Development of microsatellites and the population genetic analysis of the parasitic nematode *Teladorsagia circumcincta*

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A thesis submitted for degree of Doctor of Philosophy
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December 2005

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Declaration

I declare that this thesis consists entirely of my own work, unless specifically indicated and has been composed by myself. This thesis has not been accepted in any previous application for a degree in this or any other university.

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Abstract

Gastrointestinal parasitic nematodes of sheep and goats are of major economic importance worldwide. The control of parasitic nematodes, in both domestic livestock and man, is dependent upon the strategic use of anthelmintic drugs such as the benzimidazoles, levamisoles and ivermectins. However, for parasitic nematodes of sheep and goats, resistance to these drugs is becoming increasingly common. Anthelmintic resistance in *T. circumcincta*, a parasitic gastrointestinal nematode of sheep and goats, is of major economic importance especially in countries of the northern hemisphere, such as the U.K. However, relatively little is understood regarding how resistance develops and spreads in parasitic nematode populations, including *T. circumcincta*. In order to address these issues, an understanding of the population genetic structure is required but as yet very few studies have investigated this area. The main aim of my thesis was to investigate the population genetic structure of *T. circumcincta* using multilocus genomic markers. However there are very few molecular tools available for this parasite species and very little genomic sequence available from which to design markers. Therefore, a key first step of this project was to isolate microsatellite markers from *T. circumcincta* that were suitable for population genetic analysis.

Three approaches were used to isolate microsatellite markers from *T. circumcincta*. Hybridisation screening of small insert genomic libraries, amplification of conserved loci with previously known loci in *Haemonchus contortus* and EST database searching, yielded 45, 19 and 14 microsatellites respectively.

Five of these microsatellite markers were found to be sufficiently robust and polymorphic to be used in population genetic studies. These were used to genotype single adults, L3 stages or eggs from eighteen *T. circumcincta* populations from Scotland, France and New Zealand. Overall, the level of polymorphism for all populations investigated was high, which corresponds with previous studies of trichostrongylid nematodes which have been predominantly performed using mitochondrial DNA markers. The majority of diversity seen was within populations rather than between populations. However, population genetic analysis with the microsatellites markers supports the existence of a 'cryptic' species of *T. circumcincta* in French goats, as originally suggested by Leignel *et al.* (2002). In the FrMe population, the same individuals genetically differentiated with multilocus genotyping where found to be typed as 'cryptic' when using the previously described markers; ITS-2 and β-tubulin (Leignel *et al.* 2002). The microsatellites markers used in this study, demonstrated independent evidence that this 'cryptic' species is potentially a new species of *T. circumcincta*. In addition, moderate levels of

differentiation are seen between the New Zealand population and all the other populations in this study. From the populations analysed from the U.K., the data suggest that there is very little differentiation seen between populations regardless of the host species, geographical location or source of origin as a field or laboratory maintained isolate. Population genetic analysis using the microsatellites detected no genetic differentiation between *T. davtiani*, *T. circumcincta* and *T. trifurcata* supporting the suggestion that these may be morphological variants of the same species.

In addition to investigating the population genetic structure of *T. circumcincta*, a survey of U.K. goat farms was conducted, as the current prevalence of anthelmintic resistance in U.K. goats is unknown. For many parts of the world, drug resistance in sheep nematodes has been preceded by drug resistance in goat nematodes, suggesting that goats may be a source of resistant parasites for sheep. Therefore the survey was conducted to investigate the efficacy of parasitic control methods and as well as to identify anthelmintic resistant populations of *T. circumcincta* for use in further population genetic studies. The survey found not only farmers with poor drug efficacies, but also highlighted the need to improve the tests used to investigate and quantify parasitic nematode control.

Acknowledgements

I would like to thank John Gilleard, my supervisor, for continuous advice, discussion and enthusiasm in addition to the various useful debates. John has provided me with the knowledge and skills to allow me to become a confident scientist in my own right. I would also like to thank the University of Glasgow for funding this project. In addition, I would like to thank my assessors, Frank Jackson and Andy Tait for providing advice and discussion when required.

I would also like to thank everyone in the Veterinary Parasitology Department (Division of Infection and Immunity), past and present for their help throughout and in particular Judith Smith, Pamela McGarr, Annabelle Couthier, and Stevie Robertson for their endless laughs and practical advice in the lab, James McGolderick for his advice regarding my goat survey and allowing me to bring smelly samples into his lab, as well as doing some FEC for me. I would also like to acknowledge Willie Weir and Erica Packard for their continuous sense of humour and optimism throughout my writing up, which I believe kept me sane!

The collaboration with the Moredun Institute during this project has been highly productive not only for the endless numbers of *T. circumcincta* but providing me with the parasitology skills required for my survey. In particular, Frank and Liz Jackson, Dave Bartley and Lindsay Stenhouse for advice, discussion, and always welcoming me as one of the extended lab family, especially at tea break!

I would like to acknowledge everyone who provided *T. circumcincta* populations, including Dr Warwick Grant for the New Zealand sample, Dr Jacques Cabaret for the French populations prepared at INRA, Barbara Craig, Dr BarbaraWimmer and Dr Josephine Pemberton for the samples from Hirta and Dr Sian Mitchell for the Welsh sample. I would also like to thank all the goat farmers who took part in the survey.

I would like to thank all my friends and family for endless jokes regarding my (worm) project, in particular Babs and Roddy for support, chats, coffee and nights out when necessary and Rich for proof reading.

It has been a long stint here in Glasgow, but the love and emotional support from my parents and in particular my soon to be husband, Jason has been unfailing along the way. I would like to thank my parents in particular for their encouragement and support of their daughter's seemingly endless education!

I would like to dedicate this thesis to Jason, as he was the one who encouraged me to go after what I want, and has been so patient with the time it has taken to complete. Without him, I wouldn't have accomplished this!

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Abbrevations

AMOVA	Analysis of Molecular Variance
AFLP	Amplified Fragment Length Polymorphism
β	Beta
bp	base pair
BZ	Benzimidazole
DET	drench efficacy test
EHA	egg hatch assay
EPG	eggs per gram
FECRT	Faecal Egg Count Reduction Test
GDA	Genetic Data Analysis
H_o	Observed heterozygosity
H_e	Expected heterozygosity
HW	Hardy-Weinburg
HWE	Hardy-Weinburg equilibruim
ITS	Internal Transcribed Spacer
IVR	Ivermectin
K_2P	kimura two parameter
LEV	Levamisole
MDH-2	malate dehydrogenase
mtDNA	Mitochrondrial DNA
mya	million years ago
N_e	effective population size
ND4	nicotinamide adenine dinucleotide dehydrogenase subunit 4
PCA	Principle Coordinate Analysis
PCR	Polymerase Chain Reaction
pers. comm.	Personal communication
rDNA	ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
SNP	Single Nucleotide Polymorphism
SSCP	single-stranded conformation polymorphism
ver	version

Chapter 1: Introduction

1.1 Nematodes: An overview

The phylum Nematoda encompasses a diverse range of species which inhabit nearly all ecosystems (Sommer 2000) and has been estimated to contain up to 100 million species (Dorris *et al.* 1999). The majority of nematodes are free living, and are not reliant on other living organisms. A smaller number are parasitic and live within other organisms (hosts) to which they are specifically adapted and often cause harm (Poulin 1998). Parasitic nematodes are found in both plants and animals including humans (Jasmer *et al.* 2003). Plant parasitic nematodes affect crops worldwide, and with the increase in world trade of crops, many parasitic nematodes are no longer isolated to one country or area (Jasmer *et al.* 2003). Control of these nematodes includes breeding resistant crop varieties and the use of nematocides. Parasitic nematodes of humans infect over 1 billion people worldwide and are a major cause of morbidity and mortality (Jasmer *et al.* 2003). Intestinal nematode infections account for three of the top fourteen infectious and parasitic diseases worldwide, based on disability-adjusted life years (DALYs) (Hotez *et al.* 2004). Most domesticated grazing livestock throughout the world are infected by parasitic nematodes which results in major economic loss and often clinical disease. Control of these livestock parasites depends on the use of a limited number of anthelmintics to which many nematode species are becoming increasingly resistant. Consequently there is significant interest in new approaches to livestock parasite control such as breeding resistant livestock, feeding nematophagous fungi, and developing recombinant vaccines. However, the extent to which parasitic nematodes will adapt to novel control methods is unknown. Hence there is a need to understand the genetic variability of parasitic nematodes, how such variation is structured within and between nematode populations and how it will affect different parasitic nematode control measures.

