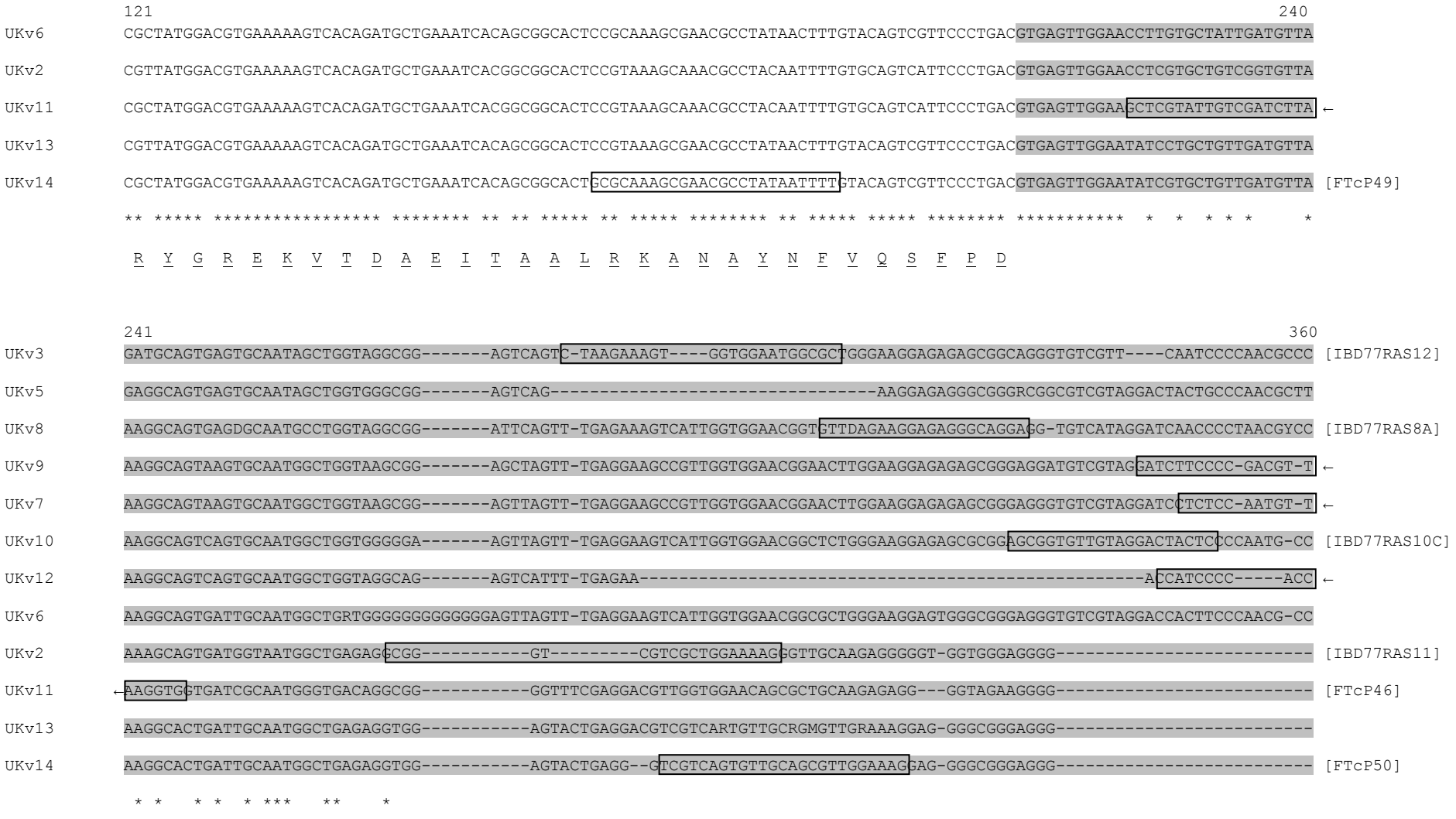
Figure 5.1 Generic Secondary Nested PCR Products from MTci2 (individuals 1-12)

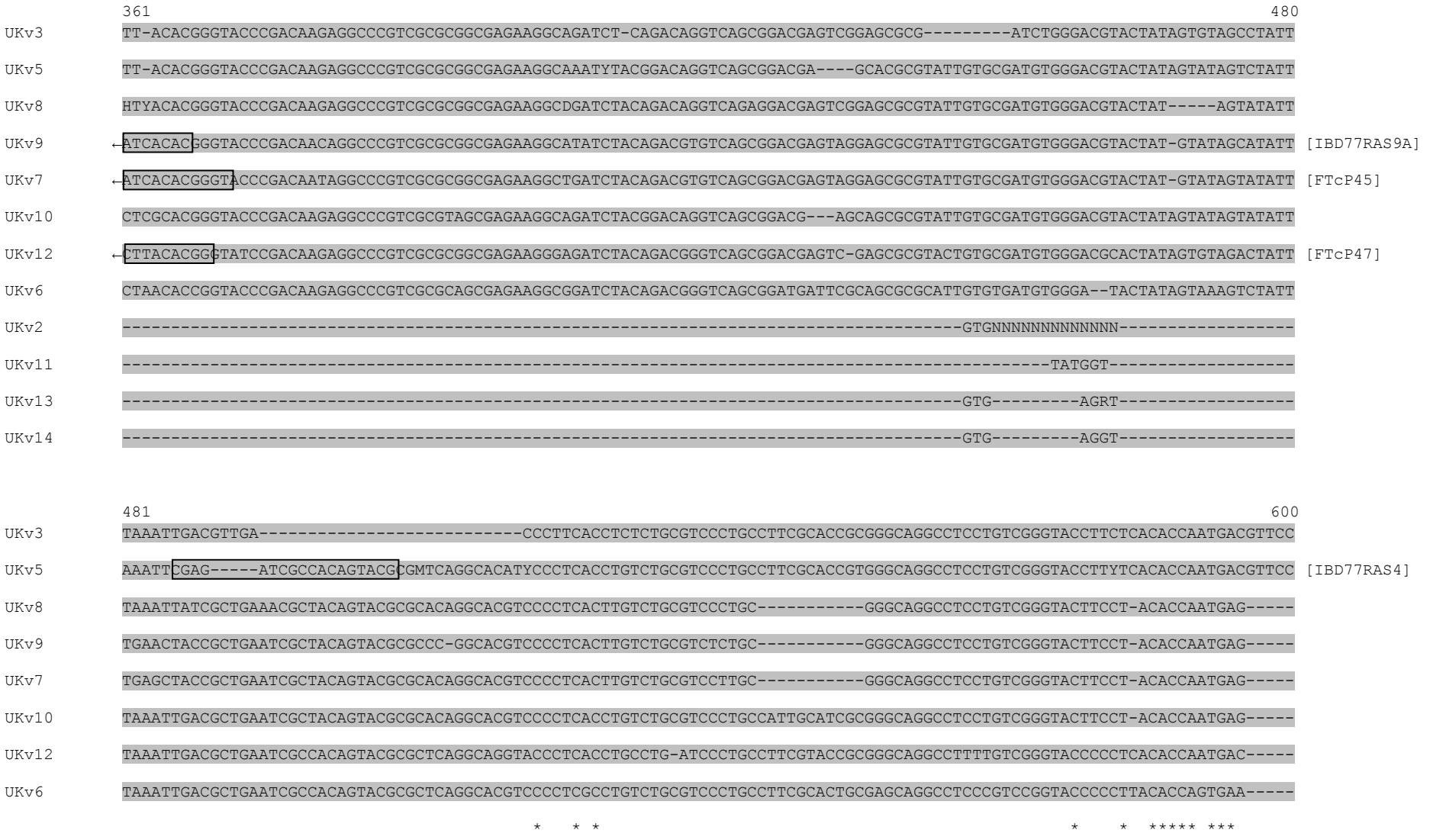
Virtual gel output from the QIAxcel Advanced Systsem showing PCR products amplified using primer pair IBD77GF4/IBD77GR3 from a selection of individuals of the MTci5 strain. The alignment markers, 15 bp and 1 kb are shown in each lane along with products of differing sizes, ranging from ~400 - 800 bp. The QX DNA Size Marker 50 – 800 bp (v2.0) is highlighted in lane H12.

Several distinct allelic variants could be distinguished within the *Tci-pgp-9-IBDA* gDNA region (Figure 5.2). The sequence data generated for the allelic variants present in UK isolates of *T. circumcincta* were aligned with the 9 NZ allelic variants previously described to check for homology. When classifying sequence variants, it was considered impractical to take into account every apparent SNP. A limited number of polymorphisms was considered as "one-off mutations" and as such were tolerated within each of the "allelic variants" groupings. In most cases, groupings were based on blocks of nucleotide substitutions, insertions or deletions within introns, which were repeatedly associated with particular allelic variants.

Seven of the nine *Tci-pgp-9-IBDA* allele variants previously identified in the NZ near-isogenic strains were found in the UK strains of *T. circumcincta*. These seven allelic variants aligned closely to NZ variants (NZv) 2, 3, 5, 7, 8, 9, and 10, sharing between 93 – 99 % identity (Table 5.2) and were annotated accordingly. Four allelic variants identified in the UK strains did not align with the previously identified NZ allelic variants, and were numbered 11-14. A phylogenetic tree was constructed to show relationships (Figure 5.3) between the allele variants found in UK and NZ strains of *T. circumcincta*, supporting the high levels of identity observed within this amplified gDNA fragment of *Tci-pgp-9-IBDA*. Allele variant NZv1 was not observed in the UK strains, although UKv11 was found to be most closely related to NZv1, sharing 89 % identity when aligned.


1 [IBD77GF5] → 120
UKv3 GAGTAGTKTCACARGAGCCHATGCTGTTCAACACGACCATTGAACAGGTTTGTTTTCGGG-GAGGTCTTATCAACAGAAAAA-----AAAAGGAAAATGGCTAATCTCATTCAGAATATC
UKv5 GAGTAGTKTCACAGGAGCCAATGCTGTTTAACACAACGATTGAACAGGTTTGTTCTCACG AGAGCTYTGATCCAAAGAAA---GAAARATGGAAAATAACTCGTTCYTTTTAGAATATC
UKv8 GAGTAGTTTCACAGGAGCCTATGCTGTTCAACACAACGATTGAACAGGTTTGTTCTCACGAGAGSTTTGATCAAAAGAAAAATGGAAAAATTGAAAATAACBCATTCCTTTCAGAATATC
UKv9 GAGTAGTTTCACAGGAACCTATGCTTTTCAACACAACGATTGAACAGGTTTGTTTTGAGGAGGTCTG--ATCAATAGAAAAAAGGAAAAATTGAAAATGACGTATCTCTTTCAGAATATT
UKv7 GAGTAGTGTCACAGGAGCCTATGCTGTTTAACACAACGATTGAACAGGTTTGTTCTCACGAGAGCTTTAATCCGAAGAAAAATGGGAAGATGGAAAATAACTCGGTCCTTTCAGAATATC
UKv10 GAGTAGTTTCACAAGAACCGATGCTCTTTAACACGACGATTGAACAGGTTCGTTCCCACGAGAGCTTTGATCAAACGAAAAATGG-AAAACTGAAAATAACCCATTCCTTTCAGAACATC
UKv12 GAGTAGTTTCACAGGAGCCKATGCTCTTTAACACAACGATTGAACAGGTTTGTTCTAACGAGAGCTTTGTTCAAAAGAAAAATG----------CTGATCTCATTCAGAATATT
UKv6 GAGTAGTTTCACAAGAGCCSATGCTGWTTAACACAACGATTGAACAGGTTTGTTCTCACGAGAGCTTTGATCCAAAGAAAAATGGAAAGATGGAAAATAACTCGTTCCTTTTAGAATATY
UKv2 GAGTAGTKTCACAGGAGCCHATGCTGTTYAACACAACGATTGAACAGGTTTGTACTCACGAAAGTTTCGATCAAAAGAAAAATGGAAAAATTAAAAATAACCCATTCCTTTCAGAATATC
UKv11 GAGTAGTKTCACAGGAGCCRATGCTGTTTAACACAACGATTGAACAGGTTTGTACTCACGAAAGTTTCGATCAAAAGAAAAATGGGAAAATTAAAAATAACCCATTCCTTTCAGAATATC
UKv13 GAGTAGTTTCACAAGAGCCTATGCTGTTTAACACAACGATTGAACAGGTTTGT-TTTTGGAGAGATCTGATGAATAGAAAAAAGGAAA-ATGGTTAAT—CTCATT------CAGAATATC
UKv14 GAGTAGTTTCACARGAACCKATGCTTTTCAACACAACGATTGAACAGGTTTGT-TTCTGAAGAGGTCTGATCAATAGAAAAATGGAAAGATGGAAAATGACTCATTCCTTTTAGAATATC
******* ***** ** ** ***** * ***** ** ********** ** * * **** * **** ** **
V V S Q E P M L F N T T I E Q N I
121 240
UKv3 CGATATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGCAAAGCAAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAATCTCGTGCTGTTGAT---A
UKv5 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTCCGCAAAGCGAACGCCTATAACTTTGTACAGTCGTTCCCTGACGTGAGTTGGAACCTCTTGCTGTTGATGTTA
UKv8 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCGAACGCCTACAATTTTGTGCAGTCGTTCCCTGACGTGAGTTGGAACCTCGTGCTGTCGACCTTA
UKv9 CGCTATGGACGTGAAAAAGTCACAGACGCTGAAATAACGGCCGCACTCCGTAAAGCAAACGCCTACAATTTTGTGCAGTCGTTCCCTGATGTGAGTTGGAACCTCATGCTGTTGACGTTA
UKv7 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTCCGCAAAGCGAACGCCTATAACTTTGTACAGTCGTTCCCTGACGTGAGTTGGAACCTCGTGCTGTTGACGTTA
UKv10 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCAGCACTCCGTAAAGCGAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAACCTCGTGTTGTCGGTGTTA
UKv12 CGTTATGGTCGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCAAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAACCTCGTGTTGTCGGCGTTA


121
240
UKv6 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTCCGCAAAGCGAACGCCTATAACTTTGTACAGTCGTTCCCTGACGTGAGTTGGAACCTTGTGCTATTGATGTTA
UKv2 CGTTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCAAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAACCTCGTGCTGTCGGTGTTA
UKv11 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCAAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAAGCTCGTATTGTCGATCTTA ←
UKv13 CGTTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTCCGTAAAGCGAACGCCTATAACTTTGTACAGTCGTTCCCTGACGTGAGTTGGAATATCCTGCTGTTGATGTTA
UKv14 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTGCGCAAAGCGAACGCCTATAATTTTGTACAGTCGTTCCCTGACGTGAGTTGGAATATCGTGCTGTTGATGTTA [FTcP49]
** ***** ********** ******** ** ** ***** ** ***** ******** ** ***** ***** ******** ********** * * * * * *
R Y G R E K V T D A E I T A A L R K A N A Y N F V Q S F P D
241
360
UKv3 GATGCAGTGAGTGCAATAGCTGGTAGGCGG-------AGTCAGTC-TAAGAAAGT----GGTGGAATGGCGCTGGGAAGGAGAGAGCGGCAGGGTGTCGTT----CAATCCCCAACGCCC [IBD77RAS12]
UKv5 GAGGCAGTGAGTGCAATAGCTGGTGGGCGG-------AGTCAG---------- AAGGAGAGGGCGGGRCGGCGTCGTAGGACTACTGCCCAACGCTT
UKv8 AAGGCAGTGAGDGCAATGCCTGGTAGGCGG-------ATTCAGTT-TGAGAAAGTCATTGGTGGAACGGTGTTDAGAAGGAGAGGGCAGGAGG-TGTCATAGGATCAACCCCTAACGYCC [IBD77RAS8A]
UKv9 AAGGCAGTAAGTGCAATGGCTGGTAAGCGG-------AGCTAGTT-TGAGGAAGCCGTTGGTGGAACGGAACTTGGAAGGAGAGAGCGGGAGGATGTCGTAGGATCTTCCCC-GACGT-T ←
UKv7 AAGGCAGTAAGTGCAATGGCTGGTAAGCGG-------AGTTAGTT-TGAGGAAGCCGTTGGTGGAACGGAACTTGGAAGGAGAGAGCGGGAGGGTGTCGTAGGATCCTCTCC-AATGT-T ←
UKv10 AAGGCAGTCAGTGCAATGGCTGGTGGGGGA-------AGTTAGTT-TGAGGAAGTCATTGGTGGAACGGCTCTGGGAAGGAGAGCGCGGAGCGGTGTTGTAGGACTACTCCCCCAATG-CC [IBD77RAS10C]
UKv12 AAGGCAGTCAGTGCAATGGCTGGTAGGCAG-------AGTCATTT-TGAGAA---------- ACCATCCCC-----ACC ←
UKv6 AAGGCAGTGATTGCAATGGCTGRTGGGGGGGGGGGGGAGTTAGTT-TGAGGAAGTCATTGGTGGAACGGCGCTGGGAAGGAGTGGGCGGGAGGGTGTCGTAGGACCACTTCCCAACG-CC
UKv2 AAAGCAGTGATGGTAATGGCTGAGAGGCGG----------GT---------CGTCGCTGGAAAAGGGTTGCAAGAGGGGGT-GGTGGGAGGGG---------- [IBD77RAS11]
UKv11 ←AAGGTGGTGATCGCAATGGGTGACAGGCGG----------GGTTTCGAGGACGTTGGTGGAACAGCGCTGCAAGAGAGG---GGTAGAAGGGG---------- [FTcP46]
UKv13 AAGGCACTGATTGCAATGGCTGAGAGGTGG----------AGTACTGAGGACGTCGTCARTGTTGCRGMGTTGRAAAGGAG-GGGCGGGAGGG----------
UKv14 AAGGCACTGATTGCAATGGCTGAGAGGTGG----------AGTACTGAGG--GTCGTCAGTGTTGCAGCGTTGGAAAGGAG-GGGCGGGAGGG---------- [FTcP50]
* * * * * *** ** *


361
480
UKv3 TT-ACACGGGTACCCGACAAGAGGCCCGTCGCGCGGCGAGAAGGCAGATCT-CAGACAGGTCAGCGGACGAGTCGGAGCGCG---------ATCTGGGACGTACTATAGTGTAGCCTATT
UKv5 TT-ACACGGGTACCCGACAAGAGGCCCGTCGCGCGGCGAGAAGGCAAATYTACGGACAGGTCAGCGGACGA----GCACGCGTATTGTGCGATGTGGGACGTACTATAGTATAGTCTATT
UKv8 HTYACACGGGTACCCGACAAGAGGCCCGTCGCGCGGCGAGAAGGCDGATCTACAGACAGGTCAGAGGACGAGTCGGAGCGCGTATTGTGCGATGTGGGACGTACTAT-----AGTATATT
UKv9 ←ATCACACGGGTACCCGACAACAGGCCCGTCGCGCGGCGAGAAGGCATATCTACAGACGTGTCAGCGGACGAGTAGGAGCGCGTATTGTGCGATGTGGGACGTACTAT-GTATAGCATATT [IBD77RAS9A]
UKv7 ←ATCACACGGGTACCCGACAATAGGCCCGTCGCGCGGCGAGAAGGCTGATCTACAGACGTGTCAGCGGACGAGTAGGAGCGCGTATTGTGCGATGTGGGACGTACTAT-GTATAGTATATT [FTcP45]
UKv10 CTCGCACGGGTACCCGACAAGAGGCCCGTCGCGTAGCGAGAAGGCAGATCTACGGACAGGTCAGCGGACG---AGCAGCGCGTATTGTGCGATGTGGGACGTACTATAGTATAGTATATT
UKv12 ←CTTACACGGGTATCCGACAAGAGGCCCGTCGCGCGGCGAGAAGGGAGATCTACAGACGGGTCAGCGGACGAGTC-GAGCGCGTACTGTGCGATGTGGGACGCACTATAGTGTAGACTATT [FTcP47]
UKv6 CTAACACCGGTACCCGACAAGAGGCCCGTCGCGCAGCGAGAAGGCGGATCTACAGACGGGTCAGCGGATGATTCGCAGCGCGCATTGTGTGATGTGGGA--TACTATAGTAAAGTCTATT
UKv2 ----------GTGNNNNNNNNNNNNN----------
UKv11 ----------TATGGT----------
UKv13 ----------GTG---------AGRT----------
UKv14 ----------GTG---------AGGT----------
481
600
UKv3 TAAATTGACGTTGA----------CCCTTCACCTCTCTGCGTCCCTGCCTTCGCACCGCGGGCAGGCCTCCTGTCGGGTACCTTCTCACACCAATGACGTTCC
UKv5 AAATTCGAG-----ATCGCCACAGTACGCGMTCAGGCACATYCCCTCACCTGTCTGCGTCCCTGCCTTCGCACCGTGGGCAGGCCTCCTGTCGGGTACCTTYTCACACCAATGACGTTCC [IBD77RAS4]
UKv8 TAAATTATCGCTGAAACGCTACAGTACGCGCACAGGCACGTCCCCTCACTTGTCTGCGTCCCTGC----------GGGCAGGCCTCCTGTCGGGTACTTCCT-ACACCAATGAG-----
UKv9 TGAACTACCGCTGAATCGCTACAGTACGCGCCC-GGCACGTCCCCTCACTTGTCTGCGTCTCTGC----------GGGCAGGCCTCCTGTCGGGTACTTCCT-ACACCAATGAG-----
UKv7 TGAGCTACCGCTGAATCGCTACAGTACGCGCACAGGCACGTCCCCTCACTTGTCTGCGTCCTTGC----------GGGCAGGCCTCCTGTCGGGTACTTCCT-ACACCAATGAG-----
UKv10 TAAATTGACGCTGAATCGCTACAGTACGCGCACAGGCACGTCCCCTCACCTGTCTGCGTCCCTGCCATTGCATCGCGGGCAGGCCTCCTGTCGGGTACTTCCT-ACACCAATGAG-----
UKv12 TAAATTGACGCTGAATCGCCACAGTACGCGCTCAGGCAGGTACCCTCACCTGCCTG-ATCCCTGCCTTCGTACCGCGGGCAGGCCTTTTGTCGGGTACCCCCTCACACCAATGAC-----
UKv6 TAAATTGACGCTGAATCGCCACAGTACGCGCTCAGGCACGTCCCCTCGCCTGTCTGCGTCCCTGCCTTCGCACTGCGAGCAGGCCTCCCGTCCGGTACCCCCTTACACCAGTGAA-----
* * * * * ***** ***

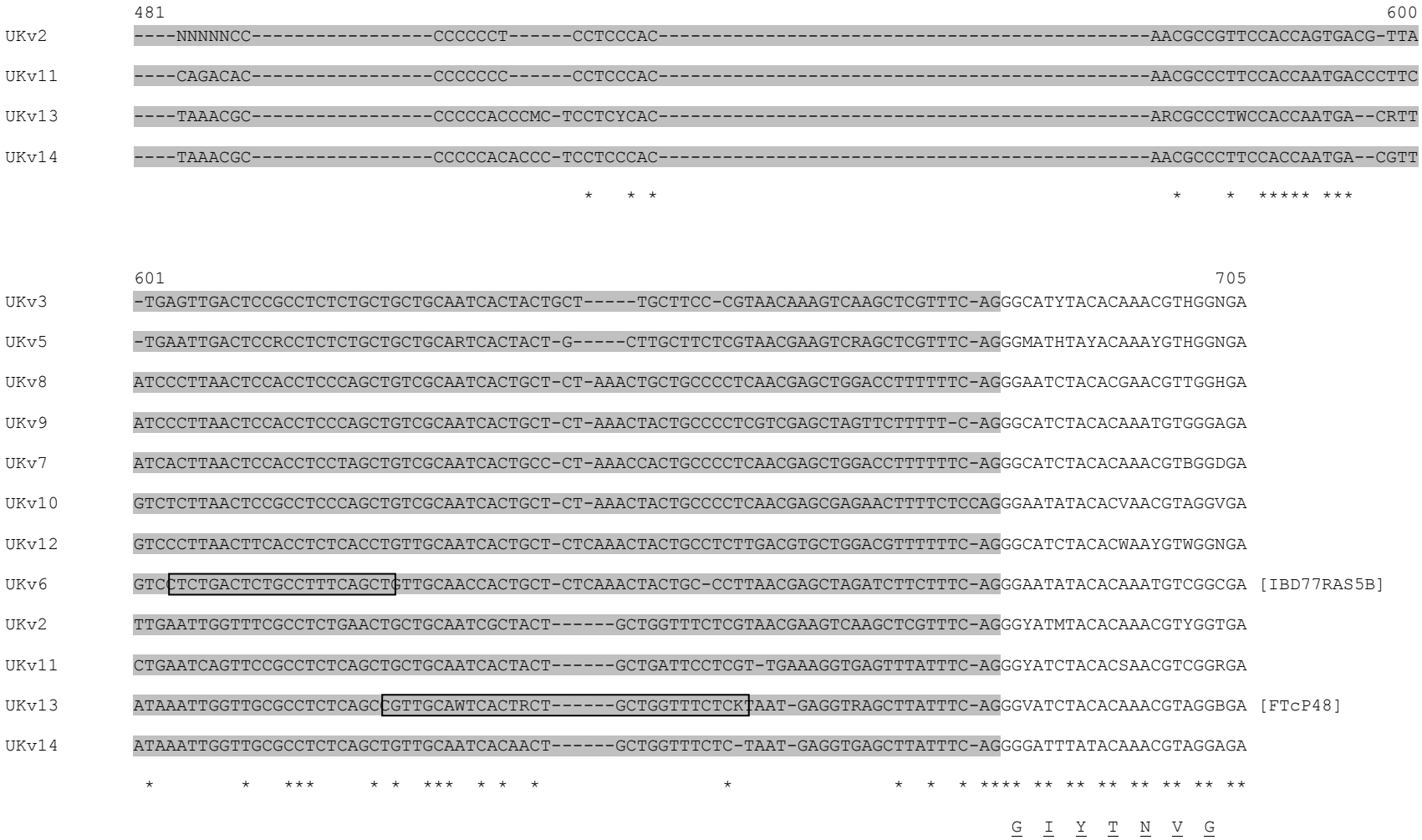


Figure 5.2 Allele-Specific Primer Locations Within the *Tci-pgp-9-IBDA* Domain

ClustalW2 multiple alignment of partial gDNA sequences of *Tci-pgp-9* allelic variants showing introns (shaded), amino acid translation (underlined) and locations of allele-specific primers (boxed) used to distinguish between allelic variants.

Table 5.2 Percentage Identity of *Tci-pgp-9-IBDA* Allelic Variants

The percentage identity between the *Tci-pgp-9-IBDA* allelic variants from the UK and NZ strains of *T. circumcincta* was calculated and shown in the table. The allele variants that shared the highest percentage of identity (shaded) formed the basis for the numbering of the UK allelic variants. The homology between the NZ isolates is shown to the right of the dashed line.

	UK v2	UK v3	UK v5	UK v6	UK v7	UK v8	UK v9	UK v10	UK v11	UK v12	UK v13	UK v14	NZ v1	NZ v2	NZ v3	NZ v5	NZ v6	NZ v7	NZ v8	NZ v9
UKv3	80																			
UKv5	81	84																		
UKv6	76	83	86																	
UKv7	76	81	84	87																
UKv8	80	83	83	85	90															
UKv9	74	82	81	83	91	88														
UKv10	79	82	84	87	87	89	86													
UKv11	83	80	80	82	80	84	79	82												
UKv12	75	79	81	85	84	85	83	86	79											
UKv13	76	80	80	81	81	79	78	77	78	77										
UKv14	78	80	80	83	81	79	80	81	80	78	91									
NZv1	76	82	80	81	79	80	80	80	89	76	82	81								
NZv2	**93**	80	81	79	79	84	76	81	85	77	79	81	79							
NZv3	80	**98**	84	83	81	84	82	83	80	79	80	80	82	81						
NZv5	80	85	**96**	87	85	85	82	85	82	82	82	82	83	84	86					
NZv6	77	83	86	**99**	87	86	83	87	82	85	81	84	82	80	83	88				
NZv7	74	79	81	82	**95**	87	86	84	78	81	79	80	79	78	80	83	83			
NZv8	81	82	82	85	89	**95**	88	90	83	86	79	81	78	84	82	83	85	86		
NZv9	73	81	80	83	91	88	**96**	86	77	84	79	83	79	77	81	81	83	87	88	
NZv10	78	82	84	87	87	88	86	**99**	82	86	77	81	81	81	82	85	87	83	89	86

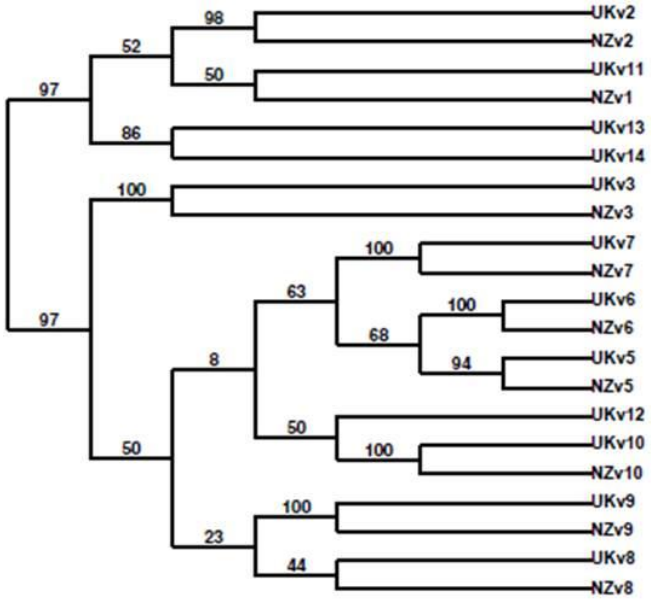


Figure 5.3 Phylogenetic Relationships of *Tci-pgp-9-IBDA* Allelic Variants

A phylogenetic tree constructed based on bootstrap (x100) alignment data to show the relationship between the allelic variants of *Tci-pgp-9-IBDA*, found in the UK and NZ strains of *T. circumcincta*. Percentage concordance based on 100 bootstraps is shown at the nodes.