The traditionally accepted classification within the phylum Nematoda has been based on morphological traits (Anderson 1992). However, the majority of nematodes have relatively few morphological features, limiting the precision by which these genetically diverse species can be classified (Dorris *et al.* 1999). More recently there is a recognition of the need to analyse nematode diversity using molecular tools and to determine true phylogenetic relationships which should permit a new understanding of strategies used by parasitic nematodes to invade hosts or avoid host defences (Dorris *et al.* 1999;Jasmer *et al.*

2003). Currently, the most accepted phylogenetic tree of the phylum Nematoda is based on the sequences of the 18S rRNA subunit (Blaxter *et al.* 1998;Dorris *et al.* 1999) (Figure 1.1). This tree is not entirely congruent with that produced by previous classical taxonomy. The plant and animal parasitic nematodes are dispersed with free-living nematodes throughout five major clades (Blaxter *et al.* 1998). This new phylogeny suggests that parasitism has appeared independently multiple times throughout evolution. Also, the phylogenetic relationship between a number of parasitic nematodes and the important model organism *Caenorhabditis elegans* has been revised. Parasites in the genus Strongyloides have traditionally been considered to be very closely related to rhabditids such as *C. elegans*. However, based on small subunit rRNA (18S) sequence, *C. elegans* appears to be more closely related to members of the order Strongylida. Many studies have used sequence from the rRNA gene cluster and / or mitochrondrial DNA (mtDNA) for phylogenetic analysis and the investigation of nematode speciation (Hoberg *et al.* 1999;Leignel *et al.* 1997;Zarlenga *et al.* 1998). However, the choice of molecular marker used to study phylogenetic relationships is a complex issue and multilocus approaches are highly desirable.

This project concentrates on the population genetics of the parasitic strongylid nematode *Teladorsagia circumcincta* which is of considerable economic importance in small ruminant livestock throughout the world, particularly in temperate regions. This chapter presents an overview of this parasite including some background information on the ever increasing problem of anthelmintic resistance. In addition, the current understanding of the population genetics of parasitic nematodes is reviewed.

1.1.1 Parasitic nematodes of veterinary importance

The superfamily Trichostrongyloidea contains an exceptionally high number of species of parasitic nematode that are of veterinary importance. They are found in a wide range of domesticated and wild animal host species including birds, marsupials and mammals (Anderson 1992;Chilton *et al.* 2001). They are of greatest economic importance in domesticated ruminants (sheep, cattle and goats) where their presence, predominantly in the gastrointestinal tract, causes a syndrome known as parasitic gastroenteritis which involves inappetance, reduced growth rates (i.e. weight loss) and diarrhoea. Severe cases can be fatal but more commonly low grade infections reduce weight gains, predispose to concurrent infections and result in poor carcass quality, all of which result in enormous economic loss to farmers worldwide. There are a large number of trichostrongylid nematode species that

can contribute to parasitic gastroenteritis, and the importance of different species varies between region and with climate (Urquhart *et al.* 1996).

1.1.2 *Teladorsagia circumcincta*: Host range and life cycle

Teladorsagia is one of the most important genera of trichostrongylid nematodes. Previously placed in the *Ostertagia* genus, a recommendation to use the genus *Teladorsagia* to distinguish these species from those of the *Ostertagia* genus was based on the morphological features; ventral ridges, bursal ray patter and absence of the proconus (ventral swelling of genital cone) (Lichtenfels *et al.* 1988). This reclassification has been generally accepted by the parasitology community although the name *Ostertagia circumcincta* is still occasionally used in some publications (Callaghan and Beh 1996;Leathwick *et al.* 1999). For the remainder of this thesis the name *Teladorsagia circumcincta* will be used. Different *Teladorsagia* species exhibit a broad host range, variable morphological characteristics and have an extensive geographical range which has been influenced by the transport of domesticated ruminant hosts by humans (Hoberg *et al.* 1999). *Teladorsagia circumcincta* has been reported in a large number of ruminant host species including sheep, goats, deer, llamas, muskoxen and antelope (Anderson 1992). However, it is unclear if the parasites identified as *T. circumcincta* on morphological criteria are a single species or a species complex. In temperate areas, such as the U.K., *Teladorsagia circumcincta* is the most economically important parasitic nematode of this genus due to its presence in sheep and goats.

T. circumcincta is a diploid organism that has a direct life cycle with obligate sexual reproduction (Figure 1.2a). Adult males and females live in the host's abomasum and females lay eggs which then pass onto the pasture in the faeces. The eggs hatch and develop to the infective L3 stage under appropriate climatic conditions. Reported temperatures required by *T. circumcincta* eggs to develop and hatch in the environment range from 4 - 34°C (Anderson 1992). Hatching can occur within 17 hrs at higher temperatures. Under optimal climatic conditions, the eggs can develop to the infective L3 stage within two weeks (Urquhart *et al.* 1996). Once developed to L3s, the parasite may survive on the pasture for up to two years depending on the weather conditions. These free-living infective L3s are ingested by the host where they exsheath in the rumen and further develop to L4 inside the abomasal glands. After a minimum time of 18 days, immature adults emerge from the glands to become sexually mature on the mucosal surface of the abomasum, completing the life cycle.

A special feature of *T. circumcincta*, and the trichostrongylids in general, is that larval stages (L3 - L4) may arrest their development for prolonged periods while within the abomasal glands. The ability to arrest allows the parasite to survive in the host for prolonged periods when external environmental conditions are unsuitable for parasite development and survival (Anderson 1992). Arrested larvae often resume their development to the adult egg-laying stages during pregnancy and lactation (known as the periparturitant rise). This strategy ensures large numbers of eggs pass onto the pasture and develop to the infective L3 stage at a time when many susceptible young hosts (lambs or kids) are present to ingest the parasite. A number of factors that may be responsible for arrested development have been recognised; including host immunity, presence and number of adults already established and environmental factors such as seasonal temperature (Anderson 1992). Arrested stages are not always effectively cleared by the use of anthelmintics, complicating parasite control measures.

1.1.3 The veterinary importance of *T. circumcincta*

The presence of *T. circumcincta* in the domesticated sheep and goat causes two clinical disease syndromes known as Type I or Type II ostertagiosis. Type I disease is the most common of the two types and occurs in young ruminants as a result of ingesting infective larvae 3 - 4 weeks previously from the pasture. Type I ostertagiosis is usually seen in the northern hemisphere from mid-July onwards, with the most common clinical sign being profuse watery diarrhoea. Type II disease can occur in all ages of animals and results from the resumption of development of arrested larvae which have accumulated in the abomasal glands following ingestion the previous grazing season. The clinical signs include inappetance, intermittent diarrhoea, weight loss and thirst. Hypoproteinemia often occurs in Type II disease due to the pathophysiological changes in the abomasal glands which result in a protein losing enteropathy (Scott *et al.* 1998). Animals may develop submandibular oedema as a consequence of the hypoproteinemia. In both diseases, animals become dull and lose considerable amounts of weight. The Type I disease tends to affect large numbers of animals but mortality is low if appropriate treatment is applied within 2 - 3 days of onset. Whereas, Type II disease tends to only affect a small proportion of the group but the mortality rate is high, often even in the face of treatment. Economic loss is inevitable to the farmer through loss of animals as well as the detrimental effects on time to finishing and on carcass weights and quality. Control of trichostrongylid nematode infections, including *T. circumcincta*, are dependant on the prophylactic use of anthelmintics. However, in recent

years control is increasingly problematic in most regions of the world due to the emergence of anthelmintic resistant parasites.

1.1.4 Anthelmintic Resistance

Anthelmintic resistance is becoming increasingly important worldwide as the use of anthelmintics continues to be the main method of parasite control. The problem is most acute in trichostrongylid nematodes of sheep and goats. Countries in the southern hemisphere are generally the most severely affected with a high prevalence of nematodes exhibiting multiple resistance (Jackson and Coop 2000) (Table 1.1). The resistance problems in some parts of the world, such as Australia and South Africa, have become so severe that some farmers have abandoned sheep and goat farming entirely. In addition, the high costs and risks associated with drug development mean that fewer pharmaceutical companies are pursuing new drugs and there is little likelihood of new classes of anthelmintic becoming available in the foreseeable future (Waller 1997).

The three main classes of anthelmintics used to control trichostrongylid nematodes are the benzimidazoles, imidazothiazoles / tetrahydropyrimidines (e.g. levamisole), and the macrocylic lactones (e.g. avermectin / ivermectin). Resistance of sheep nematodes in the U.K. is predominantly to the benzimidazoles and mainly found in *T. circumcincta* with some reports in *Haemonchus contortus* (Hong *et al.* 1992;Hong *et al.* 1996;Jackson and Coop 2000). A recent survey using an in vitro Egg Hatch Assay (EHA) found 64 % of Scottish sheep farms had evidence of benzimidazole resistant nematodes with *T. circumcincta* being the predominant species (Bartley *et al.* 2003). Resistance in sheep and goat flocks to levamisole and ivermectin (an avermectin) have also been reported in the U.K. and although less prevalent than benzimidazole resistance, recent small scale surveys suggest resistance can be detected in approximately 30% of farms (Dr F. Jackson, pers. comm.). Recently cases of multiply resistant *T. circumcincta* have been described from two sheep flocks and one fibre goat herd in Scotland (Bartley *et al.* 2004;Jackson *et al.* 1992b;Sargison *et al.* 2001) and on one sheep farm (Yue *et al.* 2003) and two commercial angora goats herds (Coles *et al.* 1996) in England. These trends follow those seen in the southern hemisphere, where the situation is more advanced. Further investigations are required to assess the extent of anthelmintic resistance in the U.K. Chapter 3 describes a small pilot survey to test the efficacy of anthelmintic treatment regimes on a number of goat farms in the U.K.

1.1.5 Genetics of anthelmintic resistance

Anthelmintic resistance can be defined as a heritable reduction in the sensitivity of a parasite population to the action of a drug (Geerts and Gryseels 2001). Reduction being expressed as a decrease in the frequency of susceptible individuals compared to the frequency of resistant individuals in the same population compared to the population upon initial or prior exposure to an anthelmintic (Condor and Campbell 1995). An understanding of the genetic basis of resistance will be crucial in order to develop control strategies that minimise the rate at which it develops.

The benzimidazole (BZ) group of anthelmintics has been the most intensively studied and it is for this group of anthelmintics that resistance is best understood. Benzimidazoles (BZ) act by interfering with tubulin polymerisation during microtubule formation causing a disruption of cellular integrity in the nematode gut (Kohler 2001). Restriction Fragment Length Polymorphism (RFLP) analysis has shown a reduction in polymorphism of the β-tubulin locus in BZ resistant populations relative to BZ susceptible populations of *H. contortus* (Roos *et al.* 1990). Further work has shown that a single amino acid substitution at position 200 (p200) of the polypeptide encoded by the β-tubulin isotype 1 gene, from tyrosine to phenylalanine, is more frequent in resistant than susceptible populations (Elard *et al.* 1996;Kwa *et al.* 1994). The ability of *H. contortus* alleles containing this polymorphism to confer resistance has been demonstrated using transfection of the parasite gene into *C. elegans ben-1* mutants (Kwa *et al.* 1995). This simple mechanism for resistance has led to the development of polymerase chain reaction (PCR) techniques capable of detecting resistance alleles in individual adult worms, allowing homozygous and heterozygous individuals to be distinguished, in parasite populations (Elard *et al.* 1999). However reliable molecular diagnosis of anthelmintic resistance will have to take into account additional mechanisms of resistance. There is evidence that a tyrosine to phenylalanine mutation at the p167 position of the β-tubulin isotype 1 gene can also confer resistance (Silvestre and Cabaret 2002). Furthermore, a second β-tubulin locus (isotype II) has also been shown to be involved in BZ resistance (Beech *et al.* 1994;Kwa *et al.* 1993). In this case a deletion of the entire locus is thought to confer resistance. Hence our understanding of BZ resistance is still incomplete. Resistant mechanisms to levamisoles and avermectins have been suggested to involve polymorphisms in a number of different genes including cholinergic receptors for levamisole resistance and glutamate-gated chloride channels and P-glycoproteins for ivermectin resistance. However, strong evidence for the

functional significance of such polymorphisms and their relative importance in populations of resistance parasites is lacking. The original studies on BZ resistance suggested that resistant alleles already exist in the susceptible populations at low frequencies and selection pressure by repeated exposure to anthelmintics increases their frequency within parasite populations over time (Jackson and Coop 2000). However Silvestre and Humbert (2002) have recently suggested that in addition to selection of pre-existing alleles, resistance can arise by more recent spontaneous mutation followed by selection. This was indicated by a large number of unique BZ resistant alleles found in closed parasite populations, alongside BZ resistant alleles which pre-existed in the parasite population from which the populations were founded. Another potential source of anthelmintic resistant alleles for a parasite population is migration from other resistant parasite populations which could occur due to host animal movement. Hence in summary, resistant alleles can potentially arise in a parasite population by selection of pre-existing alleles, selection of recent spontaneous mutations and migration of resistant alleles from other populations. Further work is still required in order to confirm which of these mechanisms are the most important in different situations. A prerequisite to this is an understanding of the genetic structure of parasite populations which is the major focus of this project.

1.2 Population Genetics of Nematodes

Population genetics has been defined as the study of the distribution and change of allele frequencies under the evolutionary forces of natural selection, genetic drift, mutation, migration and non-randoming mating (Hartl 2000). Population genetics has many applications in ecology and evolutionary biology (Frankham *et al.* 2002;Hoffman *et al.* 2003) as well as in studying the genetic basis of diseases of humans and domestic animals (Ginns *et al.* 1998;Xu *et al.* 2001). It also has important applications in the study of the epidemiology and the control of infectious diseases. Although there has been a considerable amount of work on the population genetics of viral, bacterial and protozoan pathogens there has been relatively little on helminth parasites.

An understanding of the genetic structure of parasite populations is necessary to appreciate important evolutionary processes such as adaptation to host defences, speciation and the evolution of resistance to drugs and vaccines (Blouin *et al.* 1999;Criscione *et al.* 2005). In this section the current knowledge of the population genetics of parasitic nematodes is reviewed.

1.2.1 Nematode Population Dynamics

Nematode population dynamics are extremely complicated. Nematodes, such as *T. circumcincta*, are not evenly distributed in the environment; instead there is a high degree of fragmentation and aggregation of nematode populations in the environment. How fragmented the population is depends on factors such as direct or indirect life cycles, how many host species the parasite can infect, and movement of all hosts species involved in the parasite life cycle. For parasites with direct life cycles like *T. circumcincta,* the adult stages that live in the host make up what is called the "infra-population" (Huyse *et al.* 2005;Poulin 1998). In addition to this, a large number of free–living L3 infective stages exist in the environment. The "supra-population" is made up of all the different life stages of the same species in a given ecosystem (Zander and Reimer 2002). Whereas, a "meta-population" is the assemblage of populations in a larger area, among which migration of individuals can occur (Huyse *et al.* 2005). Although the free-living infective stages are a potential source of new genotypes and should be considered, studies investigating the population structure of parasitic nematodes have mainly concentrated on the infra-population. The parasite infra-populations have a tendency to aggregate among the hosts available to them and often parasite populations are said to be over dispersed, i.e. the majority of hosts have low parasite burdens and a minority of hosts have high burdens. Precisely how the population is distributed among available hosts will depend on a number of factors including immune status, age and sex of the host, in addition to availability, size, fecundity, infectivity, and pathogenicity of the parasitic nematode, resulting in some individual parasites belonging to small infra-populations and other individuals which belong to large infra-populations. It is unknown how these factors interact and affect the population structure of parasitic nematodes.