Numerous attempts to obtain the complete sequence of UKv2 were made, but each time the sequencing was truncated at the same point in both the sense or antisense directions. Therefore, the UKv2 sequence remains incomplete, though the partial 5' and 3' sequences generated aligned closely to the NZv2 (Figure 5.4) allowing its categorisation as UKv2. From alignments with the NZv2 allele, it is likely that the loop of the hairpin is formed by 18 unknown nucleotides, consequently the UKv2 allele has been annotated with 18 'N' bases to represent the portion of unknown sequence (yellow highlighted section in Figure 5.4). The complementarities shared in different parts of the gene were visualised using mfold (Zuker, 2003), a predictive DNA structure program (Figure 5.5). Closer inspection of the sequencing data revealed the presence of a large hairpin structure in UKv2, which may impede the DNA sequencing method.

```
UKv2   GAGTAGTKTCACAGGAGCCHATGCTGTTYAACACAACGATTGAACAGGTTTGTACTCACG  60
NZv2   GAGTAGTGTCACAAGAGCCTATGCTGTTTAACACAACGATTGAACAGGTTTGTACTCACG  60
       ******* ***** ***** ******** ********************************

UKv2   AAAGTTTCGATCAAAAGAAAAATGGAAAAATTAAAAATAACCCATTCCTTTCAGAATATC  120
NZv2   AAAGTTTCGATCAAAAGAAAAATGGAAAAATTAAAAATAACCCATTCCTTTCAGAATATC  120
       ************************************************************

UKv2   CGTTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCAAAC  180
NZv2   CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCAAAC  180
       ** ********************************************************

UKv2   GCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAACCTCGTGCTGTCGGTGTTA  240
NZv2   GCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAACCTCGTGCTGTCGGTGTTA  240
       ************************************************************

UKv2   AAAGCAGTGATGGTAATGGCTGAGAGGCGGGTCGTCGCTGGAAAAGGGTTGCAAGAGGGG  300
NZv2   AAAGCAGTGATGGTAATGGCTCAGAGGCGGGTCGTCGCTGGAAAAGGGTTGCAAGAGGGG  300
       ******************** ************************************

UKv2   GTGGTGGGAGGGGGTGNNNNNNNNNNNNNNNNNNCCCCCCCCTCCTCCCACAACGCCGTT  360
NZv2   GTGGTGGGAGGGGGTGTGGTCACACCGCCCCTCCCCCCCCCCTCCTCCCACAACGCCGTT  360
       ****************                  **************************

UKv2   CCACCAGTGACGTTATTGAATTGGTTTCGCCTCTGAACTGCTGCAATCGCTACTGCTGGT  420
NZv2   CAACCAGTGACGTTATTGAATTGGTTTCGCCTCTGAACTGCTGCAATCGCTACTGCTGGT  420
       * ************************************************************

UKv2   TTCTCGTAACGAAGTCAAGCTCGTTTCAGGGYATMTACACAAACGTYGGTGA  472
NZv2   TTCTCGTAACGAAGTCAAGCTCGTTTCAGGGAATCTACACGAACGTTGGTGA  472
       ****************************** ** ***** ***** *****
```

Figure 5.4 ClustalW Alignment of UKv2 and NZv2

Alignment showing the identity between the allele variants UKv2 and NZv2. It was not possible to generate the complete UKv2 sequence, therefore an 18 nucleotide stretch (shaded) remains to be determined. The generation of sequence data at the 5' and 3' ends of this fragment was successful.

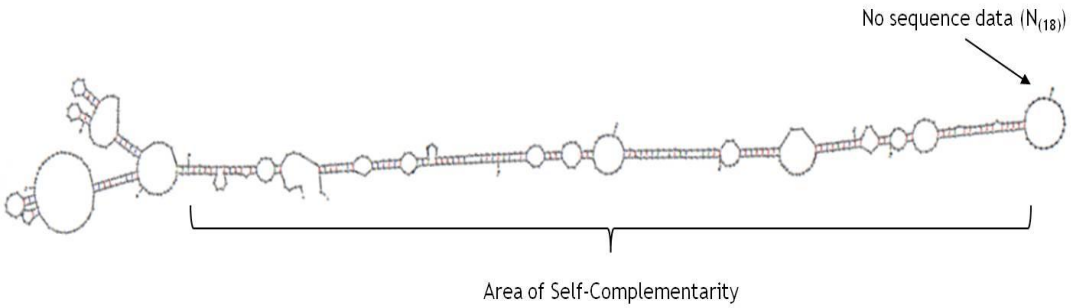


Figure 5.5 Prediction of DNA-Folding in UK Allele Variant 2

The DNA sequence data was generated for UKv2 in the sense and antisense directions. When aligned with the NZv2 sequence it revealed 18 nucleotides (arrowed) that had not been sequenced. One possible explanation for the failure to sequence through this region is offered by the high level of self complementarity of the sequence, immediately before and after the unknown nucleotides, as predicted by the mfold program (Zuker, 2003).

5.3.2 Allele-Specific Genotyping

The gDNA sequence data generated above were used to design allele-specific primers to distinguish between the allele variant(s) present in individual larvae. A series of allele-specific primers targeted unique regions of polymorphism in each allelic variant ensuring that each primer did not cross-react with other allelic variants. The specificity of the primers was confirmed in the primers listed in Table 5.1, with the exception of primers FTcP49 and FTcP50, which displayed cross-reactivity with other variants. The sequencing data generated from the amplified fragment of *Tci-pgp-9-IBDA* in Figure 5.2 revealed very few polymorphisms that were unique to UKv14, drastically limiting the sites available for the placement of an allele-specific primer. Sequencing data for UKv6 were initially generated from a single larva belonging to the MTci5 isolate. During the genotyping analysis, the rarity of the UKv6 sequence variant became apparent as UKv6 was absent in the selected larvae from the UK isolates. Using allele-specific PCR reactions, the presence or absence of each allelic variant was determined in individuals from the MTci2, MTci5 and MTci5PT UK isolates. A representative gel-output for allele-specific PCR reactions that screened for UKv5 in the MTci2 and MTci5 isolates is shown in Figure 5.6. The results for each of the other variants are recorded in Appendix 4. The procedure was repeated for the sub-populations of MTci2 and MTci5 larvae collected in Chapter 4 and recorded in Appendix 5.

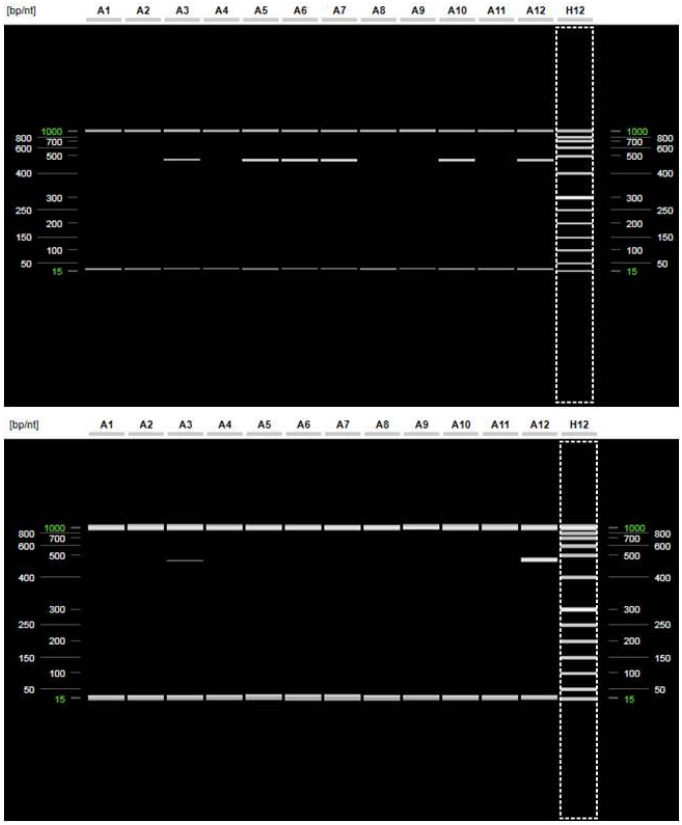


Figure 5.6 Representative Virtual Gels Showing UKv5 Allele-Specific PCR Products

Virtual gel output from the QIAxcel Advanced System showing screening for UKv5 PCR products (458 bp). The gel shows products amplified from larvae A1-A12 from MTci2 (top) and MTci5 (bottom) isolates, along with a molecular size marker in row H12.

The combination of allelic variants that each larva possessed was allocated a "haplotype" number (Appendix 6). In all, 147 different haplotypes were identified. The numbers and proportions of each of the allelic variants are listed in Tables 5.3, 5.4 and 5.5. Over all 439 individual larvae that were genotyped, the allelic variants UKv2, UKv13, UKv5 and UKv3 were most common, being recorded in 0.57, 0.45, 0.37 and 0.35 of individuals. However, given that it was impossible to distinguish homozygotes from heterozygotes and larvae with single copies from those with multiple copies, it is likely that an analysis of frequencies of individual allelic variants would be misleading.

Table 5.3 Frequency of *Tci-pgp-9* Allelic Variants in the MTci2 and MTci5 Isolates

Incidence of *Tci-pgp-9-IBDA* allele variants identified from the MTci2 and MTci5 isolates of *T. circumcincta*. The number and the percentage of individuals which possessed a particular allelic variant are described.

Allele Variant	Allele Incidence (Percentages in brackets)	
	MTci2 *(n = 84)*	MTci5 *(n = 84)*
2	42 *(50.00%)*	40 *(47.62%)*
3	9 *(10.71%)*	47 *(55.95%)*
5	50 *(59.52%)*	17 *(20.24%)*
6	0 *(0.0%)*	0 *(0.0%)*
7	8 *(9.52%)*	15 *(17.85%)*
8	45 *(53.57%)*	18 *(21.43%)*
9	37 *(45.05%)*	18 *(21.43%)*
10	31 *(36.90%)*	15 *(17.86%)*
11	13 *(15.48%)*	17 *(20.24%)*
12	39 *(46.43%)*	15 *(17.86%)*
13	48 *(57.14%)*	26 *(30.95%)*

Table 5.4 Frequency of Allelic Variants Present in Anthelmintic Susceptible UK Isolates of *T. circumcincta*

The incidence of allelic variants was measured in the MTci2 and the sub-populations of MTci2 that were collected after *in vitro* exposure to IVM.

Allele Variant	Allele Incidence (Percentages in brackets)		
	MTci2 ***(n = 84)***	**MTci2$_{(NF0.1\mu M)}$** ***(n = 43)***	**MTci2$_{(F10\mu M)}$** ***(n = 43)***
2	**42** *(50.00%)*	**40** *(93.02%)*	**34** *(79.07%)*
3	**9** *(10.71%)*	**4** *(9.30%)*	**7** *(16.28%)*
5	**50** *(59.52%)*	**32** *(74.42%)*	**24** *(55.81%)*
6	**0** *(0.0%)*	**0** *(0.0%)*	**0** *(0.0%)*
7	**8** *(9.52%)*	**3** *(6.98%)*	**2** *(4.65%)*
8	**45** *(53.57%)*	**1** *(2.33%)*	**0** *(0.00%)*
9	**37** *(45.05%)*	**7** *(16.42%)*	**7** *(16.28%)*
10	**31** *(36.90%)*	**14** *(32.56%)*	**10** *(26.26%)*
11	**13** *(15.48%)*	**1** *(2.33%)*	**3** *(6.98%)*
12	**39** *(46.43%)*	**7** *(16.28%)*	**9** *(20.93%)*
13	**48** *(57.14%)*	**26** *(60.47%)*	**27** *(62.79%)*

Table 5.5 Frequency of *Tci-pgp-9* Allelic Variants in Multiple Anthelmintic Resistant UK Isolates of *T. circumcincta*

The frequency of allelic variants in the MTci5 isolate was compared to populations that were collected after both *in vivo* exposure (MTci5PT) and *in vitro* exposure ($MTci5_{(NF0.1\mu M)}$ and $MTci5_{(F10\mu M)}$).

Allele Variant	Allele Incidence (Percentages in brackets)			
	MTci5 ***(n = 84)***	**MTci5PT** ***(n = 80)***	**$MTci5_{(NF0.1\mu M)}$** ***(n = 45)***	**$MTci5_{(F10\mu M)}$** ***(n = 47)***
2	**40** *(47.62%)*	**34** *(42.50%)*	**27** *(60.00%)*	**31** *(65.95%)*
3	**47** *(55.95%)*	**14** *(17.50%)*	**35** *(77.78%)*	**36** *(76.60%)*
5	**17** *(20.24%)*	**34** *(42.50%)*	**3** *(6.67%)*	**3** *(6.38%)*
6	**0** *(0.0%)*	**0** *(0.0%)*	**0** *(0.0%)*	**0** *(0.0%)*
7	**15** *(17.85%)*	**9** *(11.25%)*	**5** *(11.11%)*	**4** *(8.51%)*
8	**18** *(21.43%)*	**8** *(10.00%)*	**8** *(17.78%)*	**6** *(12.77%)*
9	**18** *(21.43%)*	**11** *(13.75%)*	**5** *(11.11%)*	**6** *(12.77%)*
10	**15** *(17.86%)*	**9** *(11.25%)*	**6** *(13.33%)*	**5** *(10.64%)*
11	**17** *(20.24%)*	**6** *(7.50%)*	**2** *(4.44%)*	**2** *(4.26%)*
12	**15** *(17.86%)*	**32** *(40.00%)*	**6** *(13.33%)*	**6** *(12.77%)*
13	**26** *(30.95%)*	**20** *(25.00%)*	**25** *(55.56%)*	**27** *(57.45%)*

Often more than one allelic variant was detected in an individual larva, as indicated by more than one PCR product in Figure 5.1. The results of the allele-specific PCR were collated and revealed the presence of up to ten different allelic variants in larvae from MTci2, significantly more than the MTci5 and MTci5PT isolates ($p < 0.001$). The MTci5 isolate possessed up to 7 allelic variants, and a further reduction was observed in the MTci5PT isolate which displayed up to 5 different allelic variants (Figure 5.7). Comparisons of the number of alleles in each isolate showed MTci2 had a mean of 3.8 variants, a reduction in the

mean number of allelic variants to 2.7 was observed in the MTci5 isolate, and a further reduction in the MTci5PT isolate, which displayed a mean of 2.2 variants. The one-way ANOVA analyses (Figure 5.8) revealed that the majority of larvae in the MTci2 isolate possess 2-5 allelic variants, whereas, representatives of the MTci5 and MTci5PT isolates possess 2-3 allelic variants.

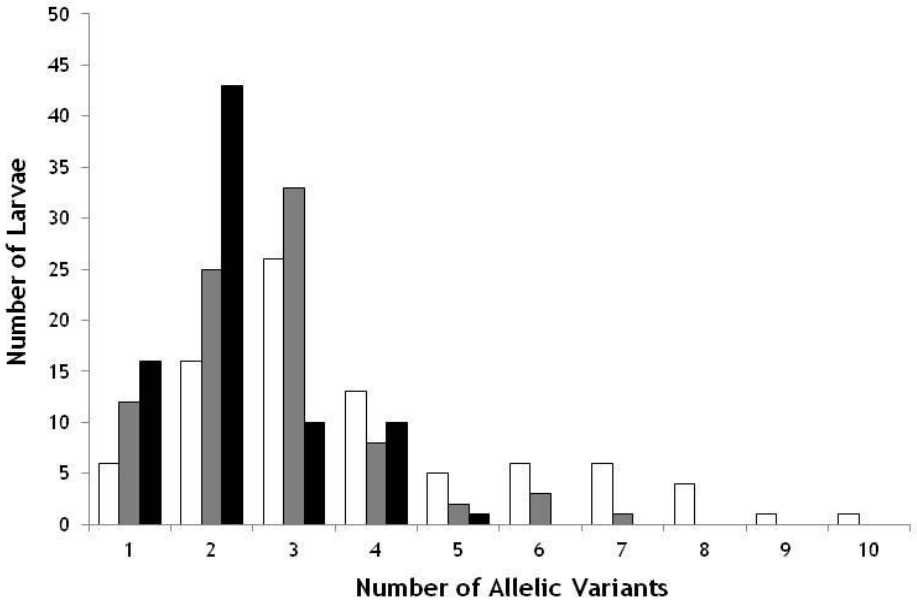
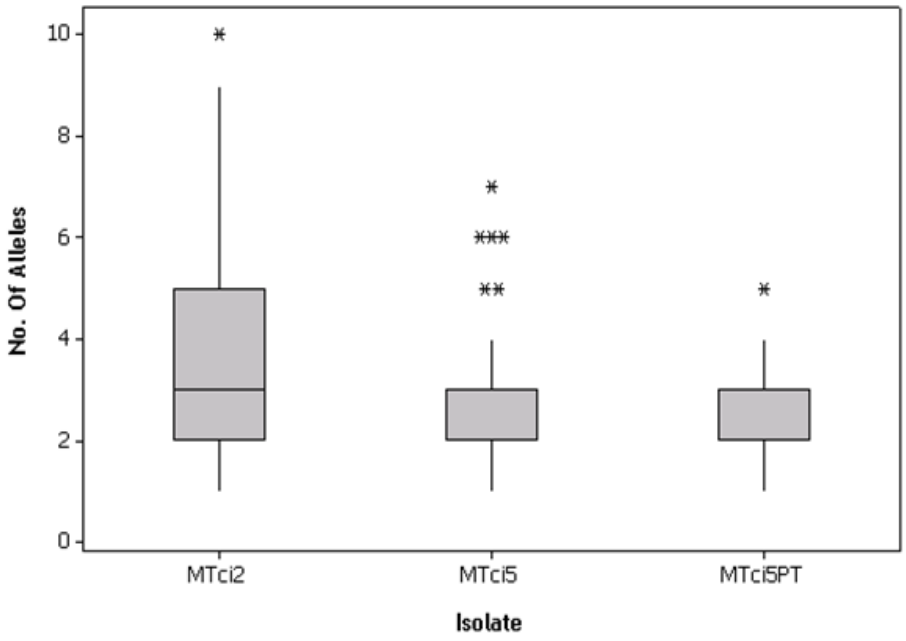


Figure 5.7 Number of Allelic Variants Identified in Individual Larvae

Larvae from the MTci2 (white), MTci5 (grey) and MTci5PT (black) were screened for the presence of the 13 different allelic variants. The total number of larvae tested was 84 for each of the MTci2 and MTci5 isolates and 80 for the MTci5PT isolate.



One-Way ANOVA:

Source	DF	SS	MS	F	P
Isolate	2	113.64	56.82	25.49	<0.001
Error	245	546.20	2.23		
Total	247	659.83			

s = 1.493 R-Sq = 17.22 % R-Sq(adj) = 16.55 %

Level	N	Mean	StDev
MTci2	84	3.833	2.053
MTci5	84	2.174	1.228
MTci5PT	80	2.212	0.951

```
        Individual 95 % CIs For Mean Based on Pooled StDev
        ---------+---------+---------+---------+
MTci2                                 (----*----)
MTci5             (----*-----)
MTci5PT (-----*----)
        ---------+---------+---------+---------+
               2.40      3.00      3.60      4.20

Pooled StDev = 1.493
```

Figure 5.8 One-way ANOVA Analysis of Numbers of Allelic Variants

The number of allelic variants present in individual larvae was analysed using one-way analysis of variance. The variation between the MTci2, MTci5 and MTci5PT isolates was analysed and the results were summarised in the form of a boxplot with stars indicating outlier points.

The number of allelic variants present in the sub-populations of MTci2 and MTci5, collected after IVM exposure *in vitro*, was analysed and revealed that these sub-populations possessed between 1-5 allelic variants (Figure 5.9). The one-way ANOVA analyses indicated that the sub-populations with inhibited feeding when exposed to 0.1 µ*M* IVM, $MTci2_{(NF0.1\mu M)}$ and $MTci5_{(NF0.1\mu M)}$, had a greater range in the number of allelic variants compared to the $MTci2_{(F10\mu M)}$ and $MTci5_{(F10\mu M)}$ sub-populations, which retained the ability to feed after exposure to 10 µ*M* IVM. No significant differences in the mean number of allelic variants were observed between the sub-populations tested, as illustrated in Figure 5.10.

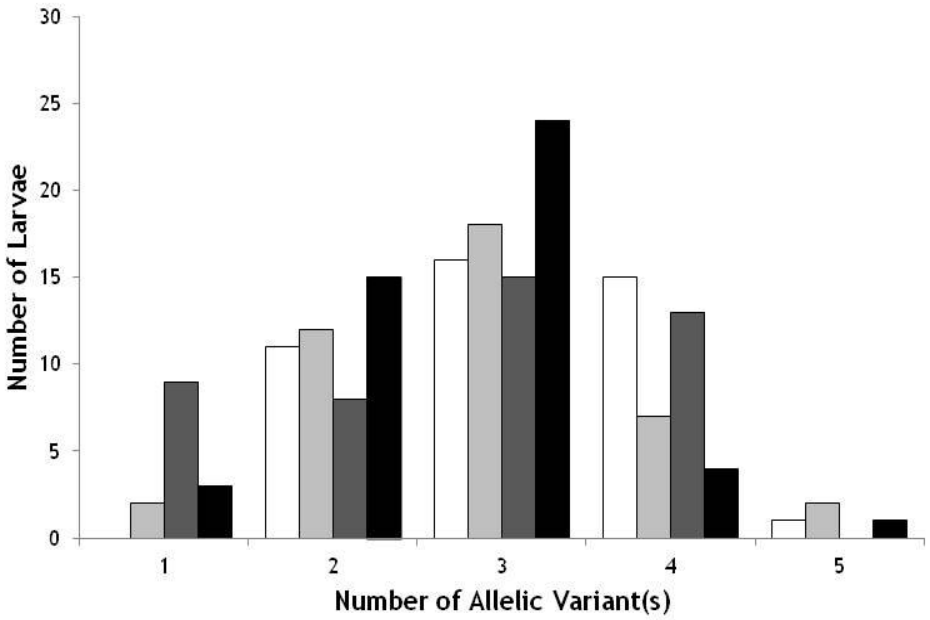
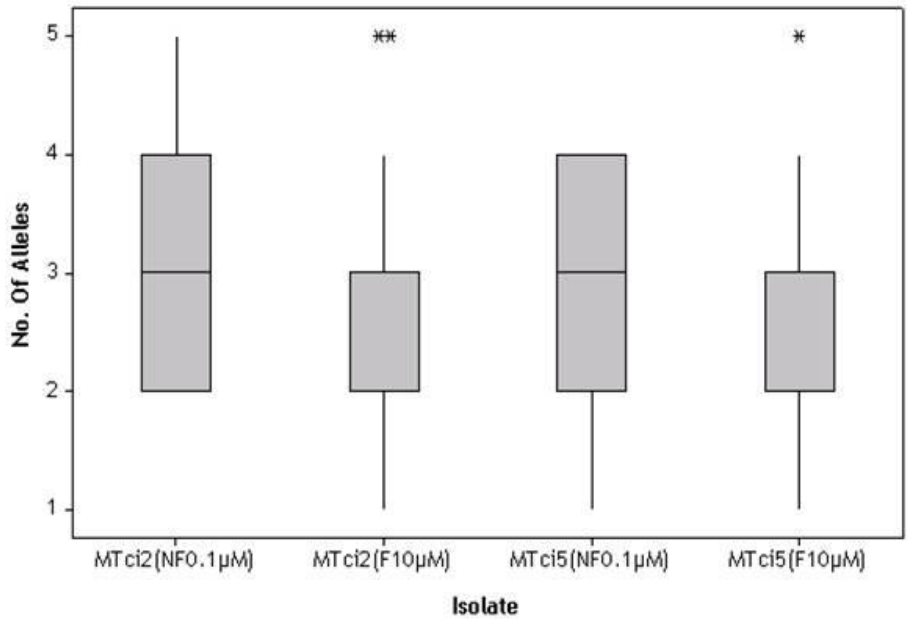


Figure 5.9 Number of Allele Variants Present in Selected Sub-populations

The number of allelic variants present in individual larvae from the $MTci2_{(NF0.1\mu M)}$ (white), $MTci2_{(F10\mu M)}$ (light grey), $MTci5_{(NF0.1\mu M)}$ (dark grey), and $MTci5_{(F10\mu M)}$ (black) sub-populations was determined. Forty-three larvae were screened from the $MTci2_{(NF0.1\mu M)}$ and $MTci2_{(F10\mu M)}$ sub-populations; 45 and 47 larvae screened in the $MTci5_{(NF0.1\mu M)}$ and $MTci5_{(F10\mu M)}$ sub-populations, respectively.

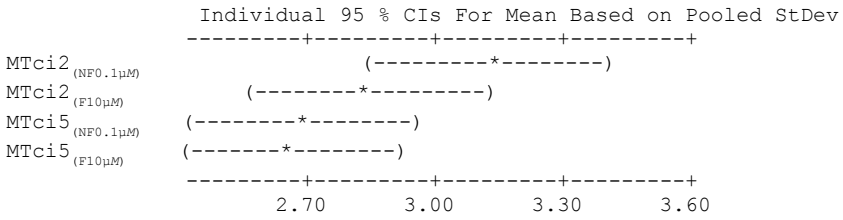


One-Way ANOVA:

Source	DF	SS	MS	F	P
Isolate	3	5.813	1.930	2.20	0.001
Error	174	147.783	0.849		
Total	177	153.596			

s = 0.9216 R-Sq = 3.78 % R-Sq(adj) = 2.13 %

Level	N	Mean	StDev
MTci2 (NF0.1μM)	43	3.1395	0.8333
MTci2 (F10μM)	43	2.8605	0.9150
MTci5 (NF0.1μM)	45	2.7111	1.1000
MTci5 (NF0.1μM)	47	2.6809	0.8104



Pooled StDev = 0.9216

Figure 5.10 One-way ANOVA Analysis of Numbers of Allelic Variants in Selected Subpopulations

The number of allelic variants present in individual larvae was analysed using one-way analysis of variance. The variation between the selected subpopulations of the MTci2, and MTci5 isolates was analysed and the results were summarised in the form of a boxplot, where stars represent outlier points.

Appendices 4 and 5 list the haplotypes seen in each of the populations. Figure 5.11 shows the distribution of haplotypes across all populations. It is clear that the majority of haplotypes were unique to individual larvae, with only a few haplotypes being seen in many. Table 5.6 shows the most frequent haplotypes (occurring in at least 5 individuals), with their representation in each population. The most common haplotype overall was 70 (UKv2, 13), which was equally common in all populations. Not surprisingly, given the large number of different haplotypes, and the small number of haplotypes represented by more than 5 individuals, there were few significant deviations from the expected distribution. However, haplotype 21 (UKv2, 3, 13) was seen twice as frequently in MTci5 larvae that fed successfully after *in vitro* treatment with IVM (χ^2 = 50.9168, 6 d.f., p <0.005). In contrast, haplotype 71 (UKv3) was most common in the MTci5 larvae that failed to feed after *in vitro* treatment with IVM (χ^2 = 20.2756, p <0.005).

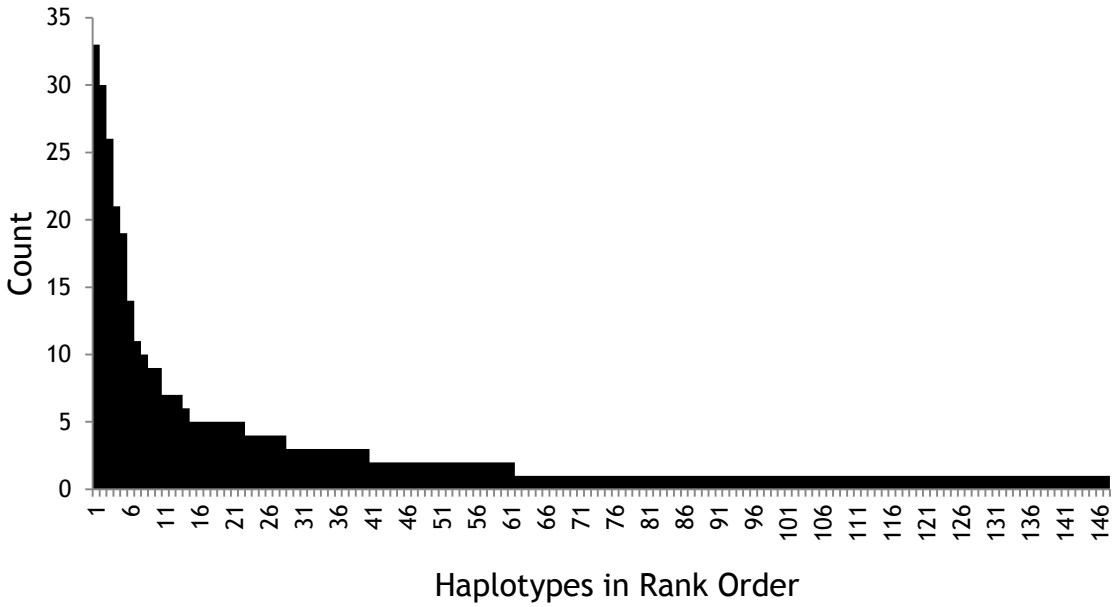


Figure 5.11 Frequency Distribution of Haplotypes Over All Populations Combined

The majority of haplotypes were seen in only one larva, with only 8 haplotypes being seen in 10 or more individual larvae.

The MTci2, MTci5, MTci5PT and the sub-populations derived from MTci2 and MTci5 were not very distinct. There were no private alleles, that is, all allelic variants were present in all populations. Nei's genetic distance matrix is shown in Table 5.7. It shows particularly short Nei genetic distances between the feeder and non-feeder samples within each population and between the source populations (MTci2 and MTci5) and their derived population. The longest Nei genetic distance (0.060) is between MTci2 and $MTci5_{(F1.0\mu M)}$ populations. The relatively small contribution of the populations to genetic variance is shown also in Table 5.8, a molecular analysis of variance. This analysis suggests that the within-population molecular variance amounts to 86 %, and the among-population variance is 14 %.

Table 5.6 Frequency of the Common *Tci-pgp-9* Haplotypes in Each Population

The frequency of haplotypes occurring at least five times in total within all populations under study: MTci2 and MTci5, and populations that were collected after *in vivo* exposure (MTci5PT) and *in vitro* exposure to IVM ($MTci5_{(NF0.1\mu M)}$ and $MTci5_{(F10\mu M)}$). Within each column (population), the most common haplotype is shaded. In those cells in which the χ^2 value ≥ 10, data are presented in order: count, percentage of column (population) of this genotype, expected count, χ^2

Haplotype									
Code	**Allelic Variant(s)**	**MTci2**	**$MTci2_{(NF0.1\mu M)}$**	**$MTci2_{(F10\mu M)}$**	**MTci5**	**$MTci5_{(NF0.1\mu M)}$**	**$MTci5_{(F10\mu M)}$**	**MTci5PT**	**All**
70	2,13	4	5	8	1	4	6	5	33
21	2,3,13	0	0	1	5	8	**16 34% 3.22 50.9168**	0	30
44	2,5,13	5	**9 19% 2.7836 13.8826**	**8**	1	0	1	2	26
71	3	0	0	0	**7**	**9 19% 2.25 20.2756**	3	2	21
124	5,12	**7**	1	2	1	0	0	**8**	19
	null	0	4	4	0	2	0	4	14
69	2,12,13	5	0	3	2	0	1	0	11
1	2	2	0	0	3	0	0	5	10
103	3,12	0	0	0	2	1	3	3	9
40	2,5,10,13	0	5	3	0	0	0	1	9
2	2,3	0	2	0	5	1	0	0	8
68	2,12	0	1	0	1	0	0	5	7
22	2,5	0	1	1	0	0	0	4	6
90	3,8	0	0	0	4	0	2	0	6
122	5,10	1	0	1	1	0	0	3	6
145	12	0	0	2	0	0	0	3	5
105	5	3	0	0	1	0	0	1	5
110	5,8	1	0	0	1	0	1	2	5
64	2,9,13	0	2	1	2	0	0	0	5
51	2,7,9,13	0	1	1	1	1	0	1	5
20	2,3,12,13	0	0	0	0	3	1	1	5

Table 5.7 Pairwise Population Matrix of Nei's Genetic Distance for the 7 Populations Understudy

Analysis based on 7 populations with 11 binary loci (rationale provided in Materials and Methods). Populations with Nei genetic distance = 0 are identical. Increasing values indicate increasing genetic distance between populations.

MTci2	MTci5	MTci5PT	$MTci2_{(NF0.1\mu M)}$	$MTci2_{(F10\mu M)}$	$MTci5_{(NF0.1\mu M)}$	$MTci5_{(F10\mu M)}$	
0.000							**MTci2**
0.034	0.000						**MTci5**
0.021	0.012	0.000					**MTci5PT**
0.031	0.043	0.030	0.000				**$MTci2_{(NF0.1\mu M)}$**
0.023	0.023	0.015	0.005	0.000			**$MTci2_{(F10\mu M)}$**
0.055	0.008	0.031	0.052	0.032	0.000		**$MTci5_{(NF0.1\mu M)}$**
0.060	0.012	0.037	0.051	0.032	0.001	0.000	**$MTci5_{(F10\mu M)}$**

Table 5.8 Molecular Analysis of Variance for the 7 Populations Understudy

Molecular analysis of variance (AMOVA) conducted using GenAlEx version 6.501 with 438 samples, 7 populations and 11 binary loci. PhiPT is analogous to F_{st} for co-dominant data (a scaled estimator for differentiation ranging from 0 = identical to 1 = no identity).

Source	Degrees of Freedom	Sum of Squares	Mean Square	Estimated Variance	Percentage
Among Populations	6	104.635	17.439	0.256	14 %
Within Populations	432	690.025	1.597	1.597	86 %
Total	438	794.661		1.853	100 %
	Value	**P(rand ≥ data)**			
PhiPT	0.138	0.001			

5.4 Discussion

The focus of this Chapter was to identify and record the allelic variation in the gDNA sequence of the N-terminal internucleotide binding domain, *Tci-pgp-9-IBDA*, of individuals from UK isolates of *T. circumcincta*. The sequences generated were compared with 9 allelic variants previously identified in the same *Tci-pgp-9-IBDA* region isolated from NZ strains of *T. circumcincta* (Bisset, 2007) (Figure 5.2). The incidences of the identified allelic variants in over 80 randomly selected larvae from each of MTci2, MTci5 and MTci5PT isolates of *T. circumcincta* were recorded and classified into 12 different "allelic variants". Eight of the nine previously identified allele variants were present in the UK isolates, with NZv1 the exception. An additional four allelic variants were sequenced and numbered 11-14 (Figure 5.3). High levels of identity (93-99 %) shared between the UK and NZ *Tci-pgp-9-IBDA* allelic variants (Table 5.2), suggested a degree of conservation of these alleles in these two geographically diverse *T. circumcincta* isolates. The results confirmed a high degree of sequence diversity in the introns, as well as synonymous SNPs in the exons of *Tci-pgp-9-IBDA*. It is conceivable that polymorphisms elsewhere in the *Tci-pgp-9* cDNA sequence may be linked to the allele variants identified in the intronic regions in the *Tci-pgp-9-IBDA* domain, although this remains to be proven. The possibility of having an allele that is capable of negating or diminishing the toxic effects of a drug is greater in genetically diverse species, such as the trichostongyloid nematodes *H. contortus* (Beech *et al.*, 1994) and *T. circumcincta* (Blackhall *et al.*, 1998). Pgps have been implicated in the molecular basis of IVM-resistance (Xu *et al.*, 1998; Molento & Prichard, 1999), and polymorphisms in certain alleles have the potential to improve drug efflux from the cell, thereby changing the drug distribution within the parasite's tissues, thus preventing anthelmintics reaching their site of action (Wolstenholme *et al.*, 2004; Prichard & Roulet, 2007).

Based on the amplification of an allele-specific PCR product, comparisons of the observed frequency of allele variants were conducted. The majority (78.6 %) of individuals in the anthelmintic susceptible isolate, MTci2, carried between 1-4 allelic variants, and 21.4 % carried >5 allelic variants of *Tci-pgp-9*. The maximum number of allelic variants observed in an individual MTci2 larva was ten, suggesting at least 5 heterozygous copies of the *Tci-pgp-9* gene were

amplified by the allele-specific PCR. Possessing up to ten different variants may provide this individual with an arsenal of *Tci-pgp-9* alleles which potentially offer a fitness advantage against a variety of xenobiotic agents. A reduction in the number of allelic variants in individuals of the anthelmintic resistant isolate, MTci5, was observed with 92.9 % of individuals carrying ≤ 4 variants and the remaining 7.1 % of larvae possessing 5-7 allelic variants. These results would suggest that purifying selection has eliminated deleterious alleles (Prince & Pickett, 2002). The further reduction in allele variants observed in the MTci5PT isolate, collected after *in vivo* exposure to IVM, supports this theory of purifying selection as 98.8 % of larvae displayed ≤ 4 variants with just one individual (1.2 %) possessing 5 allelic variants.

A proportion of individuals displaying fewer than ten alleles would be assumed to be homozygous in some or all of its copies of the *Tci-pgp-9* gene. As selection can act independently on each duplicated allelic variant, increasing its specificity (Prince & Pickett, 2002), it is conceivable that *T. circumcincta* can accumulate homozygous copies of the 'resistant allele variants'. Individuals possessing alleles that are susceptible to the action of the drug will succumb to the drug's toxic effects, removing the susceptible allele(s) from the population, thereby increasing the frequency of 'resistant' allele(s) that offer a survival advantage (Blackhall *et al.*, 1998). Therefore, changes in 'resistant' allele frequency in a population may indicate the resistance status of that population. Increases in homozygosity in a population can be a consequence of non-random mating due to inbreeding or continual drug selection (Bourguinat *et al.*, 2011) and the resistant genotype can accumulate in the parasite population until it causes the loss of drug efficacy in the target population (Prichard, 1990).

Caution must be applied when comparing the relative frequencies of allelic variant(s) in the UK isolates. The MTci2 and MTci5 isolates are not different generations of the same isolate but are unrelated field isolates, and therefore, natural genetic divergence between these isolates would be expected due to founder effect, bottleneck and genetic drift. Sequencing data were generated from both isolates with little variation between the UK isolates observed during the classification of allelic variants. Indeed, the identity shared in the *Tci-pgp-9-IBDA* variants identified in the unrelated, and geographically separate, UK and NZ isolates was >93 % (93-99 %). This is supported by the

relatively small Nei genetic distance estimates and relatively low contribution of among-population variance to total molecular genetic variance. Cloning was not exhaustive and additional allelic variants probably exist, although the most prevalent *Tci-pgp-9-IBDA* variants are likely to have been sampled and sequenced. Degenerate primers were used to increase the likelihood of amplifying the majority of *Tci-pgp-9-IBDA* sequence variants.

Due to the complexity of comparing the unrelated MTci2 and MTci5 isolates, the populations of worms that were collected after *in vivo* and *in vitro* treatment with IVM were more appropriate for comparisons with the starting isolate. The sub-populations MTci2$_{(NF0.1\mu M)}$ and MTci2$_{(F10\mu M)}$ were compared to the starting MTci2 isolate and the MTci5PT and MTci5$_{(NF0.1\mu M)}$ and MTci5$_{(F10\mu M)}$ sub-populations were compared to the starting MTci5 isolate, as these share a genetic background. One haplotype (UKv2,3,13) was significantly more common in successfully feeding MTci5 larvae after exposure to IVM *in vitro* and another (UKv3) was similarly less common in non-feeding MTci5 larvae after exposure to IVM *in vitro*.

Present results appear to be consistent with the hypothesis that polymorphisms in the *Tci-pgp-9* gene may have contributed at some level to the IVM-resistance in *T. circumcincta*. Further work is required to determine the gene copy number of each of the observed haplotypes, which should enable more precise classification of the haplotypes, and consequently, a more meaningful association of the frequency of allelic variants with resistance status. Another area for further work is to link the allelic variants identified in the *Tci-pgp-9-IBDA* (this chapter), with the SNP responsible for the amino acid substitutions identified during comparisons of *Tci-pgp-9* cDNA sequences from MTci2 and MTci5 isolates (Chapter 3).

Chapter 6

Relative Quantification of *Tci-pgp-9* Copy Number Using Real Time PCR

6.1 Introduction

Increased abundance of P-glycoprotein xenobiotic efflux pumps may result in a reduction in the interaction between anthelmintics and their molecular target within the cell, thus producing an anthelmintic resistance phenotype in parasitic nematodes. A greater abundance of a given gene product can be a result of increased *cis*- or *trans*- regulation of gene expression or gene amplification, which increases the number of the copies of a given gene in the genome. Gene duplication may provide a mechanism for increasing expression levels (Zhao *et al*., 2004).

Gene amplification events occur at frequencies that are orders of magnitude higher than the occurrence of point mutations of actively transcribed genes (Hastings *et al*., 2009). In some cases, amplification can enable mutations to persist that would otherwise be removed by purifying selection because it enables the retention of wild-type variants of the gene. The fact that amplification is also more likely to occur in areas of the genome that are subject to high levels of transcription (Hastings *et al*., 2009) means that genes coding for proteins that are involved in detoxification are likely to be subject to high rates of amplification. There are many examples of insecticide resistance in ectoparasites due to gene amplification, mostly as a result of esterases, glutathione transferases and mixed function oxidases (reviewed in Bass & Field, 2011). Gene amplification of members of the Pgp family has been implicated in drug resistance in protozoal parasites and also in resistance of cancer cell lines to chemotherapeutic drugs (Sidhu *et al*., 2006; Preechapornkul *et al*., 2009).

To date, there is evidence of increased expression of *Tci-pgp-9* specifically. Dicker *et al*. (2011b) demonstrated a significant constitutive elevation in expression of *Tci-pgp-9* NBD2 across all life-cycle stages when comparing the MTci2 and MTci5 isolates, most notably a 55.27-fold greater expression in eggs and a 17.49-fold increase in L_3 in the resistant worm population (Dicker, 2011b). Another study conducted on New Zealand isolates of *T. circumcincta* demonstrated an increase in the copy number of *Tci-pgp-9* NBD1 when multiple anthelmintic resistant worms were compared to their anthelmintic susceptible counterparts (Bisset, 2007).

Real time PCR is a routinely used method for the quantification of mRNA and, hence, of transcription of genes of interest relative to "housekeeping" genes that are not subject to variable transcription levels, and it can also be used to determine the number of copies of genes in genomic DNA relative to a gene of known copy number (Yuan *et al*., 2007). Real time PCR is used to simultaneously amplify and quantify a specific DNA target. In this study, SYBR dye was used as the reporter dye, which non-specifically intercalates into double-stranded DNA (dsDNA). The fluorescence is proportional to the concentration of dsDNA, allowing the quantification of dsDNA relative to a standard curve or relative to a reference gene. The fractional cycle number at which fluorescence crosses an arbitrarily placed threshold is defined as the C_T value (Schmittgen & Livak, 2008). The C_T value is also described as the quantification cycle (C_q) in the Minimum Information for Publication of Quantitative Real Time PCR Experiments (MIQE) guidelines (Bustin *et al*., 2009). The C_T values for both the reference gene (ß-tubulin) and the target gene (*Tci-pgp-9*) are recorded and the ΔC_T was calculated as the ratio between the reference and target genes for each larva. Comparing the mean ΔC_T values recorded in different isolates, using the comparative C_T ($\Delta\Delta C_T$) method (Livak & Schmittgen, 2001), allowed the standardisation of the expression of the target gene with respect to the non-regulated reference gene (Pfaffl *et al*, 2002). The comparative C_T method highlights the fold-change in abundance of the target gene in different isolates.

In Chapter 5, multiple allelic variants of the *Tci-pgp-9* gene were identified and larvae were shown to possess between 1 and 10 allelic variants. The differing incidence and combinations of allelic variants implies that multiple

copies of certain allelic variants exist. This Chapter investigates, using relative quantification real time PCR, the differences in abundance of the *Tci-pgp-9* gene in each of the MTci2, MTci5 and MTci5PT isolates. The methodology described in the Bisset (2007) study was closely followed. Using the larval lysates generated and analysed in Chapter 5, allowed further characterisation of the *Tci-pgp-9* gene in these individual larvae. The abundance of the *Tci-pgp-9* gene in the sub-populations of *T. circumcincta* larvae collected from the larval feeding inhibition assay (Chapter 4.3) was also investigated.

6.2 Materials & Methods

6.2.1 T. circumcincta Larvae

Further characterisation of the *Tci-pgp-9* gene was conducted on the crude gDNA lysates of individual larvae from the seven populations of *T. circumcincta* used in the analysis of allelic variants (Chapter 5). These consisted of 84 L_3 from two 'resting' populations (i.e. unexposed to anthelmintic), MTci2 and MTci5, and 80 L_3 from a population collected after *in vivo* exposure to IVM (MTci5PT). Two sub-populations of 43 L_1 from the MTci2 isolate, and 45 non-feeding L_1 and 47 feeding L_1 from the MTci5 isolate that were collected post *in vitro* IVM exposure (in Chapter 4).

6.2.2 Real Time PCR Analysis of UK Isolates of T. circumcincta

Real time PCR assays were performed using an ABI PRISM® 7500 Real Time PCR System (Applied Biosystems), using Power SYBR® green PCR mastermix (Applied Biosystems). All real time PCR reactions were carried out in MicroAmp® Optical 96-well Reaction Plates (Applied Biosystems) sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems), both of which are specifically designed for use in ABI real time PCR systems. Power SYBR® Green dye was selected due to its ability to quantify the amount of product generated during PCR by binding to double-stranded DNA and providing a fluorescent signal. The Power SYBR® mastermix contains SYBR® Green I Dye, AmpliTaq Gold® DNA polymerase, dNTPs and optimised buffer components. Each 20 µl real time PCR reaction consisted of 10 µl Power SYBR® Mastermix (2X), 0.15 µ*M* of each sense and antisense primer, and 1 µl gDNA template, made up to 20 µl with PCR grade

water (Sigma). Real time PCR thermocycling conditions were: 2 minutes at 50 °C and 10 minutes enzyme activation at 95 °C followed by 40 two-step cycles of 95 °C for 15 s (denature), and 60 °C for 1 minute (primer annealing and extension) with fluorescence detection at the end of this step. Dissociation curve analysis was conducted after the final cycle, which continuously measures fluorescence as the temperature is increased from 60 °C to 95 °C. Dissociation curve analysis displays a single defined melting curve with a narrow peak when a pure and homogenous real time PCR product has been generated. This analysis highlights the presence of primer dimers as a broader peak at relatively low temperatures compared to the expected PCR product.

The reference gene, ß-tubulin isotype-1 was selected as a reference gene because it has previously been shown to be present at a constant copy number in genomic DNA in a similar study into *Tci-pgp-9* (Bisset, 2007). A fragment of the ß-tubulin gene was generated from MTci2, MTci5 and MTci5PT isolates of *T. circumcincta* using primer pair 5'-CTTAGATGTTGTTCGTAAAGAGG-3' [TUBGF] and 5'-CATGTTCACAGCCAACTTGC-3' [TUBGR] (Bisset, 2007). The PCR product was inserted into a plasmid vector and amplified as described in Chapter 2.7.1 and used as the positive template control for ß-tubulin amplification in the real time PCR. The purified plasmid DNA was sequenced commercially by Eurofins/MWG/Operon and alignments were analysed using Lasergene® 10 bioinformatics software (DNASTAR Inc.). After confirmation that ß-tubulin isotype-1 had been amplified by primer pair TUBGF/TUBGR, primers for real time PCR were designed within this PCR fragment. To ensure the observed alleles of ß-tubulin gene were amplified from each of the UK isolates of *T. circumcincta*, a degenerate sense primer 5'-TGACGCATTCYTTGGGAGGAGG-3' [TUBRTGF2011] was paired with the antisense primer 5'-GAGAATGAAGCCATGATTCTATCCGG-3' [TUBRTGR2011], to amplify a 100 bp fragment (Appendix 7).

The *Tci-pgp-9* plasmid used as a positive template control during the real time PCR reactions was generated using the same method as for the ß-tubulin plasmid. The primers 5'-GAGTAGTKTCACARGARCCNATG-3' [IBD77GF4] and 5'-GCGCCATTCCACCACTTTCTTAG-3' [IBD77RAS12] (Appendix 8) were located in the first putative internucleotide binding domain, IBD77, of *Tci-pgp-9*. The real time PCR reaction used primers designed within a conserved region of gDNA sequence

identified during the haplotype analysis in Chapter 5. A short, 99 bp product from *Tci-pgp-9* IBD77 was amplified using the sense primer 5'-CGHTATGGACGTGAAAAAGTCACAGA-3' [IBD77RTGFdeg] along with the antisense primer 5'-CCAACTCACGTCRGGGAAYGACTG-3' [IBD77RTGRdeg], ensuring the *Tm* for each primer was closely matched, allowing use under the same thermocycling conditions. The degeneracy introduced to the IBD77RTGFdeg/IBD77RTGRdeg primer pair ensured that the known *Tci-pgp-9* allelic variants would be amplified during real time PCR. Non-template controls were included to verify the absence of gDNA contamination.

Amplification efficiencies of the ß-tubulin (TUBRTGF2011/TUBRTGR2011) and *Tci-pgp-9* (IBD77RTGFdeg/IBD77RTGRdeg) primer pairs were compared using 10-fold serial dilutions (10^{-9} to 10^{-15} grams) of the ß-tubulin and *Tci-pgp-9* plasmids produced above and a standard curve was plotted using the 7500 Sequence Detection Software Version 1.4 (Applied Biosystems). The efficiency of each primer pair was defined as $(10^{(-1/slope)}) - 1$ (Pfaffl, 2001), and for valid $\Delta\Delta C_T$ calculation, the amplification efficiencies of the target and reference genes should be approximately equal (Livak & Schmittgen, 2001). The amplification efficiency is 100 % when each PCR cycle doubles the number of amplicons and so the slope of the curve = -1 (Yuan *et al*., 2007).

Following confirmation of approximately equal amplification efficiencies of both primer sets, the reference gene (ß-tubulin), and the target gene (*Tci-pgp-9*) were amplified from each individual larva in duplicate reactions. Individual larval lysates generated in Chapter 2.5 were diluted 1:5 with PCR grade water (Sigma) and 1 µl was added as template for each reaction. The threshold was arbitrarily set at $\Delta Rn = 0.1$, where ΔRn was calculated by normalising the emission intensity of the reporter dye (SYBR) and passive reference dye (ROX) and subtracting this from the baseline fluorescence. The duplicate C_T results were averaged for the reference and target genes and the the ΔC_T for each individual larva was calculated using the following equation:

$$\Delta C_T = \text{Mean } C_{T\,(\text{target})} - \text{Mean } C_{T\,(\text{reference})}$$

The average ΔC_T for individuals from each isolate was calculated (Table 6.1) and the comparative ΔC_T method ($2^{-\Delta\Delta CT}$) was used to identify fold change differences in *Tci-pgp-9* gene abundance between isolates using the following equations:

$$\Delta\Delta C_T = \Delta C_{T\,(sample)} - \Delta C_{T\,(calibrator)},$$

$$\text{Fold Change} = 2^{-\Delta\Delta CT}$$

Investigations into the abundance of the *Tci-pgp-9* gene in the larval sub-populations collected from the LFIA (Chapter 4) were conducted. Crude gDNA lysates, generated using the method outlined in Chapter 2.5, were diluted 1:5 with with PCR grade water (Sigma) and 1 µl was added as template for each real time PCR reactions described in Chapter 6.2.1. The mean C_T and ΔC_T values for larvae from each sub-population were determined and comparative C_T analysis was conducted as described earlier (Chapter 6.2.1).

6.2.3 Data Analyses

Descriptive statistics were performed using Microsoft Excel (2007) and Minitab15 Statistical Software. Associations between ΔC_T and isolate were investigated using the Kruskal-Wallis and Mann-Whitney *U*-tests. The haplotypes, identified in Chapter 5, that were present in ≥ 5 individual larvae were plotted against the predicted copy number (using Minitab15 Statistical Software). A Kruskall-Wallis test was conducted to test for the effect of those haplotypes represented by ≥ 5 individual larvae on ΔC_T.

6.3 Results

6.3.1 Quantification of Tci-pgp-9 in UK Isolates of T. circumcincta

The initial step of quantitative real time PCR was to confirm the specificity of the primers used to amplify the reference and target genes. First the identity of the ß-tubulin plasmid construct was confirmed by comparing sequencing results with ß-tubulin sequences previously submitted to the NCBI database (available online at http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ß-tubulin plasmid sequence shared 99 % identity with ß-tubulin isotype-1 cDNA (Accession Number: Z69258.1) isolated from *T. circumcincta* (Elard *et al.*, 1996), and 93 % identity with the ß-tubulin gene (Accession Number: AJ550390.2) isolated from *O. ostertagi* (Van Zeveren *et al.*, 2007). Similarly, the *Tci-pgp-9* plasmid construction was verified, showing 98 % homology to a *Tci-pgp-9* gene (Accession number: FR691848.1) isolated from *T. circumcincta* (Dicker *et al.*, 2011b), 80 % identity with the *Hco-pgp-9* gene (Accession Number: HM635771.1) isolated from *H. contortus* (Williamson *et al.*, 2010, unpublished), and sharing 79 % identity with the IBD77 (Accession Number: AF055175.1) identified in ABC transporters of *H. contortus* (Sangster *et al.*, 1999). The high levels of identity shared with ß-tubulin and *Tci-pgp-9* sequences already submitted to the NCBI database, confirmed the specificity of the primers used in the real time PCR reactions and also showed that the respective plasmids were suitable for use as DNA template in the positive control reactions.

The next step in optimising the real time PCR reactions was matching the amplification efficiencies of the target and the reference genes to allow valid $\Delta\Delta C_T$ calculation (Livak & Schmittgen, 2001). For this, standard curves (shown in Figure 6.1 Panel *A*) were generated for ß-tubulin and *Tci-pgp-9* and amplification efficiencies compared. Ideally, each cycle results in a doubling of amplification product when the amplification is 100 % efficient (efficiency = 1). Using the standard curves, the efficiencies were defined as $(10^{(-1/\text{slope})}) - 1$ and the recorded amplification efficiencies were 0.96 and 1.01 for ß-tubulin and *Tci-pgp-9*, respectively. The amplification efficiencies are approximately equal, verifying the suitability of the primers specifically designed for the quantitative real time PCR.

Relative quantitative real time PCR was carried out in duplicate for the reference and target genes in each individual larva and the C_T values were measured and averaged. The ΔC_T was calculated by subtracting the mean $C_{T(\beta\text{-tubulin})}$ from the mean $C_{T(\textit{Tci-pgp-9})}$. Summaries of the C_T and ΔC_T values for β-tubulin and *Tci-pgp-9* amplification from the MTci2, MTci5 and MTci5PT isolates are shown in Table 6.1. The distribution of ΔC_T values for each larva along with its corresponding number of alleles identified in Chapter 5 was plotted (Figure 6.2).

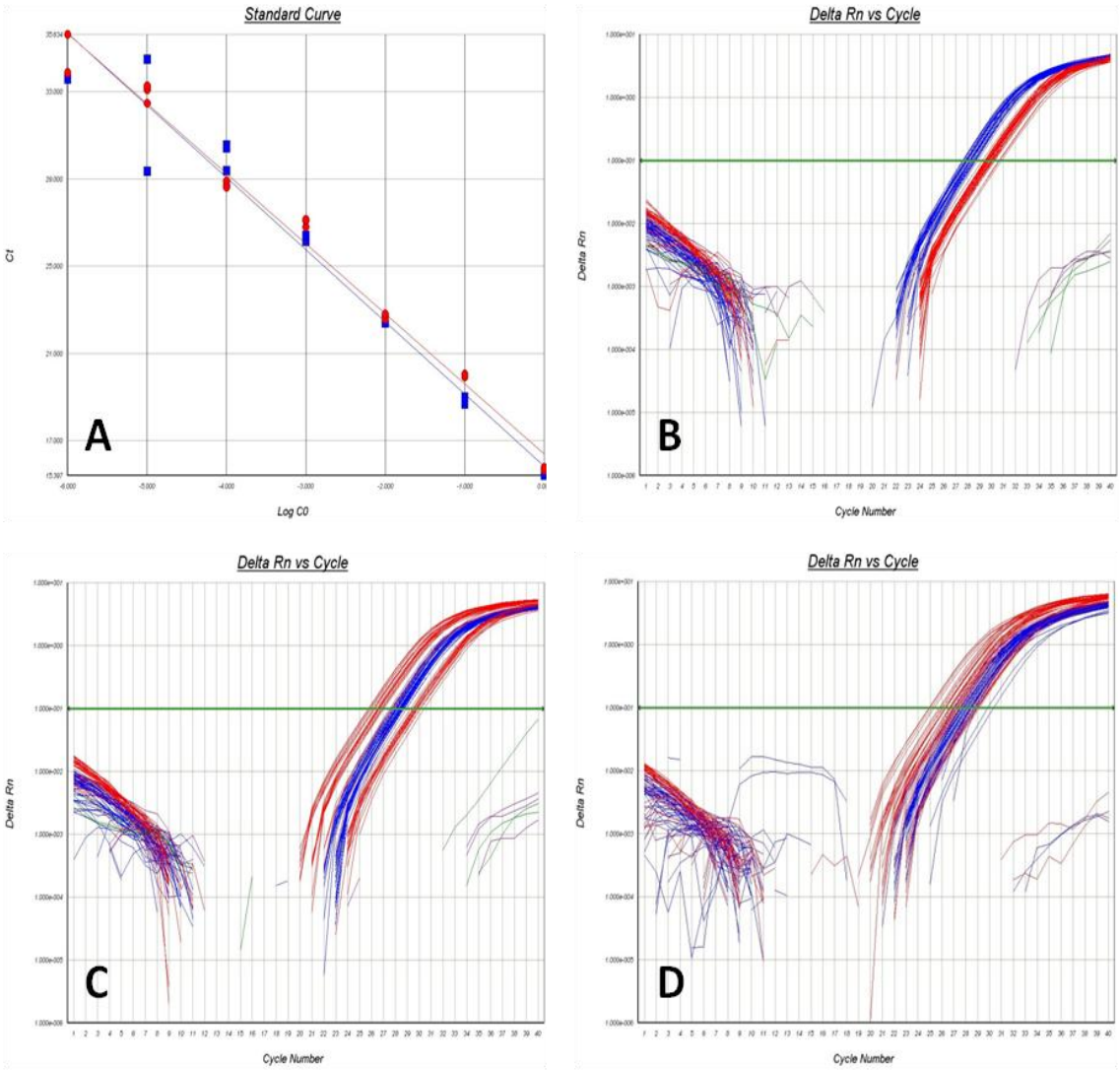


Figure 6.1 Real Time PCR Analyses of UK Isolates of *T. circumcincta*

Panel ***A*** shows serial 10-fold dilutions of ß-tubulin (blue) and *Tci-pgp-9* (red) plasmids used to calculate primer efficiencies. The slope of the ß-tubulin graph is -3.41 and the slope of the *Tci-pgp-9* graph is -3.29, these values were used to calculate the relative efficiencies of the reactions. The ß-tubulin (blue) and *Tci-pgp-9* (red) primer pairs were used in real time PCR to amplify products from the MTci2 (Panel ***B***), MTci5 (Panel ***C***), and MTci5PT (Panel ***D***) isolates. Non-template control reactions are shown for ß-tubulin (dark green) and *Tci-pgp-9* (purple) mastermixes. The C_T was set arbitrarily as ΔRn = 0.1 (Green line). Graphs generated using ABI PRISM® 7500 Real Time PCR System (Applied Biosystems).

Table 6.1 Summary of C_T Values from UK Isolates of *T. circumcincta*

C_T values were generated by real time PCR amplification of ß-tubulin and *Tci-pgp-9* gene fragments from individual larvae from each of the UK isolates of *T. circumcincta*. The target and reference genes were amplified in duplicate for each larva and the mean C_T value for each isolate was calculated along with the mean ΔC_T values. The range of values is also given in the table along with the standard error of the mean (SE).

Isolate	Mean C_T ± SE (range)		Mean ΔC_T ± SE (range)
	β-Tubulin	*Tci-pgp-9-IBDA*	
MTci2 (n = 80)	28.25 ± 0.05 (27.19 – 32.72)	29.77 ± 0.06 (28.16 – 35.00)	1.52 ± 0.03 (0.65 – 2.85)
MTci5 (n = 84)	28.39 ± 0.03 (27.04 – 29.84)	28.02 ± 0.12 (24.95 – 31.10)	-0.37 ± 0.12 (-3.47 – 2.04)
MTci5 PT (n =83)	28.40 ± 0.04 (27.13 – 30.53)	28.86 ± 0.11 (25.30 – 31.90)	0.45 ± 0.11 (-2.74 – 2.79)

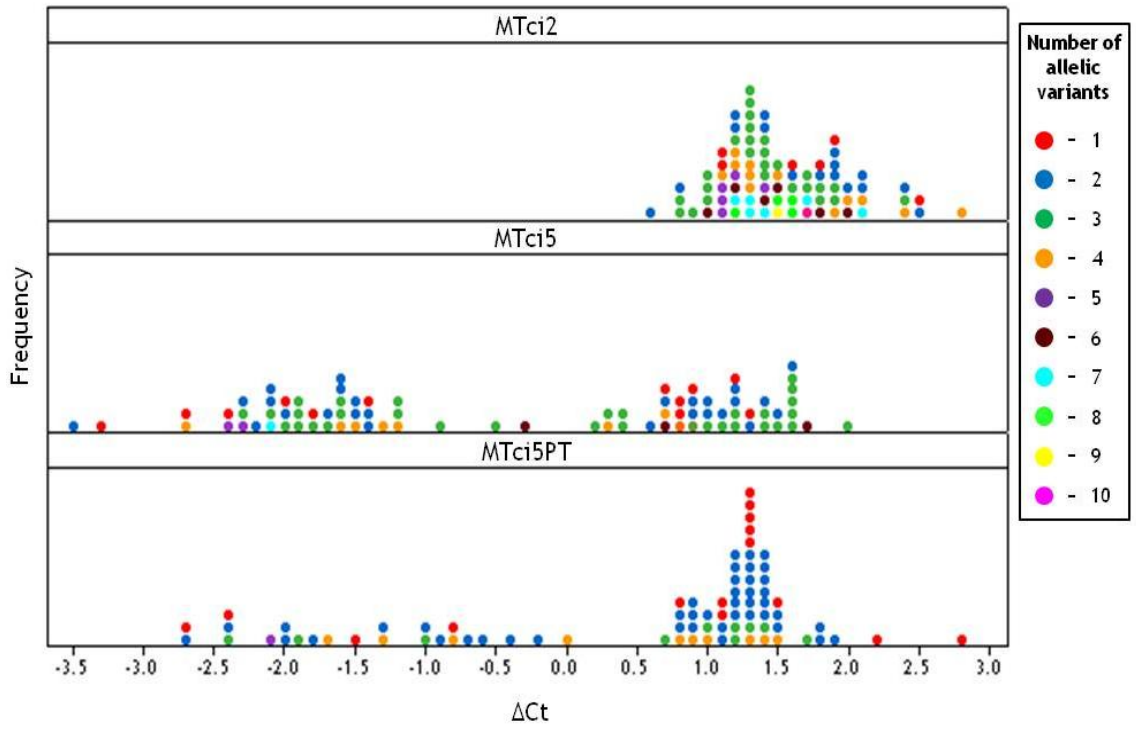


Figure 6.2 Distribution of ΔC_T Values from Each *T. circumcincta* Isolate

Dotplots showing the frequency and distribution of ΔC_T values for individual larvae in each isolate of *T. circumcincta*. Each dot represents a specific larva and the colour of the dot relates to the number of different *Tci-pgp-9* allelic variants identified within that individual. The top panel shows the MTci2 isolate, and the middle and lower dotplots show MTci5 and MTci5PT isolates, respectively. Low ΔC_T values indicate a high concentration of the target gene template relative to the reference gene, whilst high ΔC_T values indicate low concentrations of the target gene template relative to the reference gene.