1.2.2 Genetic Diversity of Nematode Populations

A variety of molecular tools have been used to investigate the nature of nematode population diversity, including mitochondrial (Anderson and Jaenike 1997;Blouin *et al.* 1992;Hugall *et al.* 1994) and nuclear DNA markers (Anderson and Jaenike 1997;Gasnier and Cabaret 1996;Hoste *et al.* 1993). A large range of diversity has been uncovered using these molecular markers (Anderson *et al.* 1998). The extent of genetic diversity of populations depends on a number of different factors. Under a model of drift-mutation equilibrium, the within population sequence diversity, π, is a simple function of sequence mutation rate, μ, and effective population size, N_e (for mitochondrial genes $\pi = N_e\mu$, while

for nuclear genes $\pi = 4 N_e\mu$) (Anderson *et al.* 1998). Therefore, increased variation within a population could be explained if either the effective population size and / or the neutral mutation rate were elevated (Beech *et al.* 1994). The effective population size may be affected by mechanisms of dispersal, life cycle, host specificity and demography (Nadler 1995). For example, some plant parasitic nematodes have been shown to have low within population diversity and highly structured populations; the overall mtDNA nucleotide diversity of 48 *Meloidogyne spp.* isolates, including both *M. javanica* and *M. arenaria* (root knot nematodes), was found to be low in Australia, with only six unique haplotypes found (Hugall *et al.* 1994). Hugall *et al.* (1994) attributed these results to their predominantly parthenogenetic mode of reproduction. A similar hypothesis has also been used to explain the low levels of sequence polymorphism found in populations of the free-living nematode *C. elegans* (Sivasundar and Hey 2003). This widespread species with relatively large populations might be expected to have relatively large levels of polymorphism. However, from the 23 strains of *C. elegans* isolated from worldwide locations, no within strain diversity was evident using 20 microsatellites (20 - 25 worms genotyped per strain) (Sivasundar and Hey 2003). Furthermore, across the 23 strains, from geographical locations as far apart as the U.K. and U.S., ten microsatellite loci were monomorphic and ten microsatellite were polymorphic, with the number of alleles ranging from 3 - 12 (Sivasundar and Hey 2003). High levels of self-fertilisation can reduce the effective population size (Pollak 1987), and therefore polymorphism within a population. The primary mode of reproduction of *C. elegans* is as a self fertilising hermaphrodite and this is consistent with the extreme lack of polymorphism found by Sivasundar and Hey (2003). This hypothesis is supported by the observation that the obligate sexually reproducing species *Caenorhabditis remanei,* had statistically greater diversity than *C.elegans* using both nuclear and mitochondrial genes (Graustein *et al.* 2002). A more recent study using ten microsatellite markers to investigate the population structure of 58 *C. elegans* strains, found evidence to support high levels of self fertilisation including the complete absence of heterozygosity within each natural strain (Haber *et al.* 2005).

Trichostrongylid nematodes that parasitise ruminants represent a very different situation to the examples described above. They have obligate sexual reproduction with separate male and female sexes and also have extremely large population sizes. For most of the trichostrongylid nematode species of ruminants, a single host can harbour more than 10,000 individual adult nematodes, each of which produces hundreds or thousands of eggs per day depending on the species (Anderson 1988;Eysker and Kooyman 1993;Urquhart *et al.* 1996).

The sequence diversity observed within trichostrongylid nematode populations generally is very high compared with other nematode species and this has been demonstrated using a variety of molecular markers (Beech *et al.* 1994;Blouin *et al.* 1992;Blouin *et al.* 1995;Leignel and Humbert 2001). For example, studies of nuclear DNA variation within populations of *Drosophilia sp.* estimated nucleotide diversity to range from 0.0003 to 0.0057 (Lynch and Crease 1990). These are low in comparison to the high DNA sequence variation found at the nuclear DNA diversity found using the isotype I ß-tubulin locus in *Haemonchus contortus* of 0.094 (Beech *et al.* 1994) and there is extremely high within population variation using mtDNA sequences (Blouin *et al.* 1992;Blouin *et al.* 1995). Furthermore when directly comparing nematode species using mtDNA ND4 sequences, the average within population nucleotide diversity was ten fold higher in the three trichostrongylids nematodes; *H. contortus, T. circumcincta* and *M. odocoilei*, being 0.023, 0.024 and 0.028 respectively, when compared to the low nucleotide diversity (0.002) seen in *Heterorhabditis marelatus* (a parasitic nematode of soil dwelling insects) from the pacific coast of North America (Blouin *et al.* 1995;Blouin *et al.* 1999).

The extremely large effective population size of trichostrongylid nematode species such as *Teladorsagia sp.*, *Trichostrongylus sp.*, and *Haemonchus* species has been considered to be the reason for their high levels of within population diversity. In addition, it has been suggested that nematode mitochondrial and nuclear DNA mutates at higher rates than is seen in other taxa which may also contribute to the high levels of sequence diversity (Anderson *et al.* 1998;Denver *et al.* 2000;Denver *et al.* 2004). Population genetic studies investigating diversity of other nematode species suggest that the effective population size is of particular relevance. For example, data from *Ascaris sp.*, which have a significantly lower effective population size, have much lower levels of sequence polymorphism than is the case for trichostrongylids (Anderson and Jaenike 1997). Similarly, lower levels of polymorphism have been demonstrated in the bovine lungworm, *Dictyocaulus viviparous*, which also has smaller infra-population sizes than other trichostrongylid species, due to both low numbers of worms in each host and also smaller supra-population sizes due to a generally lower proportion of hosts effected (Hoglund *et al.* 2004).

Another possible reason for the high within population diversity is that the sampled populations consist of a mixture of individuals from previously differentiated populations. This is referred to as admixture of populations, where migration brings highly divergent alleles into the same population. The estimate of sequence diversity increases because of the

unusually high sequence divergence within the same sample (Slatkin 1989;Strobeck 1987). A characteristic of admixture is the presence of large discontinuities in the divergence estimates between alleles (Anderson *et al.* 1993;Strobeck 1987). There was little evidence of such discontinuities in the small number of studies conducted to date, leading to the conclusion that the high within population diversity found in the trichostrongylid nematode populations examined is not due to admixture (Beech *et al.* 1994;Blouin *et al.* 1995).

1.2.3 Genetic structuring of parasitic nematode populations

Relatively few studies have looked at the genetic structure of nematode populations. Parasitic nematodes display a wide variety of life cycles and life histories, including differences in reproductive modes, host-specificity, and modes of transport (Blouin *et al.* 1999). These differences can influence how the nematode population is structured. For example, the parthenogenetic root-knot nematodes (genus *Meloidogyne*) have been shown to have low overall nucleotide diversity (0.0028) and strongly structured populations using the restriction site polymorphism of mtDNA sequences (Hugall *et al.* 1994). Hugall *et al.* (1994) investigated 48 isolates from Queensland, Australia. The mtDNA variation was geographically structured and this was suggested to be the result of rapid spread followed by local differentiation through selection. The authors suggested that the low diversity and high levels of genetic differentiation were a result of the parthenogenetic life history, with lineages arising from distinct but closely related sexual females. Blouin *et al.* (1999) demonstrated the importance of parasite modes of transport and reliance on hosts, in relation to population structure in a study investigating the insect parasitic nematode, *Heterorhabditis marelatus,* along the pacific coastal regions of California. This nematode, like many other nematodes, has limited dispersal capabilities and therefore relies on opportunities such as transportation in infected host species or by wind or water. However, transport of *H. marelatus* in infected host species is limited, as it tends to infect burying insects and hosts are killed rapidly following infection. In addition, infective juveniles of *H. marelatus* are highly susceptible to environmental factors such as UV light and lack of moisture, minimising transport of free living stages of the parasite, although some movement of *H. marelatus* may be possible in coastal regions in salt water. Consequently, it was not surprising that this species was found to have a highly structured population based on mtDNA sequences. When directly compared to the trichostrongylid nematodes, using similar sampling method and identical genetic marker, Blouin *et al.* (1999) found 86 % of the total nucleotide diversity to be distributed among *H. marelatus* populations, in contrast

only 1 % and 2 % nucleotide diversity distributed among the trichostrongylid nematodes populations of *T. circumcincta* and *H. contortus*. This contrast is even more striking because of the extensive geographical scale over which the trichostrongylids populations were collected, including populations from the far north west and far east coast of the United States (Blouin *et al.* 1995). Blouin *et al.* (1995) suggested that host movement, facilitated by humans, allowed enough gene flow between these distant populations of trichostrongylid nematodes, to prevent geographical differentiation. This hypothesis was further supported by evidence from the trichostrongylid nematode, *Mazamastrongylus odocoilei*, collected from white tailed deer (not moved by humans) over similar distances finding 31 % nucleotide diversity to be distributed among the geographically distant populations (Blouin *et al.* 1995). These studies demonstrate that host movement can affect parasite population genetic structure. However this can be complex, with no set system of transmission. For example, farming methods and movement of animals may differ within countries and between countries, with some farms remaining closed to animal imports and others continuously bringing in new animals, or mixing of animals may occur at markets.

Another parasitic nematode for which there have been population genetic studies is the human hookworm, *Necator americanus*. This strongylid parasite is of major human health concern in a number of countries including Africa, India, South-East Asia, China, and Brazil and is transmitted to humans via faecal contaminated soil. Movement of humans (and therefore hookworms) in developing countries may be infrequent and cultural practices within countries may increase or decrease the chance of transmission. For example, in rural communities, the main transmission cycle may be found to be within families (Hawdon *et al.* 2001). Whereas, in urban communities, farmers may use the community latrines to collect faeces for use on their farms (Hawdon *et al.* 2001), potentially allowing transmission between many families. Hawdon *et al.* (2001) investigated the population genetic structure of *N. americanus* in China, to facilitate understanding the spread of anthelmintic resistance and predict the geographical scale over which new vaccines would need to be tested (Hawdon *et al.* 2001). Comparisons using mtDNA cytochrome oxidase 1 gene sequences were made from 21 - 58 individual worms collected per village from four geographically distant villages. One of the four populations investigated was found to have low within population genetic variation and was highly differentiated from the three other populations examined. However, there was no correlation between geographical and genetic distances between the populations and the author suggested this to be the result of a bottleneck (significant decrease in the population size) in this population. For example, one human may

have introduced the parasite infection to an uninfected rural village, which followed by no further parasite introductions, created a founder effect from a small number of worms. Overall, *N. americanus* did not show the high level of mtDNA diversities and gene flow seen in trichostrongylids nor did it have the low diversities and highly structured populations as seen in some plant nematodes, i.e. it was intermediate between these two extreme situations. The average number of worms per host found in this study was 26. This coupled with *N. americanus* infective stages being relatively short-lived and prone to desiccation, suggests there may be no long-term reservoirs of *N. americanus* to buffer the effect of population fluctuations. Hawdon *et al.* (2001) suggested the results from *N. americanus* populations in China may be characterised by sporadic bouts of gene flow caused by random movement of infected humans and fluctuating population sizes, with some populations being more isolated than others due to both environmental or cultural influences. This study highlights how difficult it is to predict the population genetic structure of a parasitic nematode, as environmental and cultural factors have major influences on transportation of parasites and therefore gene flow.

The bovine lungworm, *Dictyocaulus viviparous,* is a trichostrongylid nematode which is quite closely related to *T. circumcincta* and *H. contortus* but has quite a different population genetic structure. *D. viviparous* populations in cattle were examined on farms in Sweden using amplified fragment length polymorphism (AFLP) and mtDNA single-stranded conformation polymorphism (SSCP) based markers (Hoglund *et al.* 2004;Hu *et al.* 2002). Hoglund *et al.* (2004) found approximately half the variation in *D. viviparous* populations was due to differences among farms (45 - 53%). This suggested lack of gene flow between farms. Hu *et al.* (2002) found similar evidence of population sub-structuring with high Fst values (0.77) using mtDNA cytochrome oxidase c subunit 1 sequences. Both studies suggest evidence that *D. viviparous* forms separate subpopulations in Sweden, based on the genetic structure found between worms from different farms, and is most likely due to low levels of gene flow between farms. These results imply that *D. viviparous* does not move between farms readily. Hoglund *et al.* (2004) hypothesised that this would decrease the speed with which the development of anthelmintic resistance could spread. This is in contrast to the situation with the other trichostrongylid nematodes, where evidence has been found for high levels of gene flow and panmictic populations suggesting that anthelmintic resistance would spread more easily.

The above examples demonstrate how differences in parasite life histories, host species, modes of transport and populations size can influence nematode population structure. However, an important point to make about the above studies is that they were conducted using single locus markers. This has generally been the case for most studies on the population genetics of parasitic nematodes. However, single locus markers may not reflect the whole of the genome and the fact that genetic evolutionary changes occur at different rates throughout the genome (Pamilo and Nei 1988). Hence, what one marker may suggest about the structure of a population may differ from what other markers suggest. The benefits of using mutlilocus markers are further discussed later in this chapter.

1.2.4 The factors affecting the genetic differentiation of parasitic nematode populations

Genetic differentiation of populations is influenced by random genetic drift, the amount and pattern of migration rate, mutation rate and the effects of selection (Hartl 2000). It is often difficult to determine the relative contributions of these competing factors on the genetic structure of a particular parasite population. Genetic drift and selective forces may act to increase the differentiation of separate populations whereas migration tends to reduce it (Nadler 1995). The rate at which each of these processes occurs is difficult to predict. Genetic drift and gene flow will be briefly discussed before reviewing a number of examples illustrating their interrelationship.

1.2.4.1 Genetic drift

Genetic drift is the process whereby a single species can diverge into two distinct species, seen as the allele frequencies drifting to different frequencies in the two daughter populations (Anderson, 1998). Random genetic drift refers to fluctuations in allele frequencies, over time, that occur by chance due to the random sampling among gametes from one generation to the next (Hartl 2000). Genetic drift will affect the probability of alleles becoming fixed in a population which in turn is proportional to the frequency of the allele in the population (Hartl 2000). For example, a new allele introduced by mutation will be at a low frequency and therefore have a low probability of fixation, whereas common alleles have a greater probability of becoming fixed. In simple terms, genetic drift tends to be inversely proportional to the effective population size which can be influenced by how the population is distributed in the environment as well as the ratio of males to females (Hartl 2000). The effective population size (N_e) is a particularly critical factor in parasite populations; the higher N_e, the slower the rate at which genetic drift between populations

occurs. In the case of parasites like *T. circumcincta* with very large effective population sizes, the rate of genetic drift is predicted to be very low. One critical question which needs to be addressed is the time period over which sufficient genetic drift would occur in order to be measurable between geographically isolated *T. circumcincta* populations. This will have an important bearing on how much genetic differentiation is likely to be seen between parasite populations and how informative the use of neutral markers, such as microsatellites, will be to infer gene flow between parasite populations. Nadler estimated that for a parasite population where $N_e = 1000$ and a generation time of 90 days, a neutral mutation will require approximately 1,000 years before fixation in the population (Nadler 1995). Consequently, if the N_e for trichostrongylid nematode populations is indeed very large then genetic differentiation of trichostrongylid nematode populations following geographical separation would require many thousands of years separation before differences were seen. Hence very little genetic differentiation between populations of parasites such as *T. circumcincta* would be expected to be observed.

However as discussed above there are many other factors that might affect parasitic nematode population dynamics such as population bottlenecks, modes of transport, environmental factors and selection pressures such as drug treatments and other parasite control strategies. Hence it is over simplistic to attempt to make generalisations on genetic differentiation of these parasite populations based on estimates of genetic drift alone.

1.2.4.2 Gene flow

Gene flow between parasite populations is dependant on the movement of individual parasites between populations which in turn is often dependant upon the movement of the host. This factor tends to reduce the genetic differentiation that is seen between separate populations. Low levels of migration (i.e. > one migrant per generation) are considered sufficient to prevent population differentiation due to random genetic drift (Slatkin 1987). The time taken to reach equilibrium will be determined by the effective population size and the proportion of the population replaced by immigrants (Slatkin 1985). Traditionally, this has been expressed in terms of Wrights (1931) Fst where $Fst = 1 / (4N_e m + 1)$, where m is the local migration rate and N_e is the effective population size. Although this method has been criticised due to the unrealistic assumptions about the populations (Wang and Whitlock 2003), it provides a starting point for determining the nature of population differentiation and the potential reasons for differentiation or similarity between populations. In the case of trichostrongylid nematodes such as *T. circumcincta*, it has been suggested that

high levels of gene flow occur due to movement of their domesticated animal hosts (Blouin *et al.* 1992;Blouin *et al.* 1995). This again would tend to reduce the rate at which separate parasite populations become genetically differentiated.