The results of the real time PCR confirmed that ß-tubulin is a stable reference gene and shows no variation in apparent copy number between each of the isolates studied, as shown by approximately equal mean C_T values. Negative ΔC_T values indicate a higher abundance of target gene compared to the reference gene, and positive ΔC_T values indicate less target gene template relative to the reference gene. This suggests that more than 1 copy of the ß-tubulin isotype-1 gene exists in *T. circumcincta* and that individuals with positive ΔC_T values possess fewer copies of *Tci-pgp-9* relative to ß-tubulin. The range of mean C_T values for *Tci-pgp-9* in MTci2 larvae fell in the range of 28.16 – 35.00, indicating that this isolate is a relatively homogeneous population compared to the range of mean C_T values of the MTci5 and MTci5PT isolates, 24.95 – 31.10 and 25.30 – 31.90, respectively. The mean C_T values of ß-tubulin in the MTci5 and MTci5PT isolates were approximately equal, demonstrating that IVM treatment had no selective effect on the ß-tubulin gene. The *Tci-pgp-9* C_T values for larvae from MTci5 and MTci5PT isolates of *T. circumcincta* showed evidence of two distinct groups within each isolate (Figure 6.1, panels *C* and *D*). On closer inspection, the distribution of ΔC_T values for these isolates showed that MTci5 and MTci5PT isolates could be divided into two groups based on their ΔC_T values. The groupings, based on their *Tci-pgp-9* ΔC_T values, were significantly different (p <0.0001) within each of the MTci5 and MTci5PT isolates. Group A, consisted of larvae with ΔC_T values ≥ 0, and Group B which included larvae with ΔC_T values < 0. The mean ΔC_T values were calculated for the sub-groupings in the MTci5 and MTci5PT isolates (Table 6.2). No obvious relationship between number of allelic variants and ΔC_T value was observed when the frequency of larvae with similar ΔC_T values was plotted along with the number of allelic variants identified in that specific larva (Figure 6.2, data recorded in Appendix 12). Individuals from the MTci2 isolate were shown to have positive ΔC_T values, and have been shown to possess between 1 and 10 allelic variants in Chapter 5. Half of the MTci5 larvae tested (42/84) had a positive ΔC_T values and possessed between 1 and 6 allelic variants. The other half showed negative ΔC_T values and possessed between 1 and 7 allelic variants. The MTci5PT isolate was split into group A, with 55 individual larvae showing positive ΔC_T values and possessing between 1 and 4 allelic variants, the remaining individuals belonged to group B, which showed negative ΔC_T values and possessed between 1 and 5 allelic variants.

The sub-groups in the MTci5 and MTci5PT isolates were significantly different from each other (p <0.0001). Further comparisons were made between the MTci2 isolate and the grouping within the MTci5 and MTci5PT isolates (Table 6.3). The ΔC_T values were used in the comparative ΔC_T method ($2^{-\Delta\Delta CT}$) to identify fold change differences in *Tci-pgp-9* abundance between isolates. The calibrator ΔC_T was subtracted from the sample ΔC_T to give the $\Delta\Delta C_T$ value, which was expressed as fold change by $2^{-\Delta\Delta CT}$ (data for each larva is shown in Appendix 9). The abundance of *Tci-pgp-9* was compared between the MTci2, MTci5 and MTci5PT isolates and also compared to the sub-groupings within the MTci5 and MTci5PT isolates (Table 6.3). Initial comparisons using the comparative $\Delta\Delta C_T$ method showed on average, MTci5 lysates have a ~3.7-fold ($2^{1.89}$) greater abundance of *Tci-pgp-9* template than lysates from their MTci2 counterparts. Lysates from larvae collected post-IVM treatment (MTci5PT) showed ~2.1-fold ($2^{1.07}$) increase in *Tci-pgp-9* template compared to those of the MTci2 lysates. A ~0.6-fold ($2^{-0.82}$) difference in abundance of *Tci-pgp-9* was observed between the MTci5PT and MTci5 larval lysates. Comparisons between MTci2 larvae and individuals attributed to Group A of each MTci5 and MTci5PT isolates, showed little variation in the abundance of *Tci-pgp-9* gene, displaying fold increases of 1.36 and 1.18 respectively. A 10.06-fold increase in *Tci-pgp-9* gene was observed in MTci5 Group B, and a 7.78-fold increase in MTci5PT Group B, relative to MTci2 larvae.

Table 6.2 Sub-grouping of MTci5 and MTci5PT Isolates

Real time PCR results of MTci5 and MTci5PT isolates of *T. circumcincta* which were split into two groups based on their ΔC_T values. Group A are those larvae with ΔC_T values ≥ 0 and Group B consists of individuals with ΔC_T values < 0. The mean and range, along with the standard error of the mean (SE), of C_T values is shown for each sub-group and the calculated ΔC_T values.

Isolate	Mean C_T ± SE (range)		Mean ΔC_T ± SE (range)
	β-Tubulin	*Tci-pgp-9-IBDA*	
MTci5 Group A (n=42)	28.41 ± 0.04 (27.35 – 29.84)	29.48 ± 0.05 (28.61 – 31.10)	1.08 ± 0.05 (0.25 – 2.04)
MTci5 Group B (n=42)	28.37 ± 0.04 (27.04 – 29.34)	26.55 ± 0.07 (25.03 – 27.90)	-1.81 ± 0.07 (-3.47 – -0.27)
MTci5 PT Group A (n =58)	28.41 ± 0.05 (27.13 – 30.53)	29.69 ± 0.05 (28.59 – 31.90)	1.28 ± 0.03 (0.75 – 2.79)
MTci5 PT Group B (n =25)	28.37 ± 0.06 (27.49 – 29.88)	26.93 ± 0.12 (25.30 – 28.60)	-1.44 ± 0.16 (-2.74 – -0.01)

Table 6.3 Fold-changes of *Tci-pgp-9* Using the Comparative ΔC_T Method

The fold-change in *Tci-pgp-9* was calculated using the comparative ΔC_T method ($2^{-\Delta\Delta CT}$). The ΔC_T values for individuals from each isolate were not normally distributed and could not be transformed, therefore, non-parametric analyses were performed. For each comparison, the Mann-Whitney *U*-test was used to test for significant differences between isolates. Rows 1-3 of the table show the comparisons of ΔC_T from the MTci2, MTci5 and MTci5PT isolates. Rows 4 and 5 of the table are comparisons between the sub-groupings within the MTci5 and MTci5 isolates. Rows 6-9 show comparisons of ΔC_T between the MTci5 isolate and the sub-groupings of the resistant isolates (MTci5 and MTci5PT).

Comparison	Fold Change ($2^{-\Delta\Delta C_T}$)	Mann-Whitney *U*-test P value
MTci2 vs MTci5	3.71 ($2^{1.89}$)	<0.0001
MTci2 vs MTci5PT	2.10 ($2^{1.07}$)	<0.0001
MTci5 vs MTci5PT	0.57 ($2^{0.82}$)	0.0008
MTci5 A vs MTci5 B	7.41 ($2^{2.89}$)	<0.0001
MTci5PT A vs MTci5PT B	6.59 ($2^{2.72}$)	<0.0001
MTci5 vs MTci5 A	0.37 ($2^{-1.45}$)	<0.0001
MTci5 vs MTci5 B	2.71 ($2^{1.44}$)	<0.0001
MTci5PT vs MTci5PT A	0.56 ($2^{0.83}$)	0.0013
MTci5PT vs MTci5PT B	3.71 ($2^{1.89}$)	<0.0001

6.3.2 Quantification of Tci-pgp-9 in Sub-populations of MTci2 and MTci5

In Section 6.3.1, the MTci5 and MTci5PT isolates were split into two distinct groups based on their ΔC_T values. Another meaningful comparison was between the sub-populations collected during the LFIA in Chapter 4, which have been separated based on their ability to feed after exposure to different IVM concentrations. Each sub-population was analysed as described in Chapter 6.3.1 (Figure 6.3), mean C_T and ΔC_T values were calculated (Table 6.4) and the fold-change in *Tci-pgp-9* abundance was established using the comparative ΔC_T method (Table 6.5). Data for individual larvae are listed in Appendices 10 and 11.

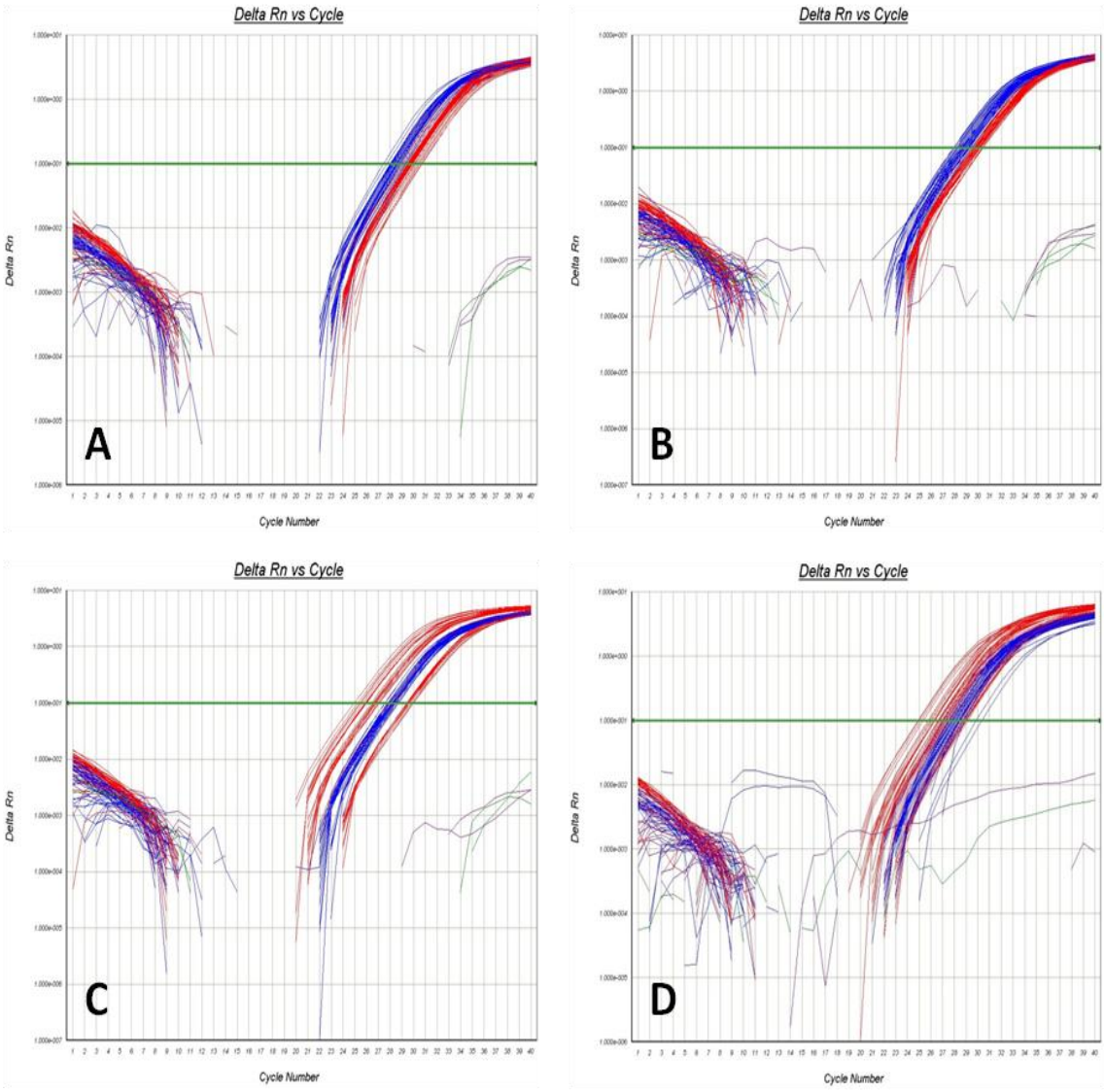


Figure 6.3 Real Time PCR Analyses of Sub-populations of MTci2 and MTci5

Larval lysates from sub-populations generated from the LFIA in Chapter 5 were used as the template gDNA for real time PCR analyses. The reference gene (ß-tubulin) is shown in blue and the target gene (*Tci-pgp-9*) is shown in red. Non-template negative controls are shown for the ß-tubulin (dark green) and *Tci-pgp-9* (purple) mastermixes. The green bar on the graph shows and arbitrarily set threshold at 0.1 ΔRn. Panel ***A*** shows $MTci2_{(NF0.1\mu M)}$, Panel ***B*** shows $MTci2_{(F10\mu M)}$, Panel ***C*** shows $MTci5_{(NF0.1\mu M)}$ and Panel ***D*** shows $MTci5_{(F10\mu M)}$. Graphs generated using ABI PRISM® 7500 Real Time PCR System (Applied Biosystems).

Table 6.4 Summary of C_T Values from Sub-populations of MTci2 and MTci5

Sub-populations of MTci2 and MTci5 isolates were collected based on their ability to feed after exposure to IVM (Chapter 4) and real time PCR was used to amplify the *Tci-pgp-9* gene. The ΔC_T values were calculated by comparing the target gene and the reference gene.

Isolate	Mean C_T ± SE (range)		Mean ΔC_T ± SE (range)
	β-Tubulin	*Tci-pgp-9-IBDA*	
$MTci2_{(NF0.1\mu M)}$ (n = 42)	28.45 ± 0.05 (27.28 – 29.95)	29.63 ± 0.05 (28.40 – 30.91)	1.18 ± 0.04 (-0.03 – 2.06)
$MTci2_{(F10\mu M)}$ (n = 43)	28.50 ± 0.04 (27.36 – 29.31)	29.63 ± 0.05 (27.37 – 30.34)	1.13 ± 0.04 (-0.05 – 1.68)
$MTci5_{(NF0.1\mu M)}$ (n = 40)	27.88 ± 0.06 (26.56 – 29.39)	27.06 ± 0.17 (24.58 – 29.82)	-0.81 ± 0.18 (-3.59 – 2.29)
$MTci5_{(F10\mu M)}$ (n = 46)	27.90 ± 0.06 (26.38 – 30.29)	26.99 ± 0.16 (24.27 – 30.18)	-0.92 ± 0.17 (-3.28 – 2.46)

The C_T values for ß-tubulin and *Tci-pgp-9* genes in individual larvae were measured for the sub-populations of the MTci2 and MTci5 isolates. The C_T values for ß-tubulin were consistent with those measured previously (Chapter 6.3.1), further supporting the use of ß-tubulin as a stable reference gene with the same copy number in all individuals examined. The ΔC_T values were calculated and plotted along with the number of allelic variants found in each individual larva (Figure 6.4). The characterisation of *Tci-pgp-9-IBDA* allelic variants and ΔC_T values for the sub-populations of MTci2 and MTci5 are recorded in Appendices 13 and 14. Individuals in the $MTci2_{(NF0.1\mu M)}$ sub-population possessed between 2 and 5 allelic variants and 42/43 larvae had positive ΔC_T values, with the exception of one larva with a ΔC_T value of -0.03. The $MTci2_{(F10\mu M)}$ sub-population displayed 1-5 allelic variants and, like the $MTci2_{(NF0.1\mu M)}$ sub-population, 42/43 larvae had positive ΔC_T values with the exception of one larvae with a ΔC_T of -0.5 measured. Between 1 and 4 allelic variants were present in the $MTci5_{(NF0.1\mu M)}$ sub-population of larvae and one third of larvae in this sub-population (14/44) had positive ΔC_T values whilst the remaining two thirds (30/44) exhibited negative ΔC_T values. Similarly, the $MTci5_{(F10\mu M)}$ sub-population possessed between 1 and 5 allelic variants, one third of the $MTci5_{(F10\mu M)}$ sub-population (15/47) displayed positive ΔC_T values and two thirds (32/47) had negative ΔC_T values.

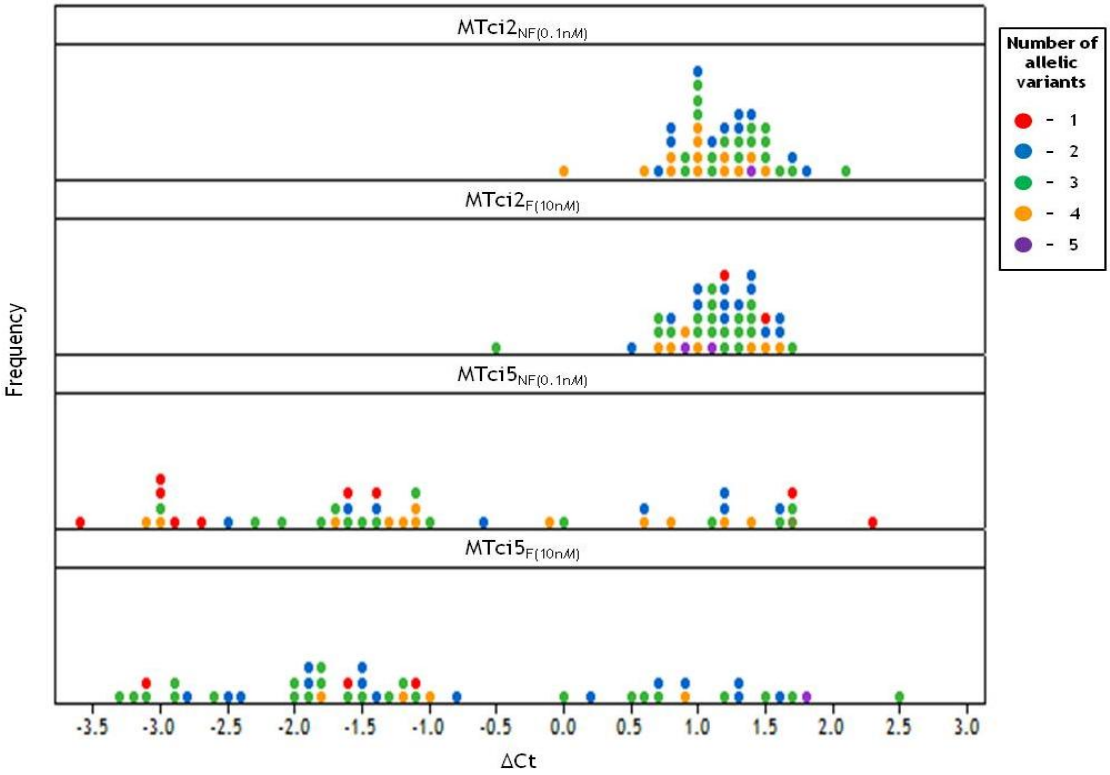


Figure 6.4 Distribution of ΔC_T values from Each *T. circumcincta* Sub-population

Dotplots showing the frequency and distribution of ΔC_T values for individual larvae in sub-populations of the MTci2 and MTci5 isolates separated using the LFIA bioassay. Each dot represents a specific larva and the colour of the dot relates to the number of different *Tci-pgp-9* allelic variants identified within that individual. The top two panels show the sub-populations of MTci2 isolate ($MTci2_{(NF0.1\mu M)}$ and $MTci2_{(F10\mu M)}$), and the lower dotplots show sub-populations of MTci5 isolate ($MTci5_{(NF0.1\mu M)}$ and $MTci5_{(F10\mu M)}$).

The comparative C_T method was used to ascertain the fold-change in the abundance of *Tci-pgp-9* between populations collected within each isolate after either *in vivo* or *in vitro* exposure to IVM. Comparisons were conducted (Table 6.5), and showed that there were significant differences (p <0.05) between sub-populations within the MTci2 isolate but not within the MTci5 isolate (p >0.05). Comparisons of the 'resting' MTci2 population with the sub-populations collected after exposure to IVM *in vitro* showed slight increases in *Tci-pgp-9* abundance with fold increase ~1. When comparing the MTci5PT worms collected after *in vivo* IVM exposure with the $MTci5_{(NF0.1\mu M)}$ and $MTci5_{(F10\mu M)}$ larvae that were exposed to IVM *in vitro*, *Tci-pgp-9* was shown to be 2.39-fold and 2.58-fold more abundant, respectively. A comparison was conducted with what were expected to be the "most resistant" MTci5 subpopulation ($MTci5_{(F10\mu M)}$) and the "least

resistant" MTci2 subpopulation ($MTci2_{(NF0.1\mu M)}$) . This comparison showed a highly significant difference (p <0.0001), and a 4.29-fold increase in *Tci-pgp-9* gDNA template abundance was observed. This increase in *Tci-pgp-9* abundance is greater than the 3.71 fold change observed when comparing the 'resting' MTci2 and MTci5 isolates. There were no significant differences (p >0.05) between the mean ΔC_T values when comparing MTci5 with $MTci5_{(NF0.1\mu M)}$, and $MTci5_{(NF0.1\mu M)}$ with $MTci5_{(F10\mu M)}$.

Table 6.5 Comparative ΔC_T values for *Tci-pgp-9* populations Derived from MTci2 and MTci5

Fold changes in the abundance of *Tci-pgp-9* template were calculated from the mean ΔC_T values in Table 6.4. Sub-populations from each isolate were compared within and isolate, and a final comparison between $MTci2_{(NF0.1\mu M)}$ and $MTci5_{(F10\mu M)}$ was made.

Comparison	Fold Change ($2^{-\Delta\Delta CT}$)	Mann-Whitney *U*-test P value
MTci2 vs $MTci2_{(NF0.1\mu M)}$	1.27 ($2^{0.34}$)	0.0004
MTci2 vs $MTci2_{(F10\mu M)}$	1.31 ($2^{0.39}$)	<0.0001
$MTci2_{(NF0.1\mu M)}$ vs $MTci2_{(F10\mu M)}$	1.04 ($2^{0.05}$)	0.0190
MTci5 vs MTci5PT	0.57 ($2^{-0.82}$)	0.0004
MTci5 vs $MTci5_{(NF0.1\mu M)}$	1.36 ($2^{0.44}$)	0.3382
MTci5 vs $MTci5_{(F10\mu M)}$	1.46 ($2^{0.55}$)	0.0889
MTci5PT vs $MTci5_{(NF0.1\mu M)}$	2.39 ($2^{1.26}$)	0.0004
MTci5PT vs $MTci5_{(F10\mu M)}$	2.58 ($2^{1.37}$)	<0.0001
$MTci5_{(NF0.1\mu M)}$ vs $MTci5_{(F10\mu M)}$	1.08 ($2^{0.11}$)	0.6767
$MTci2_{(NF0.1\mu M)}$ vs $MTci5_{(F10\mu M)}$	4.29 ($2^{2.10}$)	<0.0001

When $\Delta\Delta C_T$ values relative to ß-tubulin were back-transformed to estimate copy number, all values for which $\Delta\Delta C_T > 0$ resulted in a copy number estimate <1. Because it is theoretically not possible to have $0 < n < 1$ copies of a gene in an individual larva, it was assumed that there must be multiple copies of ß-tubulin in the *T. circumcincta* genome. The smallest multiple (number of copies of ß-tubulin) required to convert all of the values for *Tci-pgp-9* to a value ≥ 1 was 5. After this conversion, predictions of copy number ranged from 1 to 60, with a median of 2 and a mean of 7.4. Figure 6.5 is a histogram of the distribution of predicted copy number for all samples and shows that the majority of larvae were predicted to have 2 copies of *Tci-pgp-9*, while small numbers of larvae had multiple copies. Figure 6.6 is a boxplot of the predicted copy number for individuals from each of the haplotypes that was represented by ≥ 5 individual larvae. Haplotype had a significant effect on C_T and consequently on predicted copy number (Table 6.6).

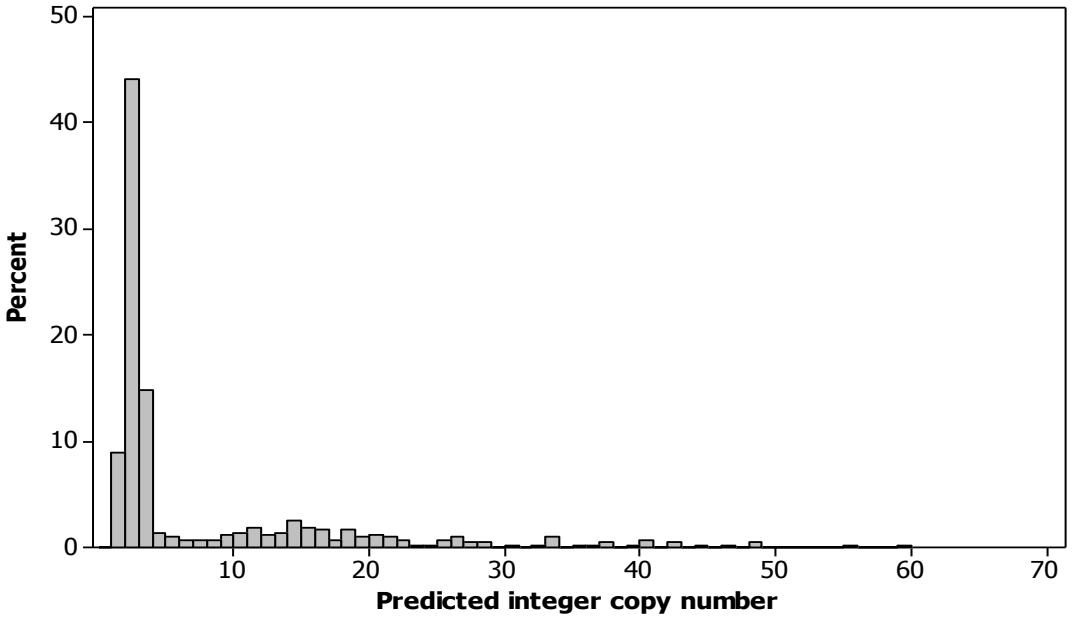


Figure 6.5 Distribution of Predicted *Tci-pgp-9* Copy Number

A histogram showing distribution of the predicted copy number of *Tci-pgp-9* from all 439 larval lysates representing UK isolates of *T. circumcincta*.

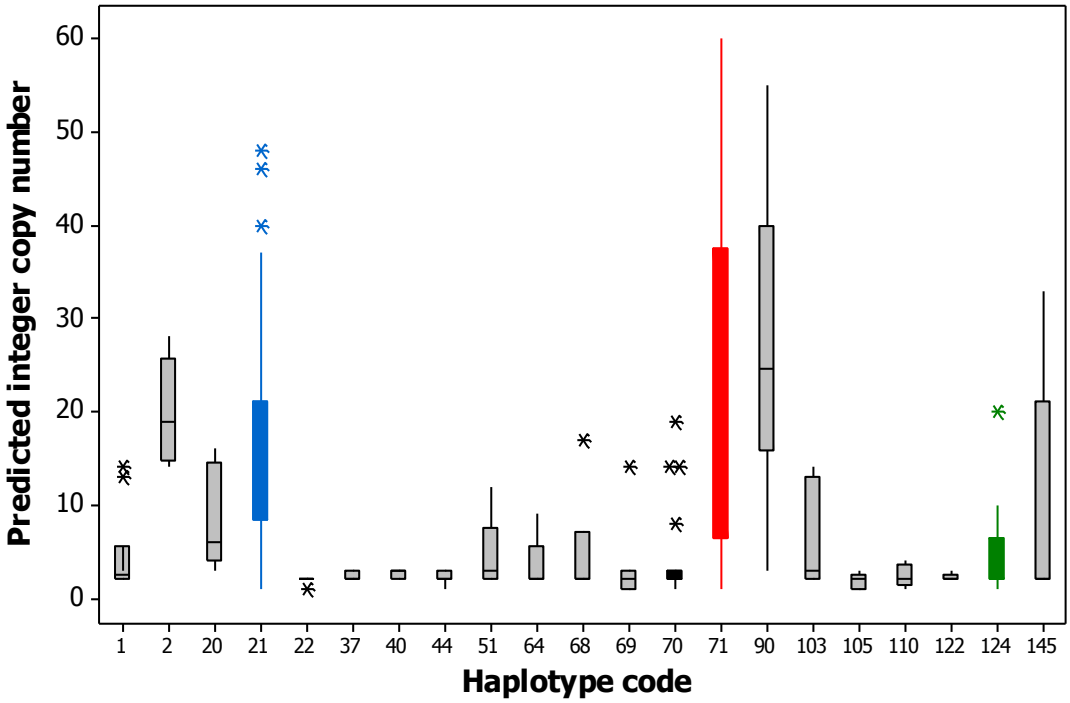


Figure 6.6 Boxplot of Predicted Copy Number for Larval Lysates of *T. circumcincta* From Haplotypes Shared by ≥ 5 Individuals

Some haplotypes of interest from Chapter 5 are highlighted. The most common haplotype over all populations combined was Haplotype 70 (UKv2, 13; black box), which was represented by 33 individuals. Haplotype 124 (UKv5, 12; 19 individuals, green box) was the most common haplotype in MTci5PT larvae. Haplotype 21 (UKv2,3,13; 30 individuals, blue box) was significantly more common in MTci5 larvae that fed successfully when exposed to IVM, whereas Haplotype 71 (UKv3; 21 individuals, red box) was present at an unexpectedly higher frequency in those that failed to feed.

Table 6.6 The Effect of Haplotypes Represented by ≥ 5 Individual Larvae on ΔC_T

All of the larval lysates generated from UK isolates of *T. circumcincta* (n = 232) were included in a Kruskall-Wallis test for the effect of those haplotypes represented by ≥ 5 individual larvae on ΔC_T.