When examining the population genetic structure of a parasite it is difficult to determine the relative contributions of genetic drift and gene flow. For example if populations show little differentiation, this may reflect recent or contemporary gene flow. However it could equally be due to insufficient time having elapsed for alternative alleles to have become fixed. Or in other words, there has not been a long enough time period for populations to have become differentiated due to genetic drift, in the absence of gene flow. A number of examples illustrating the affects of genetic drift and gene flow and the difficulties of separating these issues will now be discussed.

i) Example 1: Genetic differentiation due to Genetic Drift over extended periods of time

An example of genetic drift causing geographical genetic differentiation, in the absence of gene flow, was shown to exist in the parasitic nematode, *Heligmosomoides polygyrus*, the specific endoparasite of the wood mouse (*Apodemus sylvaticus*) (Nieberding *et al.* 2005). The *A. sylvaticus* woodmouse populations have been shown to be differentiated on the basis of isolation events which occurred during the Quaternary ice age, with re-colonisation occurring from the unaffected southern European peninsulas to northern European areas (Michaux *et al.* 1998). Nieberding *et al.*(2005) showed that *H. polygyrus* has a similar phylogeographic history, with *H. polygyrus* populations being highly differentiated between the Pleistocene refuge areas located in southern Europe, including, Iberia, Italy and the Balkan (Greece) which were isolated during the Quaternary ice age. The results of the molecular variance (AMOVA) analysis using mtDNA *cyt b* gene sequences, in which 6 to 31 individual worms were sequenced per geographical region, showed that the majority (69 %) of the total mtDNA variation is distributed among the seven geographical regions studied. The population differentiation found among the geographical regions has been established through the accumulation of mutations between allopatric populations with the disappearance of haplotypes due to random genetic drift and lack of gene flow, estimated to have occurred 2.47 – 1.55 million years ago (mya) (Nieberding *et al.* 2005). This study shows how parasite populations have managed to become differentiated over a set period of time. For trichostrongylid nematodes, there have not been any known studies for which the

populations studied have been separated for the periods of time demonstrated in the above example.

ii) Example 2: Gene flow in *Globodera pallida* and *Heterodera schachtii* populations

Studies investigating the potato cyst nematode, *Globodera pallida,* in Peru, found a large degree of genetic differentiation between regional areas (326 - 832 km apart) but not within fields (3 - 35 km apart) (Picard *et al.* 2004). Like many nematode species, dispersal mechanisms are limited for this species; transportation and therefore gene flow are dependent on other opportunities. The adult stages actively disperse in the soil but are limited to a few centimetres or decimetres and as plant nematodes, they can not rely on host transportation as a potential means of gene flow. This has potentially resulted in closely related individuals mating and may explain the heterozygote deficiency which has been observed, an indicator of inbreeding (Picard *et al.* 2004). However the cyst of this nematode, containing hundreds of infective stages, may be passively dispersed by wind, water or by being attached to potato plants by farm implements. Lack of genetic differentiation within fields suggests passive dispersal allows a level of gene flow at field level but this does extend to greater distances since high levels of genetic differentiation were seen between regional areas. In contrast, the sugar beet cyst nematode, *Heterodera schachtii,* which has a similar life history and breeding system to *G. pallida* was found to have less genetic differentiation over larger distances (up to 175 km), yet similarly showed heterozygote deficiencies and evidence of inbreeding (Plantard and Porte 2004). The lack of major biogeographical boundaries between study sites could explain the low levels of genetic differentiation for *H. schachtii,* as passive transport between sites would not have been restricted (Plantard and Porte 2004). Whereas the regionally differentiated populations of *G. pallida* (Picard *et al.* 2004) were separated by large distances, absent of potato plants, reducing the potential for gene flow. Inbreeding due to limited active dispersal of adult stages seems to conflict with limited genetic differentiation over great distances found for *H. schachtii*. However, Plantard and Porte (2004) suggests that this is explained by gene flow occurring at the cyst stage, due to passive dispersal, causing isolated adult populations to have been 'seeded' by related individuals. These plant parasitic nematode populations demonstrate the importance of understanding the nematode life histories including modes of transport when considering gene flow.

iii) Example 3: *Longistriata caudabullata* – gene flow or insufficient time for genetic drift?

A study was conducted by Brant and Orti (2003) to investigate gene flow in the intestinal parasitic trichostrongylid nematode, *Longistriata caudabullata,* between two host species of shrew, *Blarina brevicauda* and *Blarina hylophaga,* in central North America, estimated to have diverged over 4.6 mya. Individual adult nematodes were collected from both host species in sympatric and allopatric geographical locations and genetic analysis was conducted using the ND4 mitochondrial gene. The haplotype diversity was similar to that found in other trichostrongylid nematodes, with 27 haplotypes from 28 nematodes, which is suggestive that *L. caudabullata* has a high effective population size. There was no evidence of genetic differentiation between the parasite populations found in the different host species for both sympatric and allopatric populations which the authors interpreted as evidence of ongoing gene flow with no host specific boundary for *L. caudabullata.* However, the allopatric populations were widely geographically separated and yet there was equally little genetic differentiation between these as for sympatric populations. Hence it is unlikely that gene flow is the full explanation of the lack of genetic differentiation. An alternative explanation could be that, due to the large effective population size of the parasite, there as been insufficient genetic drift to produce measurable genetic differentiation; i.e. host separation may have prevented gene flow, but effective population size may be too large to allow sufficient genetic drift over 4.6 million years. However, the work on *Heligmosomoides polygyrus*, the specific endoparasite of the wood mouse described in example 1, showed that in those populations genetic drift was sufficient to allow populations to diverge over a period of 2.47 – 1.55 million years when gene flow was absent (Nieberding *et al.* 2005). This might suggest that the time period which separates *L. caudabullata* host populations (4.5 million years) should be long enough for significant genetic drift to have occurred if gene flow was indeed absent. However it is not possible to directly extrapolate between different parasite species and this third example demonstrates the difficulties in determining whether lack of genetic differentiation is due to gene flow or insufficient time for measurable genetic drift to have occurred.

1.2.5 What is a Nematode species? Implications of diversity and nematode speciation

Traditional methods have used morphological features and morphometric measurements to distinguish between nematode species. However, nematodes have relatively simple body

plans and there is increasing evidence that species defined by morphological criteria may sometimes actually consist of multiple species. These are often described as "cryptic" species which have been defined as separate species with no discernable morphological characteristics allowing differentiation (Anderson *et al.* 1998).

Defining a species is a notoriously difficult and controversial matter. There are many different species concepts in use, for example species may be defined on the basis of diagnostic traits (Cracraft, 1982 referenced by (Kunz 2002)), cladistic classification or biological concepts (Dobzhansky referenced by Anderson and Jaenike(1997)). Each of these has a particular set of advantages and disadvantages. Diagnostic traits, i.e. defining species phenotypic features, can be misleading because phenotypic differences do not necessarily reflect genotypic differences (Dame *et al.* 1993). For example, three populations of *Ostertagia ostertagi* which were characterised by arrested larval development over the summer months were compared to two populations of *O. ostertagi* which arrest over the winter months using RFLP of mtDNA, using 10 individuals from each farm (Dame *et al.* 1993). The results found 98 % of the genetic diversity was found within populations, suggesting these phenotypically different populations to be genetically the same. In addition, decisions on how many traits and the extent to which they must vary in order to define separate species are inevitably arbitrary. Cladistic classification, based on either morphology or phylogenetic markers, can be complicated due to the difficulty of determining whether groups are either mono-phylectic, para-phylectic or poly-phylectic (Anderson *et al.* 1998). The biological species concept states that different species cannot interbreed, however this is not always absolute as hybridisation between individuals considered to be separate species has been found to occur in nematodes species (Le Jambre *et al.* 1999). There are also practical difficulties with using the biological species concept, particularly for organisms such as parasitic nematodes, where experimental crosses are rarely practical.