Haplotype Code	N	Median	Ave Rank	Z
1	10	1.0145	121.0	0.22
2	6	-1.9232	33.2	-3.08
20	5	-0.1399	71.6	-1.51
21	29	-1.8187	57.2	-5.08
22	7	1.4409	185.6	2.76
37	5	1.2356	141.9	0.86
40	9	0.9804	123.8	0.33
44	26	1.3125	159.3	3.45
51	5	0.9661	131.2	0.50
64	5	1.3642	161.2	1.51
68	7	1.2130	130.4	0.56
69	11	1.4734	162.2	2.31
70	33	1.1789	137.4	1.93
71	21	-1.9521	59.4	-4.09
90	6	-2.2712	32.7	-3.10
103	9	0.8731	102.4	-0.64
105	5	1.4755	177.0	2.04
110	5	1.2064	144.6	0.95
122	5	1.1166	134.8	0.62
124	18	1.1518	133.8	1.14
145	5	1.2356	117.3	0.03
Overall	**232**		**116.5**	

H = 95.50 D.F. = 20 P = 0.000; H = 95.50 DF = 20 P = <0.001 (adjusted for ties)

6.4 Discussion

Further characterisation of the *Tci-pgp-9* gene in individual larvae from UK isolates of *T. circumcincta* was conducted. In this Chapter, relative quantification real time PCR analysis was used to measure the abundance of *Tci-pgp-9* gDNA, relative to the reference gene, ß-tubulin, in individual larvae. Two ß-tubulin isotypes had been identified in *H. contortus* (Kwa *et al.*, 1994) at the time of current study, and since then, Saunders *et* al. (2013) reported the existence of an additional two ß-tubulin isotypes in *H. contortus*. The liver fluke, *Fasciola hepatica*, has been shown to express six ß-tubulin isotypes (Ryan *et al.*, 2008). The real time data would suggest that at least 5 copies of the ß-tubulin isotype-1 gene are present in the *T. circumcincta* tested in the present study. The reference gene, B-tubulin isotype-1, was selected as it has previously been used in real time PCR analysis study into the abundance of *Tci-pgp-9* by Bisset (2007) where it showed consistent copy number under test conditions. Endogenous reference genes must be carefully selected in parasitic systems since high variability has been noted with routinely used genes such as actin and glyceraldehyde-3-phosphate dehydrogenase (Trivedi & Arasu, 2005). There are few reports of possible reference genes for analysing gene abundance in *T. circumcincta*. Baker *et al*. (2011) evaluated possible reference genes and identified elongation factor-1α and ß-tubulin as the most suitable for gene expression analysis studies in *T. circumcincta*. Comparisons of ß-tubulin ΔC_T values, observed in various isolates of *T. circumcincta* in this study, supported its use as a reference gene, due to its relatively uniform abundance in these larvae.

The abundance of *Tci-pgp-9* relative to this reference gene, the ΔC_T value, was attributed to each larva from the MTci2, MTci5 and MTci5PT isolates studied, as well as the sub-populations of MTci2 and MTc5 collected from the LFIA bioassay (Chapter 4). Negative ΔC_T values indicated a higher abundance of *Tci-pgp-9* relative to ß-tubulin within the individual larvae, whereas a positive ΔC_T value was a sign of a lower abundance *Tci-pgp-9* relative to ß-tubulin, implying that there are multiple copies of ß-tubulin present in the *T. circumcincta* genome.

Using the Comparative C_T Method (Pfaffl *et al.*, 2002), comparisons between the mean ΔC_T values from different isolates were performed. Initially the "resting" larval isolates, MTci2 and MTci5, and a post-IVM treated population of MTci5 (MTci5PT) were studied. There was a 3.7-fold higher abundance of *Tci-pgp-9* gDNA in the multiple anthelmintic resistant isolate (MTci5) when compared to the anthelmintic susceptible isolate (MTci2). This finding concurs with a previous finding in NZ, where an anthelmintic resistant isolate of *T. circumcincta* showed a 3.4-fold increase in *Tci-pgp-9* gene abundance when compared with its near-isogenic susceptible counterpart (Bisset, 2007). The higher *Tci-pgp-9* copy number may, in part, explain the 17.49-fold increase in expression of the *Tci-pgp-9* NBD2 shown in L_3 from the MTci5 relative to MTci2, observed in the Dicker *et al*. (2011b) study. Given that a full Pgp molecule consists of two NBDs, it would be expected that an increase in expression of *Tci-pgp-9* NBD2 would result in an equivalent increase in *Tci-pgp-9* IBD77 (NBD1). Direct comparisons between the copy number and mRNA expression levels were not possible within this project although an increase in copy number is likely to relate to an increased expression level of that gene.

Larvae collected post-IVM treatment (MTci5PT) displayed a 2.1-fold higher abundance of *Tci-pgp-9* gene compared to the MTci2 isolate, and a lower abundance relative to the MTci5 isolate (0.57-fold difference). Upon closer inspection of the distribution of MTci5 and MTci5PT ΔC_T values, two significantly different groups, $p < 0.0001$ became apparent (Figure 6.2). Individuals with ΔC_T values ≥ 0 were allocated to Group A and those with ΔC_T values < 0 were allocated to Group B. Members of Group A from both the MTci5 and MTci5PT isolates possessed a similar quantity of *Tci-pgp-9* gene, relative to ß-tubulin gene. The abundance of *Tci-pgp-9* in members belonging to Group B was increased 10.06-fold and 7.78-fold when compared to the Group A in the MTci5 and MTci5PT isolates, respectively.

The MTci5 isolate was previously shown to comprise of a homogeneous mixture of multiply-resistant individuals (Bartley *et al.*, 2004; Bartley *et al.*, 2005; Stenhouse, 2007). For an isolate to be considered resistant, according to the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines, the reduction in egg count, using the FECRT, has to be less than 95 % and the lower 95 % confidence interval less than 90 % (Coles *et al.*, 1992). The

MTci5 isolate, for which IVM efficacy is only 60 % (Bartley *et al.*, 2004; Bartley *et al.*, 2005), is likely to represent a heterogeneous population of *T. circumcincta* where individuals display differing degrees of anthelmintic resistance. This may explain the observation of two distinct groups within the MTci5 isolate: Group A might contain individuals in the MTci5 population that are able to survive exposure to anthelmintics utilising alternative mechanisms associated with drug metabolism to avoid the action of anthelmintics, rather than relying on the efflux facilitated by Pgp molecules. Group B may contain larvae which potentially possess higher drug efflux efficiency due to the increased abundance of *Tci-pgp-9* gene (Group B).

Sub-populations of MTci2 and MTci5 isolates were collected based on their feeding phenotype when exposed to a range of IVM concentrations *in vitro*. The sub-populations collected were individuals whose feeding was inhibited when exposed to 0.1 µ*M* IVM (annotated as $MTci2_{(NF0.1\mu M)}$ and $MTci5_{(NF0.1\mu M)}$) and individuals who retained the ability to feed when exposed to 10 µ*M* IVM (annotated as $MTci2_{(F10\mu M)}$ and $MTci5_{(F10\mu M)}$). These sub-populations were considered to be the 'least resistant and 'most resistant' individuals of the initial population and, as such, genetic comparisons were made between these phenotype-selected sub-populations at either end of the resistance spectrum. Comparisons between $MTci2_{(NF0.1\mu M)}$ and $MTci2_{(F10\mu M)}$ sub-populations showed no significant difference in the abundance of *Tci-pgp-9* gDNA, which was also observed in the MTci5 sub-populations. This finding suggests that the differences in phenotype between the non-feeding larvae exposed to 0.1 µM IVM and the larvae, from the same isolate, that retained the ability to feed when exposed to 10 µM IVM, were not due to increased abundance of the *Tci-pgp-9* gene. Comparing the most diverse sub-populations, $MTci2_{(NF0.1\mu M)}$ and $MTci5_{(F10\mu M)}$, showed a 4.29-fold increase abundance of *Tci-pgp-9* transcript. This is higher than the fold changes observed in the initial 'resting' populations of MTci5 and MTci5PT (3.71- and 2.10-fold respectively), when compared to the MTci2 isolate, suggesting that the sub-populations are more homogenous than the initial populations. Similarly, when the MTci5 and MTci5PT isolates were split into homogenous sub-groups an increase in the fold change of *Tci-pgp-9* abundance was observed when compared to the starting population.

Future work should focus upon the copy number of specific allelic variants in individual larvae. The most suitable approach would be to use TaqMan probes to increase the specificity of allele-specific quantitative PCR (von Samson-Himmelstjerna *et al*., 2003), and significantly increase in sample throughput, since no post-PCR steps are required with this procedure (Coles *et al*., 2006). The allelic variants of *Tci-pgp-9-IBDA*, identified in the present study, may form a starting point for future work of this nature in *T. circumcincta*. This may shed light on which of the *Tci-pgp-9-IBDA* allelic variant(s) have been duplicated and the number of copies of each allelic variant present within individual larva.

Chapter 7

General Discussion

P-glycoproteins in general have been implicated in the underlying genetics of IVM resistance in gastrointestinal nematodes. One specific Pgp, *Tci-pgp-9*, has been associated with IVM-resistance (and multi-drug resistance) in *T. circumcincta*, the most important gastrointestinal nematode of small ruminants in the UK. The overall aim of the present study was to further characterise this specific Pgp and its putative role in resistance in this species.

The ABC-transporter superfamily, to which P-glycoproteins belong, is an ubiquitous and evolutionarily ancient group of proteins found in species ranging from bacteria to humans (Zimniak *et al.*, 1999; Jones & George, 2005). P-glycoproteins were the first multi-drug resistance transporter molecules found to be involved in the efflux of MLs (Schinkel *et al.*, 1994; Lankas *et al.*, 1997) and are found in high quantities in the intestine and in the blood-tissue barriers, where their role is to protect the cells from exogenous and endogenous cytotoxic compounds (Kerboeuf *et al.*, 2003). Likened to a molecular "hydrophobic vacuum cleaner" by Aller *et al.* (2009), Pgps pull substrates from the plasma membrane and expel them into the extracellular compartment to circumvent or subvert drug action and promote drug resistance. Studies into anthelmintic resistance have, typically, tended to focus on the drug-target, whereas 'non-specific' mechanisms have been less extensively studied (Riou *et al.*, 2003). An understanding of Pgps, in terms of genetic polymorphisms, quantity and expression is the first step in understanding the role that these proteins play in anthelmintic resistance.

Two independent studies into Pgp expression and its association with anthelmintic resistance in *T. circumcincta* were conducted and found increased expression and polymorphisms in Pgps of multiple anthelmintic resistant *T. circumcincta* (Bisset, 2007; Dicker, 2010). Both groups showed that one Pgp in particular appeared to be associated with an IVM resistant phenotype, and classified it *Tci-pgp-9*. Bisset (2007) reported gene amplification, alternative splicing and four possible coding SNPs in *Tci-pgp-9* when comparing near-isogenic strains. Dicker (2010) identified greater constitutive expression of *Tci-pgp-9*

NBD2 in the MTci5 isolate at all lifecycle stages relative to the MTci2 isolate (Dicker *et al.*, 2011b). The present study followed on from these two projects, and focused on understanding the involvement of *Tci-pgp-9* in IVM resistance in UK isolates of *T. circumcincta*. The full-length *Tci-pgp-9* cDNA sequence was generated, for the first time, from two unrelated UK field isolates, MTci2 and MTci5. Comparisons between these isolates revealed nine non-synonymous SNPs, which altered the coding of six amino acids which were found in different frequencies in susceptible (MTci2) and resistant (MTci5) populations. Interestingly, four substitutions at amino acids 662-664 and 697 identified in the MTci5 isolate were also present in the NZ anthelmintic resistant near-isogenic strain (Bisset, 2007). However, the association of these SNPs with IVM-resistance remains unclear, due to the fact that these isolates are genetically unrelated. Before being considered as potential markers of IVM-resistance in this species, the presence of these point mutations in the *Tci-pgp-9* gene should be confirmed in additional *T. circumcincta* isolates. At this point the SNPs are unlikely to be suitable for a molecular based diagnostic test, but that was not the over-riding objective of study. To be considered as a potential diagnostic marker of anthelmintic resistance, 1) the allele must be enriched in a resistant population and the level of resistance in a resistant population should be proportional to the level of enrichment, 2) the frequency of the candidate allele would be expected to increase in the nematode population after challenge with the relevant anthelmintic, 3) changes in the sequence or level of expression of the allele that reduce its potency should have a functional effect and be supported by biochemical or pharmacological evidence, and 4) confer resistance when introduced experimentally into susceptible populations (McCavera *et al.*, 2007). Future work could focus upon the substitutions at residues 662-664 and 667 in additional isolates of *T. circumcincta* and the use of pyrosequencing assays could clarify the frequency of these SNPs within different isolates of *T. circumcincta*.

The full-length cDNA *Tci-pgp-9* sequences generated from the MTci2 and MTci5 isolates contained ~4.5 % polymorphism, suggesting that gene amplification of *Tci-pgp-9* may be responsible for the IVM-resistance phenotype, rather than the presence of non-synonymous SNPs. Increased expression of *Tci-pgp-9* has previously been observed when comparing the MTci2 and MTci5 isolates (Dicker *et al.*, 2011B). Amplification of *Tci-pgp-9* gene has also been

observed in NZ anthelmintic resistant near-isogenic strains of *T. circumcincta* (Bisset, 2007).

To allow for meaningful genetic comparisons between the UK isolates of *T. circumcincta*, sub-populations derived from MTci2 and MTci5 were collected. Firstly a population of worms representing the progeny of adult worms that survived *in vivo* exposure to IVM (MTci5PT) was coprocultured to L_3. Secondly, two sub-populations from each of the MTci2 and MTci5 isolates were collected based on their feeding phenotype after *in vitro* exposure to IVM. Individual larvae from these seven populations in total were used in subsequent genetic analyses focussing primarily on the *Tci-pgp-9-IBDA* region.

Detailed cloning and sequencing analysis of the UK isolates of *T. circumcincta* revealed twelve allelic variants of *Tci-pgp-9*, eight of which shared 93 - 99 % identity with allelic variants identified in NZ near-isogenic strains of *T. circumcincta*, and four of which were novel variants, found only in the UK isolates. Cloning could not be completely exhaustive and additional allelic variants probably exist, although the most prevalent variants are likely to have been sampled and sequenced. More than two allelic variants were observed per individual, indicating gene amplification in this diploid organism. Quantitative real time PCR results using gDNA also showed that copy number and number of alleles are consistent with gene amplification. Unique combinations of allelic variants were designated a haplotype number, and when combined with the predicted copy number, analysis showed that haplotype had a strong effect on predicted copy number. Comparisons between the abundance of *Tci-pgp-9* in MTci2 and MTci5 showed a 3.7-fold increase in the resistant isolate, which corresponds well with the 3.4-fold increase in *Tci-pgp-9* abundance reported in the NZ near-isogenic resistant strain of *T. circumcincta* (Bisset, 2007).

The copy number of the reference gene ß-tubulin, used in the real time PCR analyses, is unknown and it cannot be ruled out that ß-tubulin could be under anthelmintic selection when treated and so may not be suitable for use a reference gene. There is some evidence that BZ & ML-R are linked through ß-tubulin, at least in *Haemonchus contortus* (de Lourdes Mottier & Prichard, 2008). This interpretation was complicated in the present study because the resistant isolate, MTci5, is already demonstrably BZ-resistant (P. Skuce, pers. comm.).

The copy number for ß-tubulin was arbitrarily set at 5 copies as this was the smallest multiple required to convert all of the $\Delta\Delta C_T$ values for *Tci-pgp-9* to a value ≥ 1. Subsequent analysis indicated that the majority of larvae were predicted to have 2 copies of *Tci-pgp-9*, while small numbers of larvae had multiple copies, as many as 50 in some individuals. The most common haplotype over all populations was UKv2, 13, which had a median predicted copy number of 2, while the haplotype (UKv2, 3, 13) that was significantly more frequent in the feeding larvae of MTci5 exposed to IVM had a median predicted copy number of 19 and the haplotype (UKv3) that was significantly more common in non-feeding larvae exposed to IVM had a median predicted copy number of 18.

In summary, the present study revealed that multiple allelic variants were present in the majority of individuals, and a reduction in polymorphism, as defined by a reduced number of allelic variants, in individuals of MTci5 relative to the MTci2 isolate. A further reduction in the number of alleles present in individuals was also observed in individuals derived from an IVM treated population of MTci5, suggesting that IVM treatment applied purifying selection pressure. Quantitative real time PCR analysis showed a 3.7-fold higher *Tci-pgp-9* gene copy number in the MTci5 isolate relative to the MTci2 isolate, which was consistent with a 3.4-fold increase observed in the NZ study (Bisset, 2007). None of the common haplotypes identified appeared to be unique to any given isolate, and the relationship between haplotype and copy number was not straightforward.

To continue the work presented in this study, the next step would be to conduct allele-specific quantitative PCR using TaqMan probes (e.g. von Samson-Himmelstjerna *et al.*, 2003). This would provide insight into the number of copies of specific variants present in individual larvae and how this relates to their resistance status. The bioassays that were used as a tool in this study for separating the least and most resistant individuals within a population did not prove as useful as hoped and remain to be fully optimised for use with *T. circumcincta*. The genetic analyses in the present study focused on a specific region of *Tci-pgp-9* (*Tci-pgp-9-IBDA)*, which appeared to be under anthelmintic selection. This region represents a relatively small portion of a large polymorphic gene, and it is possible that the major genetic determinants of IVM-resistance may prove to be elsewhere in the molecule. Alternative gene splicing,

as an additional resistance mechanism, was evident in the Bisset (2007) study, but was not examined in this work, but would merit further investigation. Comprehensive genetic sequence analysis would be required to associate the allelic and splice variants with potential polymorphisms that are responsible for the resistance phenotype.

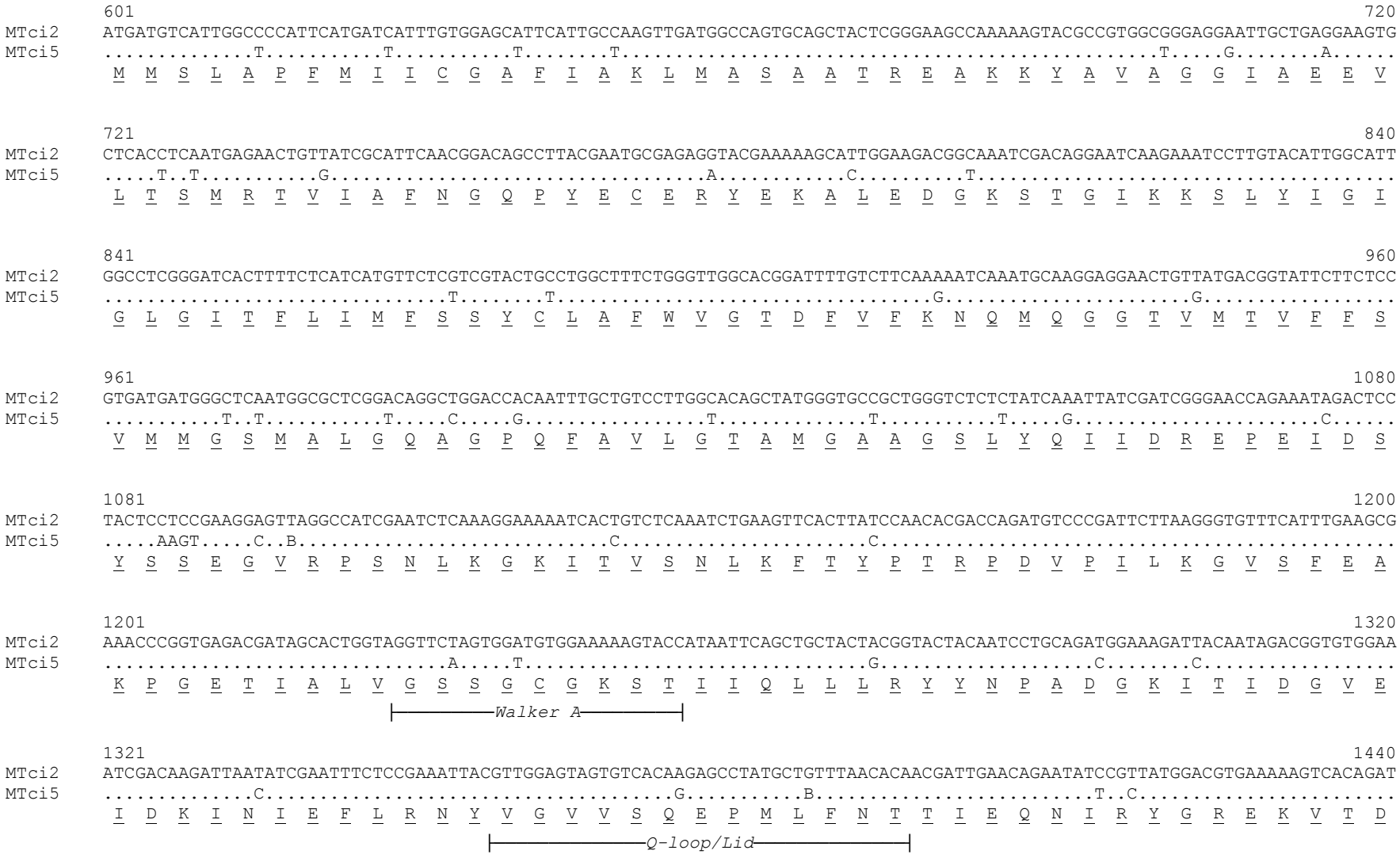
Alternative methods of worm control that are not solely reliant upon chemoprophylaxis are required as extensive use of MLs for treatment of both endo- and ecto-parasites will continue to drive selection for anthelmintic resistance. Identification of markers of anthelmintic resistance will allow the evaluation of new monitoring tools and allow evaluation of the genetic consequences of adopting management strategies aimed at delaying its spread. Resistance to BZs is mainly conferred by the recessive point mutation F200Y in the ß-tubulin gene of *T. circumcincta* (Elard *et al*., 1999), although several other mutations have been identified elsewhere in this gene (Silvestre & Cabaret, 2002; Ghisi *et al*., 2007). A study into the evolution of BZ-resistance has utilised the F200Y mutation to genotype a French strain of *T. circumcincta* (Leignel *et al*., 2010), and also to investigate the genetic consequences of different management strategies. Unfortunately, there are no equivalent molecular tests for ML-resistance, emphasising the need for further research in this area. The problem with molecular based tests, as highlighted by Coles *et al*. (2006), is that a mutation associated with resistance must be shown to be the only mutation, or at least the major genetic determinant that permits resistance to the drug under investigation, in that particular species.

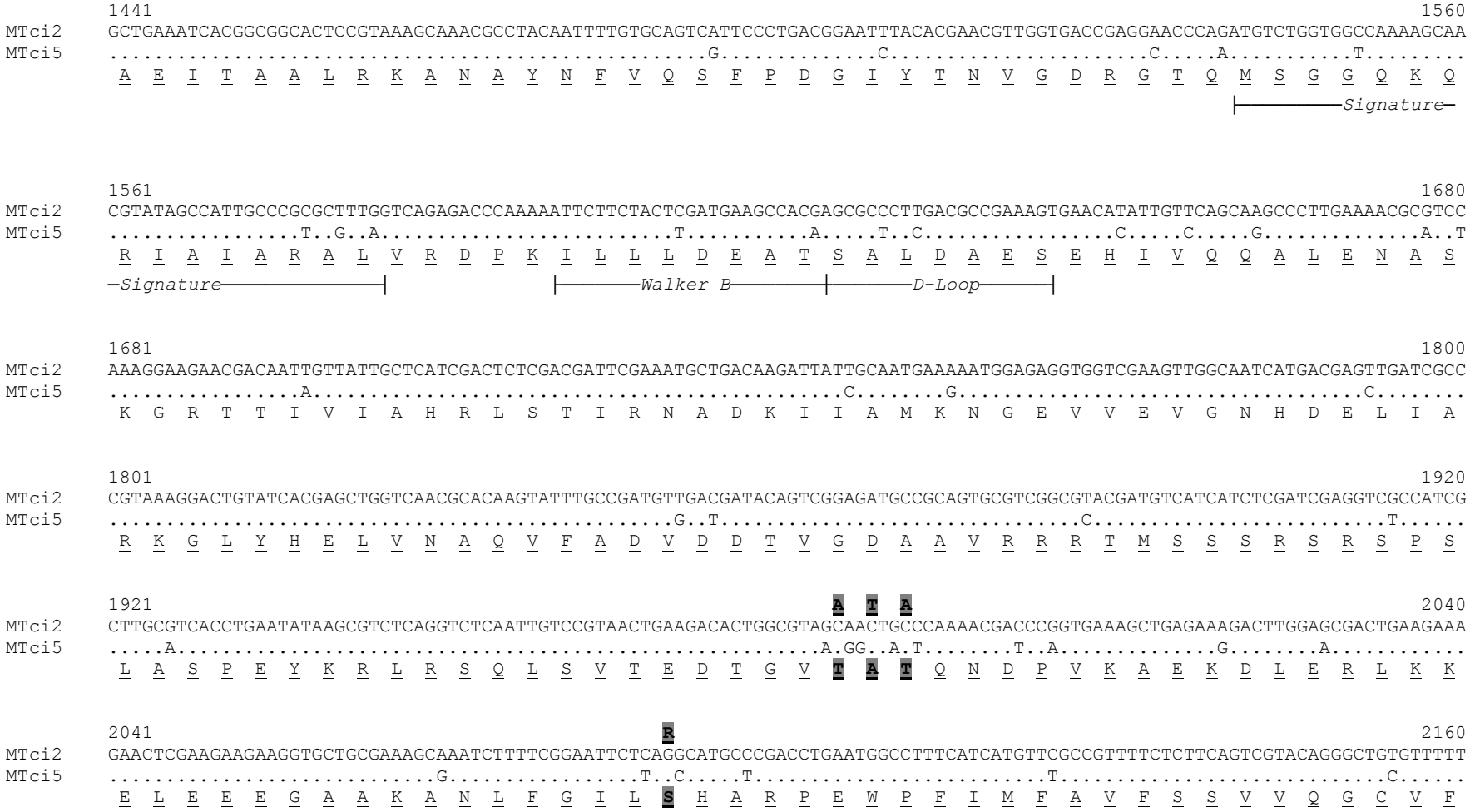
The present study further supports a role for *Tci-pgp-9* in IVM resistance in *T. circumcincta*, but was unable to define the single major genetic determinant of that resistance. The quest for genetic tests based on molecular markers of resistance remains a major research goal. This remains challenging given the highly polymorphic nature of gastrointestinal nematodes, and the fact that infections typically involve multiple species. However, the combination of rapid advances in genetic and genomic approaches, interfaced with parasite isolates of defined resistance status, should aid advancement towards this goal (Gilleard & Beech, 2007).

APPENDIX 1

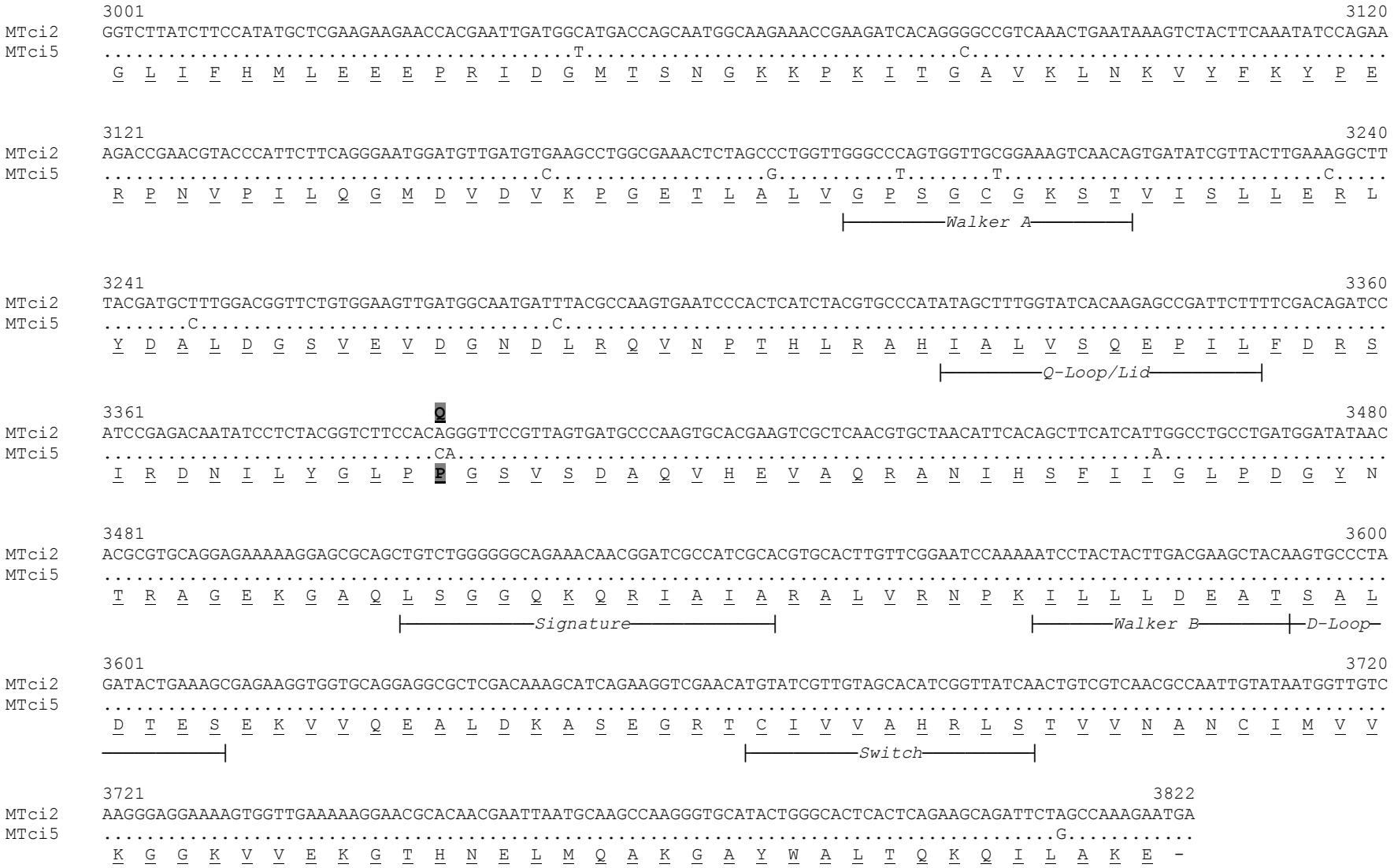
Alignment of the full-length cDNA sequence of *Tci-pgp-9* from two UK isolates of *T. circumcincta*, MTci2 susceptible to anthelmintics and MTci5 which displays multiple anthelmintic resistance. Derived from the consensus of DNA sequencing of 3 clones from each isolate, the common ABC-transporter sequence motifs are annotated below the amino acid sequence (underlined). The non-synonymous SNPs identified are shaded.

```
                                                                                         V                                  120
MTci2  ATGGGCTTCCTAAAGAAGAACGGGAAAGTGGCGGATAGCAAAGGGCAAGAAGACAGCCAGATTGAAGAAGAGAAGAAAGAGGTGGTCCCGAAAGCCAGCATCGGTCAACTGTTCCGGTAC
MTci5  ................................................................................A.A.....................C...T.......C...
        M  G  F  L  K  K  N  G  K  V  A  D  S  K  G  Q  E  D  S  Q  I  E  E  E  K  K  E  E  V  P  K  A  S  I  G  Q  L  F  R  Y

       121                                                                                                                  240
MTci2  ACAACGACCTTCGATAAGGTGCTGCTCTTGATCGGCTCAGTTGTCGCTATTGGCACCGGTATAGGACTGCCTATGATGTCTATCATTATGGGCAACATTTCACAAAACTTCATGAGTATC
MTci5  .....C........C..A........T..A..A....................T.........................................T........................
        T  T  T  F  D  K  V  L  L  L  I  G  S  V  V  A  I  G  T  G  I  G  L  P  M  M  S  I  I  M  G  N  I  S  Q  N  F  M  S  I

       241                                                                                                                  360
MTci2  ACTGGAAACACTACCTCTATCCAACAGTTCGAACATGATGTGATCCAAAACTGCCTTAAATATGTTTACCTCGGTTGCGGAGTATTCACGGCGGCAACGATTCAGGCAATGTGTTTTCTA
MTci5  .....C.....A..............A....................G.................C........C....................G........................
        T  G  N  T  T  S  I  Q  Q  F  E  H  D  V  I  Q  N  C  L  K  Y  V  Y  L  G  C  G  V  F  T  A  A  T  I  Q  A  M  C  F  L

       361                                                                                                                  480
MTci2  ACGGTATGCGAGAATCTTGTTAATCAACTCAGAAGACAGTTCTTCAAGTCGATTCTTCGTCAAGACATCACGTGGTTCGACAAAAACAATTCAGGAACTCTCGCCACAAAACTATTCGAC
MTci5  .....C.....A........G.............................A................................G.................T.....G.....G......
        T  V  C  E  N  L  V  N  Q  L  R  R  Q  F  F  K  S  I  L  R  Q  D  I  T  W  F  D  K  N  N  S  G  T  L  A  T  K  L  F  D

       481                                                                                                                  600
MTci2  AATCTGGAACGAGTCAAAGAGGGAACAGGTGACAAACTTGGCCTTATGATCCAATTCGTGGCGCAGTTTTTCGGCGGTTTCATCGTGGCGTTCACTTACGACTGGAAACTCACTCTGATC
MTci5  .......................T..C..C...........T..........................C.........................................T.........
        N  L  E  R  V  K  E  G  T  G  D  K  L  G  L  M  I  Q  F  V  A  Q  F  F  G  G  F  I  V  A  F  T  Y  D  W  K  L  T  L  I
```