For parasitic nematodes, species classification has traditionally used morphological measurements for cladistic taxonomy (Durette-Desset *et al.* 1999;Gouy de Bellocq *et al.* 2001). This approach has also been used for speciation, yet due to the lack of morphological characteristics numerous debates over speciation have arisen. This is well illustrated by the example of the minor morphological differences between *Haemonchus contortus*, found in sheep and goats, and *Haemonchus placei*, found in cattle. Over the years this has been the subject of some controversy as to whether these are actually different species. A review by

Gibbons (1979) stated that the morphological differences between the two were not substantial enough to define them as separate species whereas Lichtenfel *et al.* (1994) concluded that differences in length and number of cuticular ridges between the two were reliable criteria to classify them as two separate species. More recent molecular data, using mtDNA, rRNA ITS-2 sequences and microsatellites, has supported this latter theory, confirming *H. contortus* and *H. placei* are separate species (Blouin *et al.* 1997;Stevenson *et al.* 1995). The increased use of molecular and genetic population markers has helped confirm species identification and classification; *Ascaris spp.* and *Haemonchus spp.* (Anderson and Jaenike 1997;Blouin *et al.* 1997;Kaye *et al.* 1998;Stevenson *et al.* 1995); provided evidence of species diversity previously unrealised; Ancylostomatidae (Hu *et al.* 2003;Romstad *et al.* 1998), *Dictyocaulus sp.* (Hoglund *et al.* 1999;Hoglund *et al.* 2004), Metastrongyloidea (Conole *et al.* 1999;Leignel *et al.* 1997); and identified potentially 'cryptic' speciation in a number of nematode groups including, Cyathostominae (Hung *et al.* 1999;Kaye *et al.* 1998) in addition to Ostertagiinae (Dallas *et al.* 2000b;Gasnier and Cabaret 1996;Hoberg *et al.* 1999), *Uncinaria spp.* (Nadler *et al.* 2000) and *Dictyocaulus spp.* (Hoglund *et al.* 1999). These findings, based on genetic evidence, indicate that the population structure of many nematode species is likely to be more complicated than was once thought. Investigations have shown that evidence of cryptic speciation, species diversity, or conclusive speciation is important to the control programs for these parasites (Criscione *et al.* 2005).

A classic example of the use of molecular markers to investigate the fidelity of species designations is the work on the nematodes *Ascaris suum* and *Ascaris lumbricoides*. Traditionally these have been considered as separate species with *A. suum* infecting pigs and *A. lumbricoides* infecting humans. However these two parasites are effectively indistinguishable based on morphological criteria and so their relationship was unclear for many years (Anderson 2001). Prior to molecular studies it was not known whether these parasites have a single transmission cycle in which pigs and humans are alternative hosts or whether they have completely separate sympatric life cycles, one infecting pigs and the other infecting humans (Loreille and Bouchet 2003). In order to investigate these issues, adult *Ascaris* worms were collected from both host species in several locations worldwide and molecular investigations using both mtDNA and seven allozyme markers were conducted (Anderson and Jaenike 1997). Both approaches found that the *Ascaris* worms clustered according to host species (Anderson and Jaenike 1997). However, there were a number of mtDNA haplotypes which were found in both humans and pigs which were

identical (Anderson and Jaenike 1997). Two possible hypotheses were suggested which could potentially explain these findings: a) introgression of mitochondrial haplotypes caused by limited cross-infections or b) retention of ancestral polymorphisms (Anderson 2001). Retention of ancestral polymorphism refers to the presence of alleles, originating from the founding population, in both populations due to insufficient time having elapsed for genetic drift to have caused fixation of different alleles in each population. The results found for *Ascaris* populations were best explained by retained ancestral polymorphisms based on a number of observations. They estimated that the time required for these populations to have become fixed for different alleles was considerable (~0.3 - 1.3 million years). In addition, there was little direct evidence for hybridisation between the two host-associated species in the sympatric Guatemalan populations investigated. The authors suggested further work is required to clarify the origin of the shared mitochondrial haplotypes, by using additional genetic markers and conducting experimental genetic crosses, as the possibility of introgression might have important implications, for example the transmission genes responsible for anthelmintic resistance passing from *A. suum* to *A. lumbricoides*. These studies provide strong evidence that *A. suum* and *A. lumbricoides* are in fact separate species infecting separate hosts.

Another good example of the use of molecular markers in species investigations, are the studies looking at the host specificity of the *Dictyocaulus* lungworm species in wild cervids and cattle. This is important in order to clarify the host range and determine the extent to which wild cervids, such as deer and moose, act as a reservoir of transmission to domesticated cattle. Deer have generally been traditionally regarded as an important reservoir of the cattle lungworm, *D. viviparous*, although a separate species *D. eckerti* has been described in fallow deer (Epe *et al.* 1997). A study was conducted by Hoglund *et al.*(1999) to investigate the extent of lungworm cross-infections between host species and determine the extent of host species diversity in Sweden. Three individual lungworms per host were collected from an unspecified number of roe deer, moose and cattle, and ITS-2 sequences were compared. No intra-specific differences were found when comparing sequences of lungworms obtained from the same host species. Furthermore, there were no sequence differences between lungworms collected from roe deer and moose. However, the ITS-2 region amplified from the *D. viviparous* individuals collected from cattle were 455 bp in length compared to 474 bp from roe deer and moose. The sequence identity between the ITS-2 sequences roe deer and moose and that of *D. viviparous* of cattle was only 69 %. Furthermore, the lungworms isolated by Hoglund *et al.* (1999) from roe deer and moose in

Sweden were significantly different to the *D. erkerti* isolated from fallow deer by Epe *et al.* (Epe *et al.* 1997), with 66.5 % sequence identity between ITS-2 sequences. These differences (33.5 % - 31 %) are much greater than those found between morphologically well defined nematodes species of *Trichostrongylus* (1.3 % - 7.6 %) (Hoste *et al.* 1995). This study demonstrated the genus *Dictyocaulus* to be more complex than was thought, suggesting not only that lungworm species found in cattle to be different species to that found in wild cervids but that wild cervids in Europe are potentially parasitized by more than one species of *Dictyocaulus*.

Another good example of molecular identification of nematode species was shown by Nadler *et al.* (2000). There has been a long standing debate as to the classification and description of the hookworm species, *Uncinaria,* in fur seals and sea lions worldwide. Nadler *et al.* (2000) collected 20 hookworms from Northern fur seals and California sea lion pups where breeding sites were sympatric. Comparisons were made using sequences from the 28S rRNA subunit gene and the ITS-1 region of the rRNA cistron. The 28S sequences showed no significant differences between fur seals and sea lions, whereas hookworms from fur seals could be differentiated from those isolated from sea lions on the basis of ITS-1 sequences, with 5 fixed sequence differences. This demonstrated hookworms representing two host-associated lineages occur in two sympatric populations of seals. This example also demonstrates the requirement of analysing appropriate markers relative to the phylogenetic divergence of potentially new species.

As well as confirming species designations and identifying new species the use of molecular markers has called into question the classification of species. For example although the nematodes *Ostertagi gruehneri* and *Ostertagi arctica,* of reindeer and caribou in Norway and Svalbard, were originally considered to be separate species some workers suggested they may represent polymorphic males of the same species. Dallas *et al.* (2000a) investigated the genetic similarity of *Ostertagi gruehneri* and *Ostertagi arctica* collected from four geographically diverse regions by sequencing ITS-1 and ITS-2 regions of rDNA. The authors found virtually identical ITS-1 and ITS-2 sequences, without any sequence differences shared between *Ostertagi gruehneri* and *Ostertagi arctica,* suggesting that these are dimorphic males of the same species. However it is of course possible that they still represent separate species and multilocus approaches are necessary to resolve this.

These examples demonstrate the importance of understanding nematode population structure and identification of species using molecular markers. The enhanced level of

investigation will aid future control programs regarding host movement and treatment, in addition to controlling the spread of anthelmintic resistance. The majority of these studies were performed using a single locus on which to base their conclusions; however the *Ascaris* and *Uncinaria* research highlights the dangers of using a single locus in nematode population genetics. Leignel *et al.* (1997) also noted the limitation of using a single locus when investigating four species of lungworm of the *Metastrongylus* genus found to live sympatrically in wild boars in France. Two species, *M. salmi* and *M. confusus* were found to be identical base on ITS-2 sequences, despite clear evidence these are taxonomically distinct species based on the evidence that these species can occur independently of each other. In contrast, comparisons of ITS-2 sequences clearly distinguished between the species *M. asymmetricus* and *M. pudendotectus,* which are only found together in lung infections. In contrast, Random Amplified Polymorphic DNA assay (RAPD) analysis found all four species to be distinct. Hence the development of multilocus markers for population genetic analysis is an important priority for parasitic nematode research.