601
720
MTci2 ATGATGTCATTGGCCCCATTCATGATCATTTGTGGAGCATTCATTGCCAAGTTGATGGCCAGTGCAGCTACTCGGGAAGCCAAAAAGTACGCCGTGGCGGGAGGAATTGCTGAGGAAGTG
MTci5 .............T...........T...........T........T...T.....G.........A.....
M M S L A P F M I I C G A F I A K L M A S A A T R E A K K Y A V A G G I A E E V
721
840
MTci2 CTCACCTCAATGAGAACTGTTATCGCATTCAACGGACAGCCTTACGAATGCGAGAGGTACGAAAAAGCATTGGAAGACGGCAAATCGACAGGAATCAAGAAATCCTTGTACATTGGCATT
MTci5 .....T..T...........G.....................................A............C..........T.....................................
L T S M R T V I A F N G Q P Y E C E R Y E K A L E D G K S T G I K K S L Y I G I
841
960
MTci2 GGCCTCGGGATCACTTTTCTCATCATGTTCTCGTCGTACTGCCTGGCTTTCTGGGTTGGCACGGATTTTGTCTTCAAAAATCAAATGCAAGGAGGAACTGTTATGACGGTATTCTTCTCC
MTci5 ................................T........T.....................................G.....................G..................
G L G I T F L I M F S S Y C L A F W V G T D F V F K N Q M Q G G T V M T V F F S
961
1080
MTci2 GTGATGATGGGCTCAATGGCGCTCGGACAGGCTGGACCACAATTTGCTGTCCTTGGCACAGCTATGGGTGCCGCTGGGTCTCTCTATCAAATTATCGATCGGGAACCAGAAATAGACTCC
MTci5 ...........T..T...........T.....C.....G.................T..............T...........T.....G.....................C......
V M M G S M A L G Q A G P Q F A V L G T A M G A A G S L Y Q I I D R E P E I D S
1081
1200
MTci2 TACTCCTCCGAAGGAGTTAGGCCATCGAATCTCAAAGGAAAAATCACTGTCTCAAATCTGAAGTTCACTTATCCAACACGACCAGATGTCCCGATTCTTAAGGGTGTTTCATTTGAAGCG
MTci5 .....AAGT.....C..B.............................C.....................C...
Y S S E G V R P S N L K G K I T V S N L K F T Y P T R P D V P I L K G V S F E A
1201
1320
MTci2 AAACCCGGTGAGACGATAGCACTGGTAGGTTCTAGTGGATGTGGAAAAAGTACCATAATTCAGCTGCTACTACGGTACTACAATCCTGCAGATGGAAAGATTACAATAGACGGTGTGGAA
MTci5 ................................A.....T.................................G.....................C........C.................
K P G E T I A L V G S S G C G K S T I I Q L L L R Y Y N P A D G K I T I D G V E
Walker A
1321
1440
MTci2 ATCGACAAGATTAATATCGAATTTCTCCGAAATTACGTTGGAGTAGTGTCACAAGAGCCTATGCTGTTTAACACAACGATTGAACAGAATATCCGTTATGGACGTGAAAAAGTCACAGAT
MTci5 .............C.....................................G...........B.........................T..C.....................
I D K I N I E F L R N Y V G V V S Q E P M L F N T T I E Q N I R Y G R E K V T D
Q-loop/Lid


1441
1560
MTci2 GCTGAAATCACGGCGGCACTCCGTAAAGCAAACGCCTACAATTTTGTGCAGTCATTCCCTGACGGAATTTACACGAACGTTGGTGACCGAGGAACCCAGATGTCTGGTGGCCAAAAGCAA
MTci5 .....G.....C.....C.....A.....T.....
A E I T A A L R K A N A Y N F V Q S F P D G I Y T N V G D R G T Q M S G G Q K Q
Signature
1561
1680
MTci2 CGTATAGCCATTGCCCGCGCTTTGGTCAGAGACCCAAAAATTCTTCTACTCGATGAAGCCACGAGCGCCCTTGACGCCGAAAGTGAACATATTGTTCAGCAAGCCCTTGAAAACGCGTCC
MTci5 .....T..G..A.....T.....A.....T..C.....C.....C.....G.....A..T
R I A I A R A L V R D P K I L L L D E A T S A L D A E S E H I V Q Q A L E N A S
Signature
Walker B
D-Loop
1681
1800
MTci2 AAAGGAAGAACGACAATTGTTATTGCTCATCGACTCTCGACGATTCGAAATGCTGACAAGATTATTGCAATGAAAAATGGAGAGGTGGTCGAAGTTGGCAATCATGACGAGTTGATCGCC
MTci5 .....A.....C.....G.....C.....
K G R T T I V I A H R L S T I R N A D K I I A M K N G E V V E V G N H D E L I A
1801
1920
MTci2 CGTAAAGGACTGTATCACGAGCTGGTCAACGCACAAGTATTTGCCGATGTTGACGATACAGTCGGAGATGCCGCAGTGCGTCGGCGTACGATGTCATCATCTCGATCGAGGTCGCCATCG
MTci5 .....G..T.....C.....T.....
R K G L Y H E L V N A Q V F A D V D D T V G D A A V R R R T M S S S R S R S P S
1921
A T A
2040
MTci2 CTTGCGTCACCTGAATATAAGCGTCTCAGGTCTCAATTGTCCGTAACTGAAGACACTGGCGTAGCAACTGCCCAAAACGACCCGGTGAAAGCTGAGAAAGACTTGGAGCGACTGAAGAAA
MTci5 .....A.....A.GG..A.T.....T..A.....G.....A.....
L A S P E Y K R L R S Q L S V T E D T G V T A T Q N D P V K A E K D L E R L K K
2041
R
2160
MTci2 GAACTCGAAGAAGAAGGTGCTGCGAAAGCAAATCTTTTCGGAATTCTCAGGCATGCCCGACCTGAATGGCCTTTCATCATGTTCGCCGTTTTCTCTTCAGTCGTACAGGGCTGTGTTTTT
MTci5 .....G.....T..C.....T.....T.....C.....
E L E E E G A A K A N L F G I L S H A R P E W P F I M F A V F S S V V Q G C V F

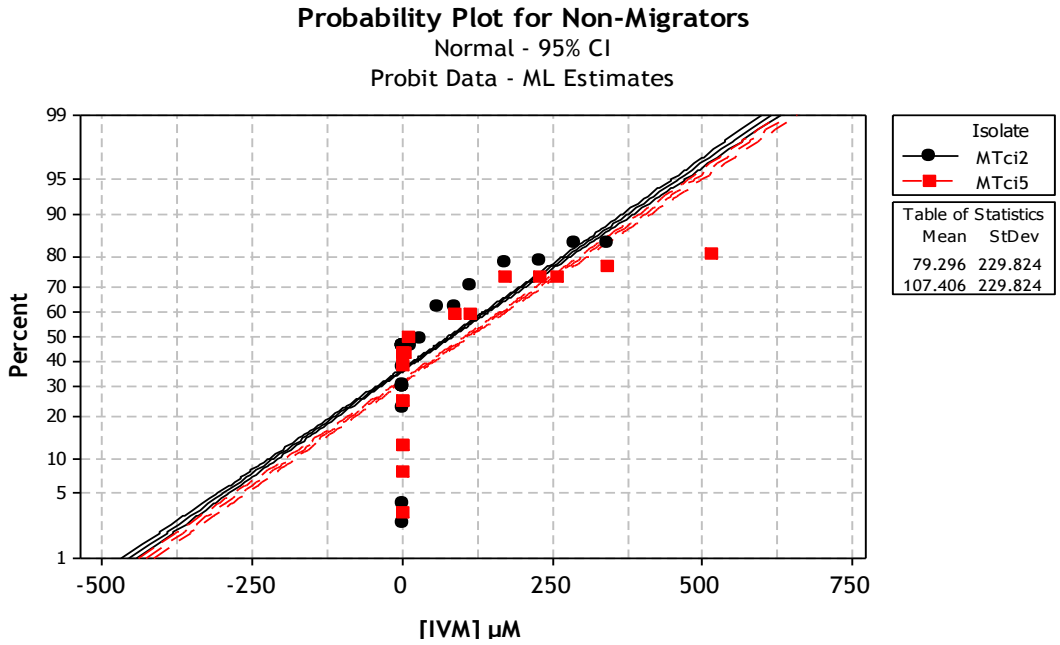

2461 2280
MTci2 CCGGCTTTCTCGCTATTCTTCTCACAAATCATCAATGTGTTCTCGAAGCAACCAGGTGATCCGACGTTAAAACAAGAGGGCCATTTTTGGGCACTGATGTTCCTCGTGTTAGGTGCTGTC
MTci5 ..........A..G.................T..........A.....G..C..........AC....G..G.....................T.....................
P A F S L F F S Q I I N V F S K Q P G D P T L K Q E G H F W A L M F L V L G A V
2281 2400
MTci2 CAAGCTACCACAATGATCATACAGTGCTTCTTCTTCGGTATGTCAGCCGAACGGCTCACAATGCGACTTCGATCCAAGATTTTCAAGAATGTTATGAGAATGGATGCCACTTATTTCGAC
MTci5 ....T...
Q A T T M I I Q C F F F G M S A E R L T M R L R S K I F K N V M R M D A T Y F D
2401 2520
MTci2 ATGCCTCGTCATTCACCTGGAAAAATCACCACTCGACTGGCTACCGATGCGCCTAACGTCAAGTCGGCTCTCGACTATCGTTTCGGTTCAGTGTTCAGTTCAGTCGTCTCCGTCTGCAGT
MTci5 .....................G...C.....................A.....
M P R H S P G K I T T R L A T D A P N V K S A L D Y R F G S V F S S V V S V C S
2521 2640
MTci2 GGTGTCGGAATCGCGCTTTATTTTGGATGGCAAATGGCAATTTTGACTATCGCCATCTTTCCCTTAGCCGCTGTTGGGCAGGCAATCCAGATGAAATTCATGTCTGGGCGTGCAACAGCT
MTci5 ....C..GC.............A..C..A.....................................
G V G I A L Y F G W Q M A I L T I A I F P L A A V G Q A I Q M K F M S G R A T A
2641 2760
MTci2 GATGCAAAAGAGATGGAAAACAGTGGAAAAGTTGCCATGGAAGCTATTGAGAACATTCGAACAGTACAAGCACTAACATTAGAACATCGACTCCACGCGCAGTTCTGTCAGCATTTGGAT
MTci5 ............................A..........C..........CA.....................G..CA.......T.....................
D A K E M E N S G K V A M E A I E N I R T V Q A L T L E H R L H A Q F C Q H L D
2761 2880
MTci2 GCACCGCACAAAACCAGCAGAAGAAAGGCTATCATCCAGGGTATTTCTTATGGATTCGCCAGCAGCATCTTCTACTTCTTATATGCGTCATGCTTCCGTTTTGGATTGTGGCTTATCGTC
MTci5 ....C..T.....................
A P H K T S R R K A I I Q G I S Y G F A S S I F Y F L Y A S C F R F G L W L I V
2881 3000
MTci2 AATGGAACTCTTCAGCCAATGAACGTCCTTAGGGTACTGTTCGCAATCTCGTTCACTGCTGGAAGCATGGGATTTGCAAGCTCTTATTTCCCCGAGTACATCAAGGCAACATTCGCTGCC
MTci5 ..........C.............T........H.............B.....................C..........C.................A..G.....T.....T
N G T L Q P M N V L R V L F A I S F T A G S M G F A S S Y F P E Y I K A T F A A


3001
3120
MTci2 GGTCTTATCTTCCATATGCTCGAAGAAGAACCACGAATTGATGGCATGACCAGCAATGGCAAGAAACCGAAGATCACAGGGGCCGTCAAACTGAATAAAGTCTACTTCAAATATCCAGAA
MTci5 .....T.....C.....
G L I F H M L E E E P R I D G M T S N G K K P K I T G A V K L N K V Y F K Y P E
3121
3240
MTci2 AGACCGAACGTACCCATTCTTCAGGGAATGGATGTTGATGTGAAGCCTGGCGAAACTCTAGCCCTGGTTGGGCCCAGTGGTTGCGGAAAGTCAACAGTGATATCGTTACTTGAAAGGCTT
MTci5 .....C.....G.....T.....T.....C.....
R P N V P I L Q G M D V D V K P G E T L A L V G P S G C G K S T V I S L L E R L
Walker A
3241
3360
MTci2 TACGATGCTTTGGACGGTTCTGTGGAAGTTGATGGCAATGATTTACGCCAAGTGAATCCCACTCATCTACGTGCCCATATAGCTTTGGTATCACAAGAGCCGATTCTTTTCGACAGATCC
MTci5 .....C.....C.....
Y D A L D G S V E V D G N D L R Q V N P T H L R A H I A L V S Q E P I L F D R S
Q-Loop/Lid
3361
Q
3480
MTci2 ATCCGAGACAATATCCTCTACGGTCTTCCACAGGGTTCCGTTAGTGATGCCCAAGTGCACGAAGTCGCTCAACGTGCTAACATTCACAGCTTCATCATTGGCCTGCCTGATGGATATAAC
MTci5 .....CA.....A.....
I R D N I L Y G L P P G S V S D A Q V H E V A Q R A N I H S F I I G L P D G Y N
3481
3600
MTci2 ACGCGTGCAGGAGAAAAAGGAGCGCAGCTGTCTGGGGGGCAGAAACAACGGATCGCCATCGCACGTGCACTTGTTCGGAATCCAAAAATCCTACTACTTGACGAAGCTACAAGTGCCCTA
MTci5 .....
T R A G E K G A Q L S G G Q K Q R I A I A R A L V R N P K I L L L D E A T S A L
Signature
Walker B
D-Loop
3601
3720
MTci2 GATACTGAAAGCGAGAAGGTGGTGCAGGAGGCGCTCGACAAAGCATCAGAAGGTCGAACATGTATCGTTGTAGCACATCGGTTATCAACTGTCGTCAACGCCAATTGTATAATGGTTGTC
MTci5 .....
D T E S E K V V Q E A L D K A S E G R T C I V V A H R L S T V V N A N C I M V V
Switch
3721
3822
MTci2 AAGGGAGGAAAAGTGGTTGAAAAAGGAACGCACAACGAATTAATGCAAGCCAAGGGTGCATACTGGGCACTCACTCAGAAGCAGATTCTAGCCAAAGAATGA
MTci5 .....G.....
K G G K V V E K G T H N E L M Q A K G A Y W A L T Q K Q I L A K E -

APPENDIX 2

Probit Analysis - Larval Migration Inhibition Assay

Probit analysis was conducted on the data generated from the LMIA. The analysis was used to estimate the concentration of IVM and its different inhibitory doses. The probability plot for non-migrators is shown below. The isolates tested were (1) MTci2 and (2) MTci5 annotated by black circles and red squares, respectively.



The inhibitory doses of IVM were estimated from the graph and are listed in the tables below along with the regression output from the Probit analysis. The regression table shows that increasing IVM concentration increases migration inhibition and that the MTci5 isolate is inhibited less than the MTci2 isolate with a negative coefficient.

Isolate	Inhibitory Dose	[IVM] (µ*M*)	SE	95 % Fiducial C.I.	
				Lower	Upper
MTci2	LMI_{10}	-215.236	3.927	-223.118	-207.713
	LMI_{50}	79.296	1.759	75.866	82.764
	LMI_{90}	373.828	4.611	365.003	383.088
	LMI_{95}	457.323	5.653	446.511	468.684
	LMI_{99}	613.947	7.657	599.310	629.345
MTci5	LMI_{10}	-187.126	4.340	-195.819	-178.798
	LMI_{50}	107.406	2.460	102.598	112.246
	LMI_{90}	401.938	4.875	392.592	411.714
	LMI_{95}	485.433	5.860	474.213	497.197
	LMI_{99}	642.057	7.796	627.145	657.724

Regression Table

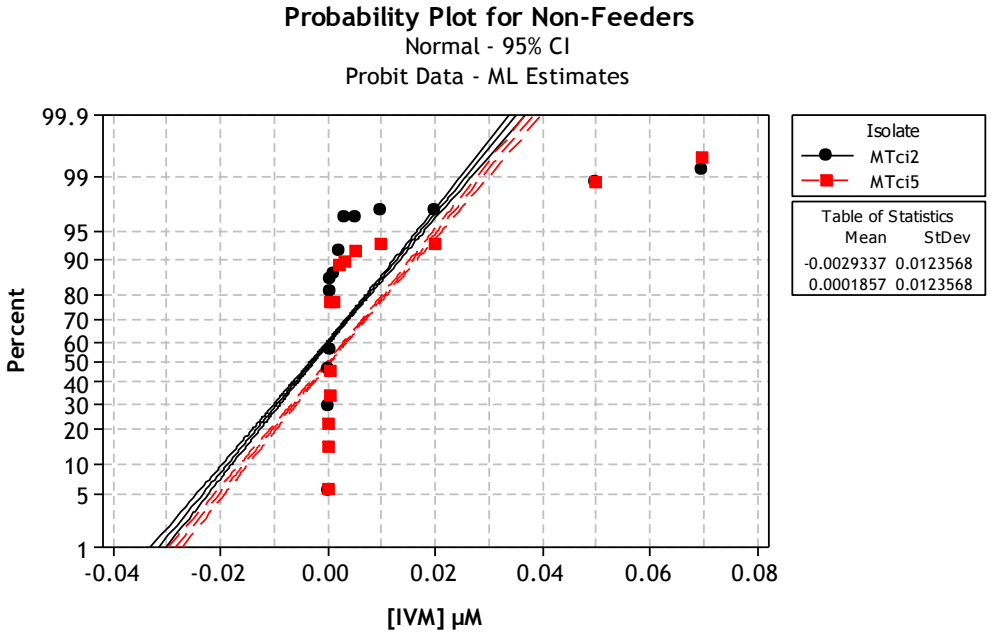
Variable	Coefficient	S.E.	Z-value	P-value
Constant	-0.34502	0.0080401	-42.91	<0.001
[IVM] (µ*M*)	0.00435	0.0000577	75.43	<0.001
MTci5	-0.12231	0.0131215	-9.32	<0.001

Chi-Square = 208.592 D.F. = 1 *P* Value = <0.001

APPENDIX 3

Probit Analysis - Larval Feeding Inhibition Assay

Probit analysis was conducted on the data generated from the LFIA. The analysis was used to estimate the concentration of IVM and its different inhibitory doses. The probability plot for non-feeders is shown below. The isolates tested were (1) MTci2 and (2) MTci5 annotated by black circles and red squares, respectively.



The inhibitory doses of IVM were estimated from the graph and are listed in the tables below along with the regression output from the Probit analysis. The regression table shows that increasing IVM concentration increases feeding inhibition and that the MTci5 isolate is inhibited less than the MTci2 isolate with a negative coefficient.

Isolate	Inhibitory Dose	[IVM] (µ*M*)	SE	95.0% Fiducial C.I.	
				Lower	Upper
MTci2	LFI_{10}	-0.0188	0.000468	-0.0197	-0.0179
	LFI_{50}	-0.0029	0.000197	-0.0033	-0.0026
	LFI_{90}	0.0129	0.000276	0.0123	0.0134
	LFI_{95}	0.0174	0.000356	0.0167	0.0181
	LFI_{99}	0.0258	0.000519	0.0248	0.0269
MTci5	LFI_{10}	-0.0156	0.000415	-0.0165	-0.0149
	LFI_{50}	0.0002	0.000168	-0.0001	0.0005
	LFI_{90}	0.0160	0.000320	0.0154	0.0167
	LFI_{95}	0.0205	0.000404	0.0197	0.0213
	LFI_{99}	0.0289	0.000570	0.0279	0.0301

Regression Table

Variable	Coefficient	S.E.	Z-value	P-value
Constant	0.2374	0.0138	17.19	<0.001
[IVM] (µ*M*)	80.9272	1.6900	47.89	<0.001
MTci5	-0.2524	0.0185	-13.63	<0.001

Chi-Square = 0.0277257 D.F. = 1 *P*-Value = 0.868

APPENDIX 4

Incidence of *Tci-pgp-9-IBDA* Variants in UK Isolates of *T. circumcincta*

Tci-pgp-9-IBDA genotypes of >80 individual L_3 were assessed by allele-specific PCR. The presence of allelic variants was recorded in individuals from the MTci2, MTci5 and MTci5PT isolates of *T. circumcincta*. The nested-PCR strategy has failed to amplify products in four MTci5PT larvae (Larval lysates E6, E8, G7 and G11). The number of allelic variants (*n*) present in each larva is also listed in the table.

Larval Lysate	MTci2		MTci5		MTci5PT	
	Allelic Variant(s)	*n*	Allelic Variant(s)	*n*	Allelic Variant(s)	*n*
A1	2, 8, 12	*3*	2, 7, 8, 10, 11, 13	*6*	3, 11, 12	*3*
A2	8, 9, 10, 13	*4*	2	*1*	3, 5, 10, 12	*4*
A3	5	*1*	3, 5, 7, 8, 9, 10, 11	*7*	2, 13	*2*
A4	2, 7, 13	*3*	2, 3, 8, 10, 13	*5*	3, 5	*2*
A5	5, 8, 9	*3*	2, 3, 8, 9, 10, 11	*6*	2, 5	*2*
A6	5, 7, 8, 12	*4*	2, 7, 9, 10, 11, 13	*6*	5, 12	*2*
A7	3, 5, 8, 9, 10, 11, 12, 13	*8*	8, 9, 10	*3*	2, 7	*2*
A8	3, 5, 8, 9, 10, 12, 13	*7*	2, 7, 9, 13	*4*	5, 12	*2*
A9	7, 9, 12	*3*	2, 3, 13	*3*	5, 11	*2*
A10	5, 8, 10	*3*	2, 3, 12	*3*	5, 8	*2*
A11	8, 10, 11, 12, 13	*5*	2, 3, 10, 12	*4*	2, 5, 12, 13	*4*
A12	2, 5, 9, 10, 12	*5*	3, 5, 11	*3*	2, 5, 13	*3*
B1	5, 8	*2*	5, 7, 9	*3*	7, 9, 11, 13	*4*
B2	5, 12	*2*	2, 3, 13	*3*	2, 5, 7, 13	*4*
B3	2, 13	*2*	3, 5, 7, 12	*4*	2, 5	*2*
B4	2, 12, 13	*3*	9	*1*	2, 13	*2*
B5	3, 5, 8, 9, 10, 11, 12	*7*	3, 8	*2*	3	*1*
B6	2, 3, 5, 7, 8, 9, 10, 11, 12, 13	*10*	3, 10, 12	*3*	5, 12	*2*
B7	5, 12	*2*	2, 12, 13	*3*	7	*1*
B8	8, 9, 10	*3*	3, 10, 12	*3*	2, 9	*2*
B9	5, 12	*2*	2, 3, 13	*3*	5, 12	*2*
B10	2, 7, 8, 9, 10, 13	*6*	9, 12	*2*	8, 12	*2*
B11	2, 13	*2*	3, 8	*2*	2	*1*
B12	8, 9, 12	*3*	3, 12	*2*	13	*1*
C1	5	*1*	2, 5, 7, 13	*4*	2, 13	*2*
C2	2, 12, 13	*3*	2, 5, 7, 13	*4*	2, 5, 13	*3*
C3	2, 13	*2*	12, 13	*2*	10, 12	*2*
C4	5, 8, 10, 12	*4*	2, 3, 5	*3*	3, 12	*2*
C5	9, 12, 13	*3*	5, 7, 9	*3*	5, 9, 12	*3*
C6	2, 5, 7, 8, 9, 10, 11, 12, 13	*9*	3, 7, 9	*3*	12	*1*
C7	5, 7, 8, 9, 10, 13	*6*	5, 8, 13	*3*	2	*1*
C8	2, 5, 13	*3*	3, 9	*2*	2, 5	*2*
C9	5, 8, 10, 12, 13	*5*	2, 5, 13	*3*	2, 5, 10	*3*
C10	5, 8, 9, 10	*4*	3	*1*	2	*1*
C11	2, 5, 8, 9, 10, 11, 13	*7*	2, 8	*2*	5, 12	*2*
C12	2, 8, 10	*3*	3, 8, 11, 13	*4*	5, 7, 9, 12	*4*

Larval Lysate	MTci2		MTci5		MTci5PT	
	Allelic Variant(s)	*n*	Allelic Variant(s)	*n*	Allelic Variant(s)	*n*
D1	5, 12	*2*	5	*1*	3, 12	*2*
D2	2, 5, 8, 13	*4*	3	*1*	3, 11, 12	*3*
D3	2, 8, 12, 13	*4*	3, 7, 10	*3*	3, 5, 8	*3*
D4	2, 5, 8, 13	*4*	7, 9, 13	*3*	2, 12	*2*
D5	2, 12, 13	*3*	2, 3, 5	*3*	2, 10	*2*
D6	2, 3, 5, 8, 9, 13	*6*	2, 3	*2*	3, 13	*2*
D7	2, 5, 8, 9, 10, 11, 12, 13	*8*	3, 7, 9, 11	*4*	2, 12	*2*
D8	2, 5, 8, 13	*4*	3	*1*	2, 3, 5, 13	*4*
D9	2, 5, 7, 8, 9, 10, 11, 13	*7*	5, 12	*2*	12	*1*
D10	8, 9, 10, 12	*4*	3	*1*	5, 8	*2*
D11	5, 8, 12	*3*	2	*1*	12	*1*
D12	2	*1*	2	*1*	3	*1*
E1	11	*1*	2, 8	*2*	2, 13	*2*
E2	2, 8, 11, 13	*4*	5, 10	*2*	5, 12	*2*
E3	2, 8, 13	*3*	3	*1*	5, 12	*2*
E4	3, 8, 9, 10, 11, 12, 13	*7*	2, 3	*2*	5, 12, 13	*3*
E5	2, 3, 9, 13	*4*	2, 3	*2*	2, 13	*2*
E6	3, 5, 8, 9, 10, 11, 12, 13	*8*	3, 5	*2*		
E7	2, 5, 13	*3*	3	*1*	5, 12	*2*
E8	5, 10	*2*	2, 13	*2*		
E9	5, 8, 9, 10, 12, 13	*6*	2, 3, 13	*3*	2, 12	*2*
E10	8, 9, 12	*3*	2, 3	*2*	2, 7, 9, 12	*4*
E11	5, 12	*2*	2, 3	*2*	5, 10	*2*
E12	2, 8, 12	*3*	3, 8	*2*	5	*1*
F1	5, 11	*2*	3, 10, 12	*3*	5, 10	*2*
F2	2, 5, 9, 13	*4*	3, 8	*2*	2, 5, 10, 13	*4*
F3	9, 12	*2*	3, 8, 13	*3*	2, 5	*2*
F4	3, 9, 13	*3*	2, 10, 13	*3*	3, 5, 8, 9, 12	*5*
F5	2, 5, 9, 10, 12, 13	*6*	2, 12, 13	*3*	2	*1*
F6	2, 5, 8, 9, 10, 12, 13	*7*	2, 9, 13	*3*	7, 9, 11	*3*
F7	5, 12	*2*	8, 11	*2*	2, 8	*2*
F8	2, 5, 8, 9, 10, 13	*6*	2, 8, 11	*3*	3, 12	*2*
F9	2, 8, 9, 10, 13	*5*	2, 3, 13	*3*	5, 9	*2*
F10	2, 5, 8, 9, 10, 12, 13	*7*	2, 9, 13	*3*	13	*1*
F11	2, 8, 9, 10, 13	*5*	3	*1*	13	*1*
F12	5, 8, 10, 13	*4*	5, 8	*2*	2, 7, 9, 13	*4*
G1	2	*1*	2, 3, 8, 10, 11	*5*	12, 13	*2*
G2	5, 9	*2*	2, 11, 13	*3*	2, 3, 12, 13	*4*
G3	5, 8, 9	*3*	2, 9, 11, 13	*4*	7, 9, 11	*3*
G4	2, 5, 13	*3*	3, 11	*2*	5, 10	*2*
G5	2, 5, 13	*3*	7, 9, 11	*3*	2	*1*
G6	2, 12, 13	*3*	7, 9, 11	*3*	2, 12	*2*
G7	5	*1*	2, 11, 13	*3*		
G8	2, 13	*2*	2, 12	*2*	2, 10	*2*
G9	5, 12	*2*	3, 5, 12	*3*	8	*1*
G10	2, 5, 13	*3*	3, 12	*2*	8, 9	*2*
G11	2, 12, 13	*3*	2, 3, 10	*3*		
G12	5, 8, 9	*3*	3, 11	*2*	2, 12	*2*

APPENDIX 5

Tci-pgp-9-IBDA Variants in Sub-populations of *T. circumcincta*

Allele-specific PCR results for individual larvae selected from the $MTci2_{NF(0.1nM)}$, $MTci2_{F(10nM)}$, $MTci5_{NF(0.1nM)}$ and $MTci5_{F(10nM)}$ sub-populations collected from the LFIA.

Larval Lysate	$MTci2_{NF(0.1nM)}$ Allelic Variants(s)	*n*	$MTci5_{NF(0.1nM)}$ Allelic Variant(s)	*n*
A1	2, 5	*2*	2, 11, 13	*3*
A2	2, 5, 10, 13	*4*	2, 7, 13	*3*
A3	2, 7, 9, 13	*4*	2, 13	*2*
A4	2, 9, 13	*3*	3	*1*
A5	2, 5, 13	*3*	2, 3, 5, 13	*4*
A6	2, 5, 9, 10	*4*	2, 3, 13	*3*
B1	2, 13	*2*	3, 12	*2*
B2	2, 10	*2*	3, 7	*2*
B3	2, 5, 7	*3*	8, 9, 12	*3*
B4			3	*1*
B5	2, 5, 10, 13	*4*	3	*1*
B6	2, 5, 10, 13	*4*	2, 3, 9, 13	*4*
C1			3, 9, 11	*3*
C2	2, 5, 13	*3*	2, 8, 10, 13	*4*
C3	2, 5, 13	*3*	2, 3	*2*
C4	2, 5, 12, 13	*4*	3	*1*
C5	2, 5, 12, 13	*4*	3, 8, 10	*3*
C6	2, 5, 13	*3*	3	*1*
D1	2, 5, 9, 10	*4*	2, 3, 13	*3*
D2	2, 12	*2*	2, 3, 13	*3*
D3	2, 5, 12	*3*	2, 3, 12, 13	*4*
D4	2, 13	*2*	2, 13	*2*
D5	2, 3	*2*		
D6	2, 5, 10, 13	*4*	3	*1*
E1	2, 3	*2*	2, 3, 12, 13	*4*
E2	2, 5, 13	*3*	2, 7, 9, 13	*4*
E3	2, 5, 10, 12	*4*	2, 3, 13	*3*
E4	2, 13	*2*	2, 3, 13	*3*
E5	2, 8, 10, 13	*4*	2, 3, 8, 10	*4*
E6	2, 9, 13	*3*	2, 13	*2*
F1	2, 5, 10, 12	*4*	2, 3, 8, 13	*4*
F2	2, 5, 13	*3*	3, 7, 9	*3*
F3	3, 5, 10	*3*	2, 13	*2*
F4	2, 5, 11, 13	*4*	2, 3, 8, 13	*4*
F5	2, 13	*2*	3, 8, 10	*3*
F6	2, 5, 7, 9, 10	*5*	3	*1*
G1	5, 12	*2*	2, 3, 12, 13	*4*
G2	2, 5, 9, 10	*4*	2, 3, 13	*3*
G3				
G4	2, 13	*2*	2, 3, 5, 13	*4*
G5	2, 3, 5	*3*	3, 5	*2*
G6	3, 5	*2*	3	*1*
H1	2, 5, 13	*3*	3, 7, 9, 12	*4*
H2	2, 5, 13	*3*	2, 3, 13	*3*
H3			3	*1*
H4	2, 5, 13	*3*	2, 3, 13	*3*
H5	2, 5, 10, 13	*4*	2, 8, 10, 13	*4*

Larval Lysate	$MTci2_{F(10nM)}$ Allelic Variants(s)	*n*	$MTci5_{F(10nM)}$ Allelic Variant(s)	*n*
A7	2, 5, 10, 11	*4*	2, 3, 12, 13	*4*
A8			3, 7, 9	*3*
A9			2, 3, 13	*3*
A10	2, 5, 13	*3*	2, 3, 13	*3*
A11	2, 12, 13	*3*	2, 3, 13	*3*
A12	2, 13	*2*	3, 12	*2*
B7	2, 13	*2*	2, 3, 13	*3*
B8	5, 12	*2*	2, 3, 13	*3*
B9	2, 12, 13	*3*	2, 3, 13	*3*
B10	2, 5, 10	*3*	2, 13	*2*
B11			2, 3, 13	*3*
B12	2, 5, 13	*3*	2, 12, 13	*3*
C7	2, 5, 10, 13	*4*	3, 7, 9	*3*
C8	2, 5,13	*3*	5, 8, 10	*3*
C9			2, 3, 10	*3*
C10	2, 3, 5	*3*	3	*1*
C11	2, 5, 10	*3*	3, 12	*2*
C12	5, 9	*2*	2, 3, 13	*3*
D7	2, 12, 13	*3*	3	*1*
D8	2, 9, 13	*3*	2, 3, 13	*3*
D9	2, 3, 5, 9	*4*	2, 3, 8, 10	*4*
D10	2, 5	*2*	2, 3, 13	*3*
D11	2, 5, 10, 13	*4*	2, 3, 13	*3*
D12	2, 5, 13	*3*	2, 13	*2*
E7	3, 9	*2*	3, 8, 10, 11	*4*
E8	2, 13	*2*	3, 10	*2*
E9	2, 13	*2*	2, 3, 13	*3*
E10	2, 5, 13	*3*	3	*1*
E11	2, 3, 13	*3*	2, 3, 12	*3*
E12	2, 3, 5, 10, 13	*5*	2, 3, 13	*3*
F7	5, 12	*2*	3, 9	*2*
F8	2, 5, 10, 13	*4*	2, 3, 7, 9, 13	*5*
F9	2, 5, 13	*3*	3, 9	*2*
F10	12	*1*	5, 8	*2*
F11	2, 5, 9, 10	*4*	2, 13	*2*
F12	2, 5, 13	*3*	3, 8	*2*
G7	2, 5, 10	*3*	2, 7, 9	*3*
G8	12	*1*	2, 3, 13	*3*
G9	5, 10	*2*	2, 3, 13	*3*
G10	2, 7, 9, 13	*4*	2, 3, 11, 13	*4*
G11	2, 5, 13	*3*	2, 3, 13	*3*
G12	2, 13	*2*	2, 13	*2*
H7	2, 13	*2*	3, 8	*2*
H8	2, 13	*2*	2, 13	*2*
H9	2, 13	*2*	2, 5, 13	*3*
H10	3, 7, 9, 11, 12	*5*	3, 12	*2*
H11	3, 11, 12	*3*	2, 13	*2*

APPENDIX 6

Allocated Haplotype Numbers

Combinations of *Tci-pgp-9-IBDA* allelic variants were allocated a "haplotype number" and their incidences are listed for each of the larval populations tested.

Haplotype Number	Allelic Variants	Incidence in Isolates			Incidence in Sub-populations			
		MTci2	MTci5	MTci5PT	MTci2 (NF0.1μM)	MTci2 (F10μM)	MTci5 (NF0.1μM)	MTci5 (F10μM)
1	2	2	3	5	0	0	0	0
2	2, 3	0	5	0	2	0	1	0
3	2, 3, 5	0	2	0	1	1	0	0
4	2, 3, 5, 7, 8, 9, 10, 11, 12, 13	1	0	0	0	0	0	0
5	2, 3, 5, 8, 9, 13	1	0	0	0	0	0	0
6	2, 3, 5, 9	0	0	0	0	1	0	0
7	2, 3, 5, 10, 13	0	0	0	0	1	0	0
8	2, 3, 5, 13	0	0	1	0	0	2	0
9	2, 3, 7, 9, 13	0	0	0	0	0	0	1
10	2, 3, 8, 9, 10, 11	0	1	0	0	0	0	0
11	2, 3, 8, 10	0	0	0	0	0	1	1
12	2, 3, 8, 10, 11	0	1	0	0	0	0	0
13	2, 3, 8, 10, 13	0	1	0	0	0	0	0
14	2, 3, 8, 13	0	0	0	0	0	2	0
15	2, 3, 9, 13	1	0	0	0	0	1	0
16	2, 3, 10	0	1	0	0	0	0	1
17	2, 3, 10, 12	0	1	0	0	0	0	0
18	2, 3, 11, 13	0	0	0	0	0	0	1
19	2, 3, 12	0	1	0	0	0	0	1
20	2, 3, 12, 13	0	0	1	0	0	3	1
21	2, 3, 13	0	5	0	0	1	8	16
22	2, 5	0	0	4	1	1	0	0
23	2, 5, 7	0	0	0	1	0	0	0
24	2, 5, 7, 8, 9, 10, 11, 12, 13	1	0	0	0	0	0	0
25	2, 5, 7, 8, 9, 10, 11, 13	1	0	0	0	0	0	0
26	2, 5, 7, 9, 10	0	0	0	1	0	0	0
27	2, 5, 7, 13	0	2	1	0	0	0	0
28	2, 5, 8, 9, 10, 11, 12, 13	1	0	0	0	0	0	0
29	2, 5, 8, 9, 10, 11, 13	1	0	0	0	0	0	0
30	2, 5, 8, 9, 10, 12, 13	2	0	0	0	0	0	0
31	2, 5, 8, 9, 10, 13	1	0	0	0	0	0	0
32	2, 5, 8, 13	3	0	0	0	0	0	0
33	2, 5, 9, 10	0	0	0	3	1	0	0
34	2, 5, 9, 10, 12	1	0	0	0	0	0	0
35	2, 5, 9, 10, 12, 13	1	0	0	0	0	0	0
36	2, 5, 9, 13	1	0	0	0	0	0	0
37	2, 5, 10	0	0	1	0	3	0	0
38	2, 5, 10, 11	0	0	0	0	1	0	0
39	2, 5, 10, 12	0	0	0	2	0	0	0
40	2, 5, 10, 13	0	0	1	5	0	0	0
41	2, 5, 11, 13	0	0	0	1	3	0	0
42	2, 5, 12	0	0	0	1	0	0	0
43	2, 5, 12, 13	0	0	1	2	0	0	0
44	2, 5, 13	5	1	2	9	8	0	1
45	2, 7	0	0	1	0	0	0	0
46	2, 7, 8, 9, 10, 13	1	0	0	0	0	0	0
47	2, 7, 8, 10, 11, 13	0	1	0	0	0	0	0
48	2, 7, 9	0	0	0	0	0	0	1
49	2, 7, 9, 10, 11, 13	0	1	0	0	0	0	0
50	2, 7, 9, 12	0	0	1	0	0	0	0
51	2, 7, 9, 13	0	1	1	1	1	1	0
52	2, 7, 13	1	0	0	0	0	1	0
53	2, 8	0	2	1	0	0	0	0
54	2, 8, 9, 10, 13	2	0	0	0	0	0	0
55	2, 8, 10	1	0	0	0	0	0	0
56	2, 8, 10, 13	0	0	0	1	0	2	0
57	2, 8, 11	0	1	0	0	0	0	0
58	2, 8, 11, 13	1	0	0	0	0	0	0
59	2, 8, 12	2	0	0	0	0	0	0
60	2, 8, 12, 13	1	0	0	0	0	0	0
61	2, 8, 13	1	0	0	0	0	0	0
62	2, 9	0	0	1	0	0	0	0
63	2, 9, 11, 13	0	1	0	0	0	0	0
64	2, 9, 13	0	2	0	2	1	0	0
65	2, 10	0	0	2	1	0	0	0
66	2, 10, 13	0	1	0	0	0	0	0
67	2, 11, 13	0	2	0	0	0	1	0
68	2, 12	0	1	4	1	0	0	0
69	2, 12, 13	5	2	0	0	3	0	1
70	2, 13	4	1	5	5	8	4	6
71	3	0	7	2	0	0	9	3

Haplotype Number	Allelic Variants	Incidence in Isolates			Incidence in Sub-populations			
		MTci2	MTci5	MTci5PT	MTci2 (NF0.1μM)	MTci2 (F10μM)	MTci5 (NF0.1μM)	MTci5 (F10μM)
72	3, 5	0	1	1	1	0	1	0
73	3, 5, 7, 8, 9, 10, 11	0	1	0	0	0	0	0
74	3, 5, 7, 12	0	1	0	0	0	0	0
75	3, 5, 8	0	0	1	0	0	0	0
76	3, 5, 8, 9, 10, 11, 12	1	0	0	0	0	0	0
77	3, 5, 8, 9, 10, 11, 12, 13	2	0	0	0	0	0	0
78	3, 5, 8, 9, 10, 12, 13	1	0	0	0	0	0	0
79	3, 5, 8, 9, 12	0	0	1	0	0	0	0
80	3, 5, 10	0	0	0	1	0	0	0
81	3, 5, 10, 12	0	0	1	0	0	0	0
82	3, 5, 11	0	1	0	0	0	0	0
83	3, 5, 12	0	1	0	0	0	0	0
84	3, 7	0	0	0	0	0	1	0
85	3, 7, 9	0	1	0	0	0	1	2
86	3, 7, 9, 11	0	1	0	0	0	0	0
87	3, 7, 9, 11, 12	0	0	0	0	1	0	0
88	3, 7, 9, 12	0	0	0	0	0	1	0
89	3, 7, 10	0	1	0	0	0	0	0
90	3, 8	0	4	0	0	0	0	2
91	3, 8, 9, 10, 11, 12, 13	1	0	0	0	0	0	0
92	3, 8, 10	0	0	0	0	0	2	0
93	3, 8, 10, 11	0	0	0	0	0	0	1
94	3, 8, 11, 13	0	1	0	0	0	0	0
95	3, 8, 13	0	1	0	0	0	0	0
96	3, 9	0	1	0	0	1	0	2
97	3, 9, 11	0	0	0	0	0	1	0
98	3, 9, 13	1	0	0	0	0	0	0
99	3, 10	0	0	0	0	0	0	1
100	3, 10, 12	0	3	0	0	0	0	0
101	3, 11	0	2	0	0	0	0	0
102	3, 11, 12	0	0	2	0	1	0	0
103	3, 12	0	2	3	0	0	1	3
104	3, 13	0	0	1	0	0	0	0
105	5	3	1	1	0	0	0	0
106	5, 7, 8, 9, 10, 13	1	0	0	0	0	0	0
107	5, 7, 8, 12	1	0	0	0	0	0	0
108	5, 7, 9	0	2	0	0	0	0	0
109	5, 7, 9, 12	0	0	1	0	0	0	0
110	5, 8	1	1	2	0	0	0	1
111	5, 8, 9	3	0	0	0	0	0	0
112	5, 8, 9, 10	1	0	0	0	0	0	0
113	5, 8, 9, 10, 12, 13	1	0	0	0	0	0	0
114	5, 8, 10	1	0	0	0	0	0	1
115	5, 8, 10, 12	1	0	0	0	0	0	0
116	5, 8, 10, 12, 13	1	0	0	0	0	0	0
117	5, 8, 10, 13	1	0	0	0	0	0	0
118	5, 8, 12	1	0	0	0	0	0	0
119	5, 8, 13	0	1	0	0	0	0	0
120	5, 9	1	0	1	0	1	0	0
121	5, 9, 12	0	0	1	0	0	0	0
122	5, 10	1	1	3	0	1	0	0
123	5, 11	1	0	1	0	0	0	0
124	5, 12	7	1	8	1	2	0	0
125	5, 12, 13	0	0	1	0	0	0	0
126	7	0	0	1	0	0	0	0
127	7, 9, 11	0	2	2	0	0	0	0
128	7, 9, 11, 13	0	0	1	0	0	0	0
129	7, 9, 12	1	0	0	0	0	0	0
130	7, 9, 13	0	1	0	0	0	0	0
131	8	0	0	1	0	0	0	0
132	8, 9	0	0	1	0	0	0	0
133	8, 9, 10	1	1	0	0	0	0	0
134	8, 9, 10, 12	1	0	0	0	0	0	0
135	8, 9, 10, 13	1	0	0	0	0	0	0
136	8, 9, 12	2	0	0	0	0	1	0
137	8, 10, 11, 12, 13	1	0	0	0	0	0	0
138	8, 11	0	1	0	0	0	0	0
139	8, 12	0	0	1	0	0	0	0
140	9	0	1	0	0	0	0	0
141	9, 12	1	1	0	0	0	0	0
142	9, 12, 13	1	0	0	0	0	0	0
143	10, 12	0	0	1	0	0	0	0
144	11	1	0	0	0	0	0	0
145	12	0	0	3	0	2	0	0
146	12, 13	0	1	1	0	0	0	0
147	13	0	0	3	0	0	0	0

APPENDIX 7

Consensus DNA sequence for β-tubulin

β-tubulin PCR product generated from UK isolates of *T. circumcincta*. Underlined are the primers designed to amplify a 687 bp generic β-tubulin product and shaded are the nested primers used for real time PCR to amplify a 100 bp product. Identity shared with β-tubulin isotype 1 from *T. circumcincta* (97 %) accession number: GQ910863 (Silvestre *et al.*, 2009).

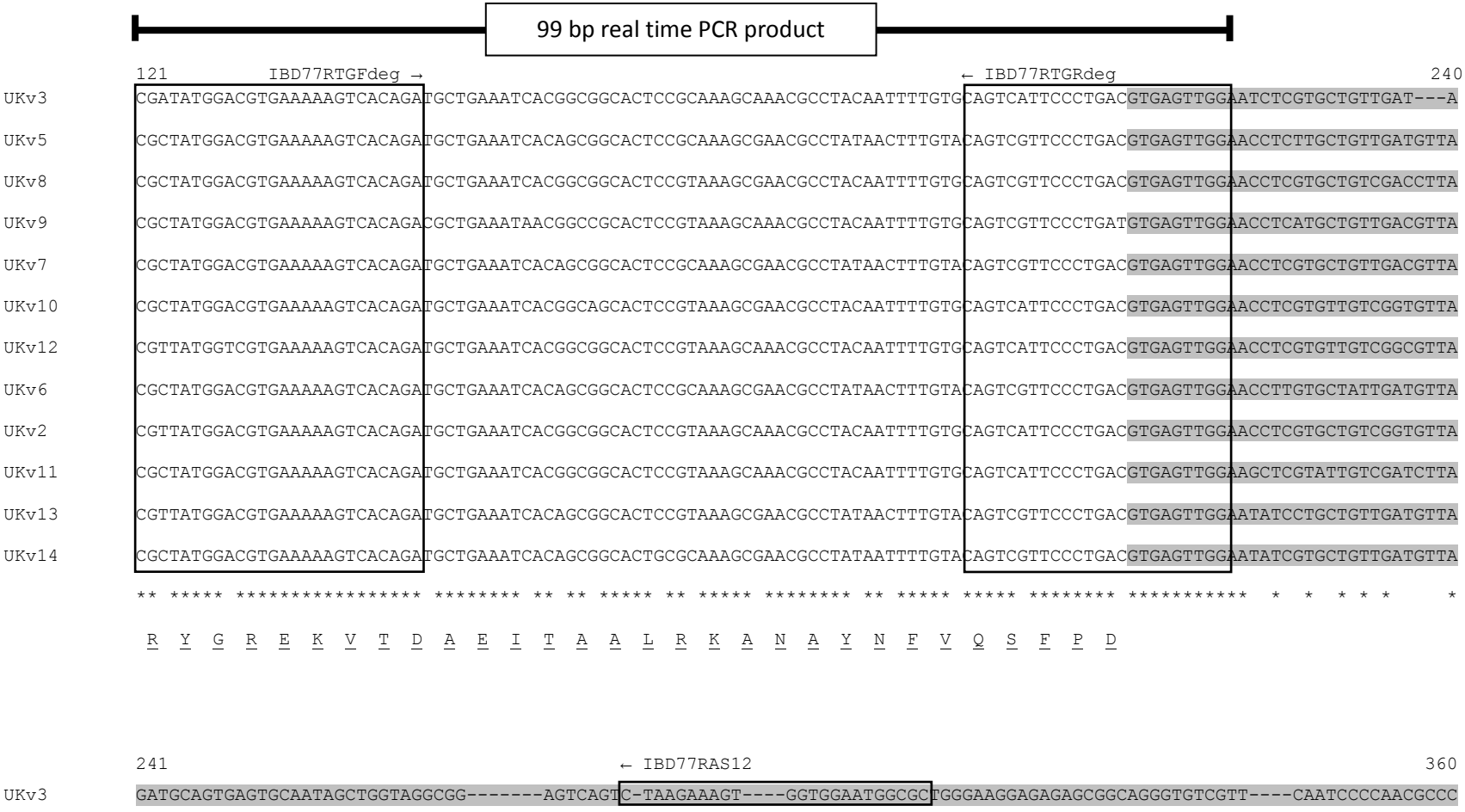
```
1                 TUBGF                                          60
CTTAGATGTTGTTCGTAAAGAGGCAGAGGGTTGCGATTGCCTTCAGGTAGTTTTTCGTCG

61                                                               120
CACTTCGGCTGTCATTAAACCGATGATCAAATATAATAATCTGAATTTAGGGCTTCCAAT

121        TUBRTGF2011                                           180
TGACGCATTCTTTGGGAGGAGGTACTGGTTCGGGTATGGGCACTTTGCTCATCTCAAAAA

181                           TUBRTGR2011                        240
TTCGCGAGGAGTATCCGGATAGAATCATGGCTTCATTCTCCGTTGTTCCATCACCAAAGG

241                                                              300
TAATGTATCCCTAACAGTAGTCCTTTTTGAGATCGTATGTACAGGTTCTCGAGGTGCAAT

301                                                              360
CCATTTACGAACTCTTCACATTGATGCGCAACTGTGAAATGTGCGAAGAAGTTATGTTTA

361                                                              420
TAGGTTTCCGATACCGTTGTGGAACCTTACAATGCCACTCTTTCTGTACACCAGTTGGTT

421                                                              480
GAAAATACCGATGAAACATTCTGCATCGATAATGAAGCTCTGTACGATATCTGCTTCCGC

481                                                              540
ACCTTAAAACTCACAAATCCAACTTACGACGATCTCAATCACTTAGGTGAGGTTTTATAT

541                                                              600
AGGTTTTATGAGTATTAGCTTACTGCCATCTTTTTAAATGTATTGTATTGTGGTTTTGCA

601                                                              660
GTGTCTGTCACAGTGTCTGGAGTCACGACCTGCCTTCGATTCCCTGGACAGTTGAATGCT

661    TUBGR            687
GATCTTCGCAAGTTGGCTGTGAACATG
```

APPENDIX 8

Consensus DNA sequence for *Tci-pgp-9* IBD77

ClustalW2 alignment of *Tci-pgp-9-IBDA* allelic variants showing introns (shaded), amino acid translation (underlined). A 99 bp PCR product was generated from UK isolates of *T. circumcincta* using real time PCR with the *Tci-pgp-9* gene specific primer pair IBD77RTGFdeg and IBD77RTGRdeg (boxed) which were designed so to amplify products from all of the identified allelic variants. A 291bp fragment of UKv3 that included the target site for the real time PCR primer pair was generated using the IBD77GF4 and IBD77RAS12 primers (boxed) and included as template DNA in the positive control wells.

```
            1           IBD77GF4 →                                                                                                  120
UKv3        GAGTAGTKTCACARGAGCCHATGCTGTTCAACACGACCATTGAACAGGTTTGTTTTCGGG-GAGGTCTTATCAACAGAAAAA-----AAAAGGAAAATGGCTAATCTCATTCAGAATATC
UKv5        GAGTAGTKTCACAGGAGCCAATGCTGTTTAACACAACGATTGAACAGGTTTGTTCTCACG AGAGCTYTGATCCAAAGAAA---GAAARATGGAAAATAACTCGTTCYTTTTAGAATATC
UKv8        GAGTAGTTTCACAGGAGCCTATGCTGTTCAACACAACGATTGAACAGGTTTGTTCTCACGAGAGSTTTGATCAAAAGAAAAATGGAAAAATTGAAAATAACBCATTCCTTTCAGAATATC
UKv9        GAGTAGTTTCACAGGAACCTATGCTTTTCAACACAACGATTGAACAGGTTTGTTTTGAGGAGGTCTG--ATCAATAGAAAAAAGGAAAAATTGAAAATGACGTATCTCTTTCAGAATATT
UKv7        GAGTAGTGTCACAGGAGCCTATGCTGTTTAACACAACGATTGAACAGGTTTGTTCTCACGAGAGCTTTAATCCGAAGAAAAATGGGAAGATGGAAAATAACTCGGTCCTTTCAGAATATC
UKv10       GAGTAGTTTCACAAGAACCGATGCTCTTTAACACGACGATTGAACAGGTTCGTTCCCACGAGAGCTTTGATCAAACGAAAAATGG-AAAACTGAAAATAACCCATTCCTTTCAGAACATC
UKv12       GAGTAGTTTCACAGGAGCCKATGCTCTTTAACACAACGATTGAACAGGTTTGTTCTAACGAGAGCTTTGTTCAAAAGAAAAATG----------------CTGATCTCATTCAGAATATT
UKv6        GAGTAGTTTCACAAGAGCCSATGCTGWTTAACACAACGATTGAACAGGTTTGTTCTCACGAGAGCTTTGATCCAAAGAAAAATGGAAAGATGGAAAATAACTCGTTCCTTTTAGAATATY
UKv2        GAGTAGTKTCACAGGAGCCHATGCTGTTYAACACAACGATTGAACAGGTTTGTACTCACGAAAGTTTCGATCAAAAGAAAAATGGAAAAATTAAAAATAACCCATTCCTTTCAGAATATC
UKv11       GAGTAGTKTCACAGGAGCCRATGCTGTTTAACACAACGATTGAACAGGTTTGTACTCACGAAAGTTTCGATCAAAAGAAAAATGGGAAAATTAAAAATAACCCATTCCTTTCAGAATATC
UKv13       GAGTAGTTTCACAAGAGCCTATGCTGTTTAACACAACGATTGAACAGGTTTGT-TTTTGGAGAGATCTGATGAATAGAAAAAAGGAAA-ATGGTTAAT—CTCATT------CAGAATATC
UKv14       GAGTAGTTTCACARGAACCKATGCTTTTCAACACAACGATTGAACAGGTTTGT-TTCTGAAGAGGTCTGATCAATAGAAAAATGGAAAGATGGAAAATGACTCATTCCTTTTAGAATATC
            ******* ***** ** ** *****  * ***** ** ************ **           *    *     ****                   *           **** **

              V  V  S  Q  E  P  M  L  F  N  T  T  I  E  Q                                                          N  I
```


99 bp real time PCR product
121
IBD77RTGFdeg →
← IBD77RTGRdeg
240
UKv3 CGATATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGCAAAGCAAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAATCTCGTGCTGTTGAT---A
UKv5 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTCCGCAAAGCGAACGCCTATAACTTTGTACAGTCGTTCCCTGACGTGAGTTGGAACCTCTTGCTGTTGATGTTA
UKv8 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCGAACGCCTACAATTTTGTGCAGTCGTTCCCTGACGTGAGTTGGAACCTCGTGCTGTCGACCTTA
UKv9 CGCTATGGACGTGAAAAAGTCACAGACGCTGAAATAACGGCCGCACTCCGTAAAGCAAACGCCTACAATTTTGTGCAGTCGTTCCCTGATGTGAGTTGGAACCTCATGCTGTTGACGTTA
UKv7 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTCCGCAAAGCGAACGCCTATAACTTTGTACAGTCGTTCCCTGACGTGAGTTGGAACCTCGTGCTGTTGACGTTA
UKv10 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCAGCACTCCGTAAAGCGAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAACCTCGTGTTGTCGGTGTTA
UKv12 CGTTATGGTCGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCAAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAACCTCGTGTTGTCGGCGTTA
UKv6 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTCCGCAAAGCGAACGCCTATAACTTTGTACAGTCGTTCCCTGACGTGAGTTGGAACCTTGTGCTATTGATGTTA
UKv2 CGTTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCAAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAACCTCGTGCTGTCGGTGTTA
UKv11 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACGGCGGCACTCCGTAAAGCAAACGCCTACAATTTTGTGCAGTCATTCCCTGACGTGAGTTGGAAGCTCGTATTGTCGATCTTA
UKv13 CGTTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTCCGTAAAGCGAACGCCTATAACTTTGTACAGTCGTTCCCTGACGTGAGTTGGAATATCCTGCTGTTGATGTTA
UKv14 CGCTATGGACGTGAAAAAGTCACAGATGCTGAAATCACAGCGGCACTGCGCAAAGCGAACGCCTATAATTTTGTACAGTCGTTCCCTGACGTGAGTTGGAATATCGTGCTGTTGATGTTA
** ***** ********** ******** ** ** ***** ** ***** ******** ** ***** ***** ******** ********** * * * * * *
R Y G R E K V T D A E I T A A L R K A N A Y N F V Q S F P D
241
← IBD77RAS12
360
UKv3 GATGCAGTGAGTGCAATAGCTGGTAGGCGG-------AGTCAGTC-TAAGAAAGT----GGTGGAATGGCGCTGGGAAGGAGAGAGCGGCAGGGTGTCGTT----CAATCCCCAACGCCC

APPENDIX 9

Mean C_T values for B-tubulin and *Tci-pgp-9* genes in UK Isolates of *T. circumcincta*

Table showing the mean C_T values calculated from duplicate reactions from individual larvae from each UK isolate studied. The ΔC_T was calculated for each larva.

Larval Lysate	MTci2			MTci5			MTci5PT		
	Mean ß-tubulin C_T	Mean *Tci-pgp-9* C_T	ΔC_T	Mean ß-tubulin C_T	Mean *Tci-pgp-9* C_T	ΔC_T	Mean ß-tubulin C_T	Mean *Tci-pgp-9* C_T	ΔC_T
A1	28.22	29.54	1.33	28.45	29.12	0.67	28.19	25.77	-2.41
A2				28.49	27.11	-1.38	27.96	26.29	-1.66
A3	27.59	30.10	2.52	28.81	26.69	-2.12	28.04	29.22	1.18
A4	27.92	29.36	1.44	28.68	26.35	-2.32	28.21	25.48	-2.74
A5	27.95	29.57	1.62	28.17	27.90	-0.27	28.12	29.34	1.22
A6	28.55	29.78	1.24	27.87	29.53	1.66	28.36	27.62	-0.74
A7	28.16	29.65	1.49	28.61	29.63	1.02	28.25	29.19	0.93
A8	28.21	29.53	1.32	28.59	27.28	-1.31	28.54	29.38	0.84
A9	28.12	29.44	1.32	28.46	26.90	-1.56	28.48	30.41	1.93
A10	28.28	29.28	1.00	29.59	30.76	1.17	28.58	29.37	0.79
A11	28.03	29.08	1.06	27.17	25.94	-1.22	28.55	27.24	-1.31
A12	28.25	29.40	1.15	28.37	26.61	-1.76	28.23	29.65	1.43
B1	28.83	31.28	2.45	28.42	29.31	0.89	28.50	29.37	0.88
B2	29.30	30.88	1.58	28.82	26.93	-1.89	28.30	29.08	0.77
B3	28.08	30.15	2.08	29.30	26.59	-2.71	28.10	29.61	1.51
B4	27.64	29.58	1.94	28.11	29.36	1.25	28.45	30.29	1.84
B5	28.15	29.82	1.67	27.79	25.61	-2.18	28.57	26.21	-2.35
B6	27.97	29.65	1.68	28.20	26.32	-1.89	28.20	27.30	-0.90
B7	28.28	29.49	1.21	28.47	30.03	1.56	27.99	30.18	2.19
B8	28.83	29.81	0.98	28.47	28.71	0.25	28.28	29.44	1.16
B9	28.21	29.03	0.82	28.17	29.38	1.21	27.54	26.52	-1.02
B10	28.06	29.29	1.23	28.05	29.27	1.21	28.59	26.62	-1.97
B11	28.48	29.13	0.65	28.56	29.51	0.96	29.03	29.84	0.81
B12	28.25	29.41	1.16	28.22	29.24	1.02	27.36	30.15	2.79
C1	28.10	29.95	1.86	28.71	28.99	0.28	27.88	29.35	1.47
C2	28.87	30.68	1.81	28.68	27.20	-1.48	28.42	29.59	1.16
C3	28.23	30.11	1.88	28.89	29.84	0.95	28.02	27.41	-0.61
C4	28.21	29.55	1.34	28.25	26.59	-1.66	28.68	29.55	0.87
C5	27.87	29.75	1.88	28.49	30.05	1.56	27.83	28.87	1.04
C6	27.99	29.44	1.46	28.28	26.19	-2.09	28.24	25.52	-2.72
C7	28.23	29.71	1.47	28.22	29.85	1.63	28.62	29.76	1.15
C8	28.19	29.50	1.31	28.11	26.00	-2.11	28.30	29.69	1.39
C9	28.21	29.35	1.13	28.12	29.73	1.61	28.14	29.48	1.35
C10	28.02	29.30	1.27	28.24	26.44	-1.80	28.48	26.97	-1.50
C11	28.05	29.31	1.27	28.49	27.05	-1.44	28.52	28.31	-0.22
C12	27.95	29.28	1.33	28.39	29.07	0.68	28.57	29.60	1.03
D1	27.66	29.42	1.76	28.27	29.11	0.84	28.30	26.95	-1.35
D2	28.63	31.06	2.43	28.56	26.21	-2.36	28.18	27.17	-1.01
D3	28.28	30.17	1.89	28.90	27.34	-1.57	28.02	26.11	-1.91
D4	28.04	30.02	1.98	28.70	29.63	0.93	28.27	26.49	-1.78

Larval Lysate	Mean β-tubulin C_T	Mean *Tci-pgp-9* C_T	ΔC_T	Mean β-tubulin C_T	Mean *Tci-pgp-9* C_T	ΔC_T	Mean β-tubulin C_T	Mean *Tci-pgp-9* C_T	ΔC_T
D5	28.23	29.70	1.47	28.31	25.97	-2.34	28.29	29.69	1.40
D6	27.69	29.67	1.98	28.10	25.77	-2.33	28.86	30.18	1.33
D7	28.77	30.35	1.58	28.44	26.84	-1.60	28.19	27.75	-0.45
D8	28.21	29.33	1.13	28.08	29.39	1.32	28.35	27.53	-0.82
D9	28.15	29.37	1.22	28.28	29.84	1.56	28.18	29.45	1.27
D10	28.27	29.73	1.46	28.29	25.03	-3.26	27.92	29.28	1.36
D11	28.07	29.36	1.29	28.39	29.15	0.75	28.26	27.44	-0.82
D12	28.03	29.17	1.13	28.47	29.37	0.89	28.45	29.72	1.28
E1	31.70	33.52	1.82	28.69	29.38	0.69	28.69	29.57	0.88
E2	28.58	30.72	2.15	28.97	29.53	0.55	28.77	26.79	-1.98
E3	27.99	29.82	1.83	28.64	29.34	0.71	28.13	29.43	1.30
E4	28.20	29.60	1.39	28.22	26.71	-1.51	28.31	29.99	1.69
E5	27.95	29.24	1.29	27.75	26.04	-1.71	28.45	29.72	1.26
E6	27.92	29.48	1.56	28.18	26.55	-1.63	29.63	28.46	-1.17
E7	28.45	29.77	1.32	28.32	25.63	-2.69	28.61	29.74	1.13
E8	28.21	29.59	1.38	28.14	29.49	1.35	28.37	29.26	0.89
E9	28.28	29.71	1.43	28.39	26.50	-1.88	28.40	29.61	1.21
E10	28.19	29.20	1.01	28.10	26.52	-1.58	27.22	28.67	1.45
E11	27.20	28.37	1.17	28.40	26.26	-2.14	28.52	29.54	1.02
E12	28.18	28.97	0.79	28.75	26.74	-2.01	28.67	30.15	1.48
F1	28.00	29.98	1.99	28.85	29.27	0.42	28.25	29.37	1.12
F2	28.15	31.00	2.85	28.85	25.38	-3.47	28.30	29.56	1.26
F3	27.91	29.78	1.87	28.78	27.57	-1.20	28.73	30.17	1.44
F4	28.08	29.44	1.36	27.99	29.50	1.50	28.54	26.44	-2.10
F5	27.38	29.13	1.75	27.54	29.58	2.04	28.47	29.80	1.33
F6	28.15	30.25	2.10	28.37	27.46	-0.91	28.87	29.62	0.75
F7	28.09	30.44	2.36	27.78	29.29	1.51	28.30	29.67	1.37
F8	28.34	29.37	1.03	28.22	27.69	-0.53	28.34	29.63	1.29
F9	28.13	29.56	1.44	28.04	26.08	-1.97	27.92	29.72	1.81
F10	28.36	29.53	1.17	28.20	29.64	1.44	29.11	30.23	1.12
F11	28.41	29.61	1.20	28.49	26.54	-1.95	29.05	30.32	1.28
F12	28.36	29.58	1.22	28.47	29.67	1.21	28.33	29.78	1.45
G1	31.92	33.56	1.64	28.52	26.16	-2.35	28.82	26.41	-2.41
G2	28.50	30.56	2.06	28.57	28.94	0.37	28.42	28.41	-0.01
G3	27.71	30.07	2.37	28.66	29.50	0.84	28.42	29.58	1.17
G4	27.76	29.48	1.73	28.22	29.30	1.08	28.58	29.88	1.30
G5	28.12	28.89	0.77	28.16	29.24	1.08	28.06	29.37	1.30
G6	28.10	29.81	1.71	28.32	29.68	1.37	28.78	29.94	1.16
G7	28.11	29.25	1.14	28.61	30.24	1.63	29.16	30.16	1.00
G8	28.19	30.04	1.85	28.64	29.91	1.27	28.29	29.47	1.18
G9	28.29	29.65	1.36	28.61	28.90	0.29			
G10	28.30	29.70	1.39	27.87	26.38	-1.48	28.16	29.52	1.36
G11	28.35	29.27	0.92	28.13	26.98	-1.16	30.44	31.82	1.38
G12	28.24	29.63	1.39	28.48	27.11	-1.37	28.75	30.00	1.25
Mean	28.25	29.77	1.52	28.39	28.02	-0.37	28.40	28.86	0.46
S.E.	0.05	0.06	0.03	0.03	0.12	0.12	0.04	0.11	0.11
Range	27.19 - 32.72	28.16 - 35.00	0.65 - 2.85	27.04 - 29.84	24.95 - 31.10	-3.47 - 2.04	27.13 - 30.53	25.30 - 31.90	-2.74 - 2.79

APPENDIX 10

Mean C_T values for sub-populations $MTci2_{NF(0.1nM)}$ and $MTci2_{F(10nM)}$

Mean C_T values for two sub-populations of MTci2, $MTci2_{NF(0.1nM)}$ and $MTci2_{F(10nM)}$, which have been separated based on their phenotype using the LFIA. The ΔC_T value is also shown.

Larval Lysate	$MTci2_{NF10}$ Mean ß-tubulin C_T	Mean *Tci-pgp-9* C_T	ΔC_T
A1	28.34	30.10	1.76
A2	28.27	29.50	1.23
A3	28.31	29.72	1.42
A4	28.31	29.68	1.36
A5	28.39	29.35	0.97
A6	28.33	29.78	1.45
B1	29.18	30.01	0.83
B2	28.85	29.77	0.92
B3	28.13	29.65	1.52
B5	28.80	29.40	0.59
B6	28.60	29.58	0.98
C2	28.26	29.43	1.17
C3	28.25	29.73	1.48
C4	27.65	28.61	0.96
C5	28.21	29.33	1.12
C6	28.44	29.77	1.33
D1	28.87	29.67	0.79
D2	28.20	29.87	1.67
D3	28.79	29.64	0.86
D4	28.19	29.30	1.11
D5	28.22	29.49	1.27
D6	28.86	29.90	1.04
E1	28.49	29.28	0.79
E2	28.33	30.07	1.74
E3	28.37	29.69	1.31
E4	29.47	30.17	0.70
E5	28.46	29.50	1.04
E6	28.01	29.46	1.45
F1	29.84	29.81	-0.03
F2	28.32	29.63	1.32
F3	27.56	29.13	1.58
F4	28.06	29.14	1.09
F5	28.20	29.40	1.20
F6	28.41	29.84	1.44
G1	29.13	30.14	1.02
G2	28.20	29.38	1.18
G4	28.65	29.93	1.28
G5	28.58	29.56	0.98
G6	28.97	30.37	1.40
H1	28.67	29.65	0.99
H2	28.09	30.15	2.06
H4	28.03	29.54	1.50
H5	28.21	29.06	0.85
Mean	28.45	29.63	1.18
S.E.	0.05	0.05	0.04
Range	27.28 - 29.95	28.40 - 30.91	-0.03 - 2.06

Larval Lysate	$MTci2_{F90}$ Mean ß-tubulin C_T	Mean *Tci-pgp-9* C_T	ΔC_T
A7	28.98	29.66	0.69
A10	28.48	29.46	0.98
A11	28.83	29.54	0.70
A12	28.50	29.67	1.17
B7	28.97	29.50	0.53
B8	28.38	29.94	1.56
B9	28.45	29.50	1.05
B10	28.48	29.89	1.41
B12	28.48	29.30	0.83
C7	28.67	29.46	0.80
C8	28.34	29.62	1.28
C10	28.77	29.91	1.14
C11	28.67	29.36	0.69
C12	28.19	29.58	1.40
D7	28.89	29.95	1.06
D8	28.31	29.64	1.33
D9	27.96	29.55	1.59
D10	28.44	29.92	1.48
D11	28.65	29.57	0.92
D12	28.56	29.55	0.99
E7	28.72	29.88	1.17
E8	28.58	29.99	1.40
E9	28.37	30.00	1.62
E10	28.47	29.61	1.14
E11	27.88	27.38	-0.50
E12	27.52	28.66	1.14
F7	28.93	29.72	0.79
F8	28.52	29.91	1.38
F9	28.53	29.78	1.25
F10	28.40	29.63	1.24
F11	28.27	29.77	1.49
F12	28.30	29.69	1.39
G7	28.67	29.90	1.24
G8	28.34	29.82	1.47
G9	27.88	29.20	1.32
G10	28.46	29.42	0.97
G11	28.44	29.86	1.42
G12	28.47	29.76	1.29
H7	28.72	29.75	1.03
H8	28.64	29.86	1.22
H9	28.84	29.87	1.03
H10	29.26	30.18	0.92
H11	28.21	29.89	1.68
	28.50	29.63	1.13
	0.04	0.05	0.04
	27.36 - 29.31	27.37 - 30.34	-0.50 - 1.68

APPENDIX 11

Mean C_T values for sub-populations $MTci5_{NF(0.1nM)}$ and $MTci5_{F(10nM)}$

Mean C_T values for two sub-populations of MTci5, $MTci5_{NF(0.1nM)}$ and $MTci5_{F(10nM)}$, which have been separated based on phenotype using the LFIA. The ΔC_T value is also shown.

Larval Lysate	$MTci5_{NF(0.1nM)}$ Mean ß-tubulin C_T	$MTci5_{NF(0.1nM)}$ Mean *Tci-pgp-9* C_T	$MTci5_{NF(0.1nM)}$ ΔC_T
A1	28.21	29.27	1.06
A2	27.91	29.52	1.61
A3	27.90	26.47	-1.43
A4	28.65	25.79	-2.86
A5	27.61	28.96	1.35
A6	26.89	28.63	1.74
B1	28.34	29.55	1.21
B2	28.14	26.49	-1.64
B3	28.12	29.80	1.68
B4	28.49	25.52	-2.97
B5	28.06	25.33	-2.73
B6	26.82	25.76	-1.05
C1	28.16	26.49	-1.67
C2	28.07	29.31	1.24
C3	28.10	25.60	-2.50
C4	29.03	25.44	-3.59
C5	28.18	26.74	-1.44
C6	26.85	25.24	-1.61
D1	27.90	25.83	-2.08
D2	28.12	26.51	-1.62
D3	28.08	27.94	-0.14
D4	28.19	28.80	0.61
D5			
D6	26.96	25.59	-1.37
E1	28.22	26.89	-1.33
E2	28.45	29.29	0.84
E3	27.76	26.71	-1.04
E4	27.16	27.12	-0.04
E5	27.95	26.84	-1.11
E6	26.92	26.32	-0.59
F1	28.10	24.96	-3.15
F2	28.10	26.56	-1.53
F3	28.20	29.40	1.20
F4	27.67	24.69	-2.98
F5	27.55	25.28	-2.27
F6	26.85	28.59	1.73
G1	27.42	25.75	-1.67
G2	28.26	25.26	-3.00
G3			
G4	27.81	28.40	0.58
G5	27.78	29.34	1.56
G6	26.82	29.11	2.29
H1			
H2	29.08	27.26	-1.82
H3	28.46	25.44	-3.02
H4	27.67	26.57	-1.10
H5	27.54	26.36	-1.18
Mean	27.88	27.06	-0.81
S.E.	0.06	0.17	0.18
Range	26.56 - 29.39	24.58 - 29.82	-3.59 - 2.29

Larval Lysate	$MTci5_{F(10nM)}$ Mean ß-tubulin C_T	$MTci5_{F(10nM)}$ Mean *Tci-pgp-9* C_T	$MTci5_{F(10nM)}$ ΔC_T
A7	28.15	29.03	0.88
A8	28.06	28.75	0.69
A9	27.66	25.62	-2.04
A10	30.07	27.17	-2.91
A11	28.56	26.95	-1.62
A12	27.93	27.14	-0.79
B7	28.14	26.32	-1.82
B8	27.39	26.19	-1.19
B9	27.66	24.38	-3.28
B10	28.07	26.14	-1.93
B11	27.74	28.95	1.21
B12	27.88	26.43	-1.45
C7	27.36	28.84	1.47
C8	27.20	29.66	2.46
C9	27.77	24.69	-3.08
C10	28.24	27.18	-1.07
C11	27.55	28.82	1.27
C12	27.53	27.50	-0.03
D7	28.13	25.07	-3.05
D8	27.36	25.36	-2.00
D9	27.53	26.54	-1.00
D10	28.64	26.07	-2.57
D11	27.27	25.98	-1.29
D12	28.01	28.75	0.73
E7	27.89	26.05	-1.84
E8	27.51	25.64	-1.87
E9	27.61	25.79	-1.82
E10	28.47	26.84	-1.63
E11	28.04	26.94	-1.09
E12	28.21	25.01	-3.20
F7	27.41	25.94	-1.47
F8	27.19	28.97	1.77
F9	27.94	25.45	-2.49
F10	28.61	28.85	0.24
F11	27.92	26.41	-1.51
F12	27.94	25.13	-2.81
G7	28.25	28.79	0.54
G8	27.64	24.76	-2.88
G9	27.45	29.13	1.68
G10	28.50	27.31	-1.19
G11	28.16	26.29	-1.87
G12	27.83	28.70	0.87
H7	28.06	25.69	-2.37
H8	27.68	29.30	1.62
H9	27.89	28.49	0.60
H10	28.22	26.86	-1.37
H11	27.15	28.48	1.33
	27.90	26.99	-0.92
	0.06	0.16	0.17
	26.38 - 30.29	24.27 - 30.18	-3.28 - 2.46

APPENDIX 12

Further characterisations of *Tci-pgp-9* in UK isolates of *T. circumcincta*

The allelic variants identified in MTci2, MTci5 and MTci5PT larval lysates are listed along with the corresponding ΔC_T value. Real time PCR Amplification failed in two individuals (MTci2 A2 & MTci5PT G9; annotated with *). No allele specific PCR product and no real time PCR products were observed in larvae E6, E8, G7 and G11 from the MTci5PT isolate.

Larval Lysate	MTci2		MTci5		MTci5PT	
	Allelic Variant(s)	ΔC_T	Allelic Variant(s)	ΔC_T	Allelic Variant(s)	ΔC_T
A1	2, 8, 12	1.33	2, 7, 8, 10, 11, 13	0.67	3, 11, 12	-2.41
A2	8, 9, 10, 13	*	2	-1.38	3, 5, 10, 12	-1.66
A3	5	2.52	3, 5, 7, 8, 9, 10, 11	-2.12	2, 13	1.18
A4	2, 7, 13	1.44	2, 3, 8, 10, 13	-2.32	3, 5	-2.74
A5	5, 8, 9	1.62	2, 3, 8, 9, 10, 11	-0.27	2, 5	1.22
A6	5, 7, 8, 12	1.24	2, 7, 9, 10, 11, 13	1.66	5, 12	-0.74
A7	3, 5, 8, 9, 10, 11, 12, 13	1.49	8, 9, 10	1.02	2, 7	0.93
A8	3, 5, 8, 9, 10, 12, 13	1.32	2, 7, 9, 13	-1.31	5, 12	0.84
A9	7, 9, 12	1.32	2, 3, 13	-1.56	5, 11	1.93
A10	5, 8, 10	1.00	2, 3, 12	1.17	5, 8	0.79
A11	8, 10, 11, 12, 13	1.06	2, 3, 10, 12	-1.22	2, 5, 12, 13	-1.31
A12	2, 5, 9, 10, 12	1.15	3, 5, 11	-1.76	2, 5, 13	1.43
B1	5, 8	2.45	5, 7, 9	0.89	7, 9, 11, 13	0.88
B2	5, 12	1.58	2, 3, 13	-1.89	2, 5, 7, 13	0.77
B3	2, 13	2.08	3, 5, 7, 12	-2.71	2, 5	1.51
B4	2, 12, 13	1.94	9	1.25	2, 13	1.84
B5	3, 5, 8, 9, 10, 11, 12	1.67	3, 8	-2.18	3	-2.35
B6	2, 3, 5, 7, 8, 9, 10, 11, 12, 13	1.68	3, 10, 12	-1.89	5, 12	-0.90
B7	5, 12	1.21	2, 12, 13	1.56	7	2.19
B8	8, 9, 10	0.98	3, 10, 12	0.25	2, 9	1.16
B9	5, 12	0.82	2, 3, 13	1.21	5, 12	-1.02
B10	2, 7, 8, 9, 10, 13	1.23	9, 12	1.21	8, 12	-1.97
B11	2, 13	0.65	3, 8	0.96	2	0.81
B12	8, 9, 12	1.16	3, 12	1.02	13	2.79
C1	5	1.86	2, 5, 7, 13	0.28	2, 13	1.47
C2	2, 12, 13	1.81	2, 5, 7, 13	-1.48	2, 5, 13	1.16
C3	2, 13	1.88	12, 13	0.95	10, 12	-0.61
C4	5, 8, 10, 12	1.34	2, 3, 5	-1.66	3, 12	0.87
C5	9, 12, 13	1.88	5, 7, 9	1.56	5, 9, 12	1.04
C6	2, 5, 7, 8, 9, 10, 11, 12, 13	1.46	3, 7, 9	-2.09	12	-2.72
C7	5, 7, 8, 9, 10, 13	1.47	5, 8, 13	1.63	2	1.15
C8	2, 5, 13	1.31	3, 9	-2.11	2, 5	1.39
C9	5, 8, 10, 12, 13	1.13	2, 5, 13	1.61	2, 5, 10	1.35
C10	5, 8, 9, 10	1.27	3	-1.80	2	-1.50
C11	2, 5, 8, 9, 10, 11, 13	1.27	2, 8	-1.44	5, 12	-0.22
C12	2, 8, 10	1.33	3, 8, 11, 13	0.68	5, 7, 9, 12	1.03
D1	5, 12	1.76	5	0.84	3, 12	-1.35
D2	2, 5, 8, 13	2.43	3	-2.36	3, 11, 12	-1.01
D3	2, 8, 12, 13	1.89	3, 7, 10	-1.57	3, 5, 8	-1.91
D4	2, 5, 8, 13	1.98	7, 9, 13	0.93	2, 12	-1.78

Larval Lysate	MTci2		MTci5		MTci5PT	
	Allelic Variant(s)	ΔC_T	Allelic Variant(s)	ΔC_T	Allelic Variant(s)	ΔC_T
D5	2, 12, 13	1.47	2, 3, 5	-2.34	2, 10	1.40
D6	2, 3, 5, 8, 9, 13	1.98	2, 3	-2.33	3, 13	1.33
D7	2, 5, 8, 9, 10, 11, 12, 13	1.58	3, 7, 9, 11	-1.60	2, 12	-0.45
D8	2, 5, 8, 13	1.13	3	1.32	2, 3, 5, 13	-0.82
D9	2, 5, 7, 8, 9, 10, 11, 13	1.22	5, 12	1.56	12	1.27
D10	8, 9, 10, 12	1.46	3	-3.26	5, 8	1.36
D11	5, 8, 12	1.29	2	0.75	12	-0.82
D12	2	1.13	2	0.89	3	1.28
E1	11	1.82	2, 8	0.69	2, 13	0.88
E2	2, 8, 11, 13	2.15	5, 10	0.55	5, 12	-1.98
E3	2, 8, 13	1.83	3	0.71	5, 12	1.30
E4	3, 8, 9, 10, 11, 12, 13	1.39	2, 3	-1.51	5, 12, 13	1.69
E5	2, 3, 9, 13	1.29	2, 3	-1.71	2, 13	1.26
E6	3, 5, 8, 9, 10, 11, 12, 13	1.56	3, 5	-1.63		
E7	2, 5, 13	1.32	3	-2.69	5, 12	1.13
E8	5, 10	1.38	2, 13	1.35		
E9	5, 8, 9, 10, 12, 13	1.43	2, 3, 13	-1.88	2, 12	1.21
E10	8, 9, 12	1.01	2, 3	-1.58	2, 7, 9, 12	1.45
E11	5, 12	1.17	2, 3	-2.14	5, 10	1.02
E12	2, 8, 12	0.79	3, 8	-2.01	5	1.48
F1	5, 11	1.99	3, 10, 12	0.42	5, 10	1.12
F2	2, 5, 9, 13	2.85	3, 8	-3.47	2, 5, 10, 13	1.26
F3	9, 12	1.87	3, 8, 13	-1.20	2, 5	1.44
F4	3, 9, 13	1.36	2, 10, 13	1.50	3, 5, 8, 9, 12	-2.10
F5	2, 5, 9, 10, 12, 13	1.75	2, 12, 13	2.04	2	1.33
F6	2, 5, 8, 9, 10, 12, 13	2.10	2, 9, 13	-0.91	7, 9, 11	0.75
F7	5, 12	2.36	8, 11	1.51	2, 8	1.37
F8	2, 5, 8, 9, 10, 13	1.03	2, 8, 11	-0.53	3, 12	1.29
F9	2, 8, 9, 10, 13	1.44	2, 3, 13	-1.97	5, 9	1.81
F10	2, 5, 8, 9, 10, 12, 13	1.17	2, 9, 13	1.44	13	1.12
F11	2, 8, 9, 10, 13	1.20	3	-1.95	13	1.28
F12	5, 8, 10, 13	1.22	5, 8	1.21	2, 7, 9, 13	1.45
G1	2	1.64	2, 3, 8, 10, 11	-2.35	12, 13	-2.41
G2	5, 9	2.06	2, 11, 13	0.37	2, 3, 12, 13	-0.01
G3	5, 8, 9	2.37	2, 9, 11, 13	0.84	7, 9, 11	1.17
G4	2, 5, 13	1.73	3, 11	1.08	5, 10	1.30
G5	2, 5, 13	0.77	7, 9, 11	1.08	2	1.30
G6	2, 12, 13	1.71	7, 9, 11	1.37	2, 12	1.16
G7	5	1.14	2, 11, 13	1.63		
G8	2, 13	1.85	2, 12	1.27	2, 10	1.18
G9	5, 12	1.36	3, 5, 12	0.29	8	*
G10	2, 5, 13	1.39	3, 12	-1.48	8, 9	1.36
G11	2, 12, 13	0.92	2, 3, 10	-1.16		
G12	5, 8, 9	1.39	3, 11	-1.37	2, 12	1.25

APPENDIX 13

Further characterisations of *Tci-pgp-9* in MTci2 sub-populations of *T. circumcincta*

The alleles identified in larvae from the MTci2 sub-populations are listed along with their ΔC_T value. Real time PCR Amplification failed in two larva from the $MTci2_{NF(0.1nM)}$ sub population (annotated with *) and rows where the initial lysis step has failed are shown with (*) in both the allelic variants and ΔCT columns.

$MTci2_{NF(0.1nM)}$		
Larval Lysate	Allelic Variants(s)	ΔC_T
A1	2, 5	1.76
A2	2, 5, 10, 13	1.23
A3	2, 7, 9, 13	1.42
A4	2, 9, 13	1.36
A5	2, 5, 13	0.97
A6	2, 5, 9, 10	1.45
B1	2, 13	0.83
B2	2, 10	0.92
B3	2, 5, 7	1.52
B4	*	*
B5	2, 5, 10, 13	0.59
B6	2, 5, 10, 13	0.98
C1	*	*
C2	2, 5, 13	1.17
C3	2, 5, 13	1.48
C4	2, 5, 12, 13	0.96
C5	2, 5, 12, 13	1.12
C6	2, 5, 13	1.33
D1	2, 5, 9, 10	0.79
D2	2, 12	1.67
D3	2, 5, 12	0.86
D4	2, 13	1.11
D5	2, 3	1.27
D6	2, 5, 10, 13	1.04
E1	2, 3	*
E2	2, 5, 13	1.74
E3	2, 5, 10, 12	1.31
E4	2, 13	0.70
E5	2, 8, 10, 13	1.04
E6	2, 9, 13	1.45
F1	2, 5, 10, 12	*
F2	2, 5, 13	1.32
F3	3, 5, 10	1.58
F4	2, 5, 11, 13	1.09
F5	2, 13	1.20
F6	2, 5, 7, 9, 10	1.44
G1	5, 12	*
G2	2, 5, 9, 10	1.18
G3	*	*
G4	2, 13	1.28
G5	2, 3, 5	0.98
G6	3, 5	1.40
H1	2, 5, 13	0.99
H2	2, 5, 13	2.06
H3	*	*
H4	2, 5, 13	1.50
H5	2, 5, 10, 13	0.85

$MTci2_{F(10nM)}$		
Larval Lysate	Allelic Variants(s)	ΔC_T
A7	2, 5, 10, 11	0.69
A8	*	*
A9	*	*
A10	2, 5, 13	0.98
A11	2, 12, 13	0.70
A12	2, 13	1.17
B7	2, 13	0.53
B8	5, 12	1.56
B9	2, 12, 13	1.05
B10	2, 5, 10	1.41
B11	*	*
B12	2, 5, 13	0.83
C7	2, 5, 10, 13	0.80
C8	2, 5,13	1.28
C9	*	*
C10	2, 3, 5	1.14
C11	2, 5, 10	0.69
C12	5, 9	1.40
D7	2, 12, 13	1.06
D8	2, 9, 13	1.33
D9	2, 3, 5, 9	1.59
D10	2, 5	1.48
D11	2, 5, 10, 13	0.92
D12	2, 5, 13	0.99
E7	3, 9	1.17
E8	2, 13	1.40
E9	2, 13	1.62
E10	2, 5, 13	1.14
E11	2, 3, 13	-0.50
E12	2, 3, 5, 10, 13	1.14
F7	5, 12	0.79
F8	2, 5, 10, 13	1.38
F9	2, 5, 13	1.25
F10	12	1.24
F11	2, 5, 9, 10	1.49
F12	2, 5, 13	1.39
G7	2, 5, 10	1.24
G8	12	1.47
G9	5, 10	1.32
G10	2, 7, 9, 13	0.97
G11	2, 5, 13	1.42
G12	2, 13	1.29
H7	2, 13	1.03
H8	2, 13	1.22
H9	2, 13	1.03
H10	3, 7, 9, 11, 12	0.92
H11	3, 11, 12	1.68

APPENDIX 14

Further characterisations of *Tci-pgp-9* in MTci5 sub-populations of *T. circumcincta*

The alleles identified in larvae from the MTci5 sub-populations are listed along with their ΔC_T value. The initial lysis step failed in two individuals (annotated with * in both the allelic variant(s) and ΔC_T columns) from the $MTci5_{NF(0.1nM)}$ sub-population. The real time PCR amplification failed in two larvae (H1 and H2) in the $MTci5_{NF(0.1nM)}$ sub-population.

$MTci5_{NF(0.1nM)}$		
Larval Lysate	Allelic Variant(s)	ΔC_T
A1	2, 11, 13	1.06
A2	2, 7, 13	1.61
A3	2, 13	-1.43
A4	3	-2.86
A5	2, 3, 5, 13	1.35
A6	2, 3, 13	1.74
B1	3, 12	1.21
B2	3, 7	-1.64
B3	8, 9, 12	1.68
B4	3	-2.97
B5	3	-2.73
B6	2, 3, 9, 13	-1.05
C1	3, 9, 11	-1.67
C2	2, 8, 10, 13	1.24
C3	2, 3	-2.50
C4	3	-3.59
C5	3, 8, 10	-1.44
C6	3	-1.61
D1	2, 3, 13	-2.08
D2	2, 3, 13	-1.62
D3	2, 3, 12, 13	-0.14
D4	2, 13	0.61
D5	*	*
D6	3	-1.37
E1	2, 3, 12, 13	-1.33
E2	2, 7, 9, 13	0.84
E3	2, 3, 13	-1.04
E4	2, 3, 13	-0.04
E5	2, 3, 8, 10	-1.11
E6	2, 13	-0.59
F1	2, 3, 8, 13	-3.15
F2	3, 7, 9	-1.53
F3	2, 13	1.20
F4	2, 3, 8, 13	-2.98
F5	3, 8, 10	-2.27
F6	3	1.73
G1	2, 3, 12, 13	-1.67
G2	2, 3, 13	-3.00
G3	*	*
G4	2, 3, 5, 13	0.58
G5	3, 5	1.56
G6	3	2.29
H1	3, 7, 9, 12	*
H2	2, 3, 13	*
H3	3	-3.02
H4	2, 3, 13	-1.10
H5	2, 8, 10, 13	-1.18

$MTci5_{F(10nM)}$		
Larval Lysate	Allelic Variant(s)	ΔC_T
A7	2, 3, 12, 13	0.88
A8	3, 7, 9	0.69
A9	2, 3, 13	-2.04
A10	2, 3, 13	-2.91
A11	2, 3, 13	-1.62
A12	3, 12	-0.79
B7	2, 3, 13	-1.82
B8	2, 3, 13	-1.19
B9	2, 3, 13	-3.28
B10	2, 13	-1.93
B11	2, 3, 13	1.21
B12	2, 12, 13	-1.45
C7	3, 7, 9	1.47
C8	5, 8, 10	2.46
C9	2, 3, 10	-3.08
C10	3	-1.07
C11	3, 12	1.27
C12	2, 3, 13	-0.03
D7	3	-3.05
D8	2, 3, 13	-2.00
D9	2, 3, 8, 10	-1.00
D10	2, 3, 13	-2.57
D11	2, 3, 13	-1.29
D12	2, 13	0.73
E7	3, 8, 10, 11	-1.84
E8	3, 10	-1.87
E9	2, 3, 13	-1.82
E10	3	-1.63
E11	2, 3, 12	-1.09
E12	2, 3, 13	-3.20
F7	3, 9	-1.47
F8	2, 3, 7, 9, 13	1.77
F9	3, 9	-2.49
F10	5, 8	0.24
F11	2, 13	-1.51
F12	3, 8	-2.81
G7	2, 7, 9	0.54
G8	2, 3, 13	-2.88
G9	2, 3, 13	1.68
G10	2, 3, 11, 13	-1.19
G11	2, 3, 13	-1.87
G12	2, 13	0.87
H7	3, 8	-2.37
H8	2, 13	1.62
H9	2, 5, 13	0.60
H10	3, 12	-1.37
H11	2, 13	1.33

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