1.2.6 Using microsatellites to study nematode population structure

As discussed above, many of the studies to date investigating the population genetic structure of nematodes have been based on molecular approaches using single markers. This is particularly true for the trichostrongylids, with the majority of investigations using the ITS-2 (Chilton *et al.* 2001;Hoste *et al.* 1998;Stevenson *et al.* 1995;Stevenson *et al.* 1996) and mtDNA markers (Blouin *et al.* 1992;Blouin *et al.* 1995;Blouin *et al.* 1997). However, Anderson (2001) demonstrated that for species or populations which have only recently diverged, analysis using a single locus can cause misleading information regarding species or population differences as discussed above. This is of particular relevance for distinguishing species of nematodes for which there are often no clear morphological differences. When one species splits into two, the genomes of each daughter species will diverge, eventually reaching a state when each new species become fixed for alternative alleles (Anderson *et al.* 1998). Prior to reaching this state of fixed alternative alleles, all loci may be in a state of polyphyly or paraphyly and as a result no one marker can be used as a diagnostic for differentiating between populations (Anderson *et al.* 1998). However, if many independent loci are used differentiation between populations will be more accurate and reliable (Pamilo and Nei 1988).

Microsatellite markers are ideal as they occur in multiple numbers fairly evenly spread throughout the genome, are polymorphic and generally considered to be neutral markers.

(Bennett 2000;Tautz 1989). They can be used for multilocus genotyping allowing powerful analysis of population differentiation and species fingerprinting. One major advantage is that, in principle, large numbers of informative markers can be isolated and used for population genetic analysis. In addition, the mutation rate of microsatellites is significantly higher than base substitution rates, ranging from 10^{-6} to 10^{-2} per generation (Schlotterer 2000) and therefore they tend to have a higher number of alleles per locus than other markers. Being a PCR-based marker, once flanking sequences are known, microsatellites can be easily PCR amplified from small quantities of DNA, followed by the relatively easy assignment of alleles. Furthermore, they are relatively easy to isolate from organisms for which no genome sequence is available, being more cost effective than other multilocus techniques such as single nucleotide polymorphisms (SNPs) (Schlotterer 2004). Microsatellites have been used to investigate the population structure of a wide range of species, most relevant are the various studies examining the population structure of species with large population sizes such as mosquitoes (*Culicidae*) (Ravel *et al.* 2001;Temu *et al.* 2004), mites (*Sarcoptes*) (Walton *et al.* 1999;Walton *et al.* 2004) and fruit flies (*Tephritidae*) (Baliraine *et al.* 2004).

A nice example of the use of microsatellites to study invertebrate population genetics comes from *Ceratitis rosa, C. fasciventris* and *C. capitata* (Medfly). These are afro-tropical fruit flies considered a major agricultural pest, of which *C. capitata* has been shown to have spread from it's east African origin to attain an almost worldwide spread, (Baliraine *et al.* 2004). Recent studies, using microsatellites, were undertaken to investigate the population structure of *C. rosa* and *C. fasciventris* in Kenya, Uganda and their corresponding islands. These are closely related to *C. capita* (Medfly) but are found in a more restricted geographical distribution. Despite high levels of within population diversity seen for both *C. rosa* and *C. fasciventris* (> 85.96 % and > 93.68 % respectively), a relatively large amount of genetic differentiation was evident between mainland and island populations of *C. rosa* and to a lesser extent found between countries on the mainland for both species (Baliraine *et al.* 2004). Significant correlation between genetic differentiation and geographical distance in addition to lower diversity levels found on the islands, Baliraine *et al.* (2004) supported predictions that these species have a similar population behaviour to the medfly with potential to colonise new areas previously void of these species.

Microsatellites allowed not only analysis of population structure but also demonstrated how species can be easily compared. Studies investigating the population genetic structure of the

mite *Sarcoptes scabiei,* validate the use of microsatellites, as compared to single locus markers, for increased resolution of population structure. Studies using mtDNA markers (16S gene) and the nuclear ribosomal marker, ITS-2, showed evidence for geographical sub-structuring but found no evidence of host association when mites were sampled from a number of locations and host species (Berrilli *et al.* 2002;Walton *et al.* 2004). Whereas, two studies utilising microsatellites found that scabies mites on people were genetically distinct from those on dogs when genotyping individuals from sympatric populations (Walton *et al.* 1999;Walton *et al.* 2004). In the study conducted by Walton *et al.* (2004), the authors directly compared the phylogenetic results from mtDNA sequence versus population genetic analysis using microsatellites and found that using mtDNA, there was evidence of host and geographical structuring but it was not as clear cut as the results from the microsatellites which more clearly separated populations of mites by both location and host species. This has important implications for future control and diagnostics.

There have been relatively few studies on microsatellites in nematodes. The most detailed information comes from studies in the free-living nematode *C. elegans* due to the availability of its full genome sequence. The most recent database search for studies either isolating or utilising microsatellites of nematodes species, found eleven species studied in eighteen papers, four papers predating 2000. These studies include three plant parasitic nematode species; *Heterodera schachtii* (Plantard and Porte 2003;Plantard and Porte 2004), *Globodera pallida* (Picard *et al.* 2004;Thiery and Mugniergy 2000) and *Meloidogyne artiellia* (De Luca *et al.* 2002); one free living nematode; *C. elegans* (Barriere and Felix 2005;Haber *et al.* 2005;Sivasundar and Hey 2003); and the remainder being animal parasitic nematodes including; *Strongyloides ratti* (Fisher and Viney 1996), *Brugia malayi* (Underwood *et al.* 2000), *Haemonchus contortus* (Hoekstra *et al.* 1997;Otsen *et al.* 2000;Roos *et al.* 2004), *Metastrongylus elongates, M. pudendotectus,* and *M. salmi* (Conole *et al.* 2001), *Trichnella pseudospiralis* (Zarlenga *et al.* 1996), *Ascaris lumbricoides* (Anderson *et al.* 2003) *Echinococcus multilocularis* (Nakao *et al.* 2003) and *Trichuris trichiura* (Barker and Bundy 2000).

1.2.7 Aims of this project

There is relatively little known about the population genetic structure of *T. circumcincta,* with all previous investigations using either allozyme, mtDNA or ITS-2 markers (Blouin *et al.* 1992;Blouin *et al.* 1995;Gasnier and Cabaret 1996;Stevenson *et al.* 1996). This project aims to use a multilocus approach to investigate the population genetic structure of

T. circumcincta and compare these results to those of previous studies. There have been no previous studies of this species, or indeed any strongylid nematode species, using microsatellite markers. Therefore, the project has focused on the isolation and characterisation of microsatellite markers from *T. circumcincta* and their use to answer a number of fundamental questions regarding population genetic structure. Examples of the questions that have been addressed include: Does any population sub-structuring exist? Are there differences between infra-populations obtained from hosts grazing the same area? Is there evidence of cryptic speciation? Are there geographical differences? Answers to these questions are critical to allow future studies regarding more complex questions concerning the extent and rate of gene flow between parasitic nematode populations and the origin and spread of anthelmintic resistance genes.

Figure 1.1: Phylogenetic tree of the nematode phyla

A simplified version of that produced by Blaxter *et al.* 1998, based on 18S rDNA sequences. * indicates species referred to within this thesis. [a] = animal parasitic nematode, [p] = plant parasitic nematode, [f] = free-living nematode and [i] = invertebrate parasitic nematode.

Figure 1.1: Phylogenetic tree of the nematode phylum

Adapted from: Blaxter *et al.* 1998, Dorris *et al.*. 1999

Figure 1.2a: Life cycle of *T. circumcincta*

Following sexual mating, adult female *T. circumcincta,* found in the host's abomasum, produce eggs which pass onto the pasture in the faeces. These develop to the free-living stages L1, then L2 and finally the infective stage L3, under optimal climatic conditions in approximately 3 weeks. L3s can survive on the pasture for extended periods of time. L3s are ingested by the grazing host animal. L3s exsheath within the host and develop to L4 and then adults to complete the life cycle.

Figure 1.2b: Comparison of adult trichostrongylid sizes

Comparison of adult stages of the three trichostrongylid nematodes, *Haemonchus, Teladorsagia* and *Trichostrongylus.*

Figure 1.2a

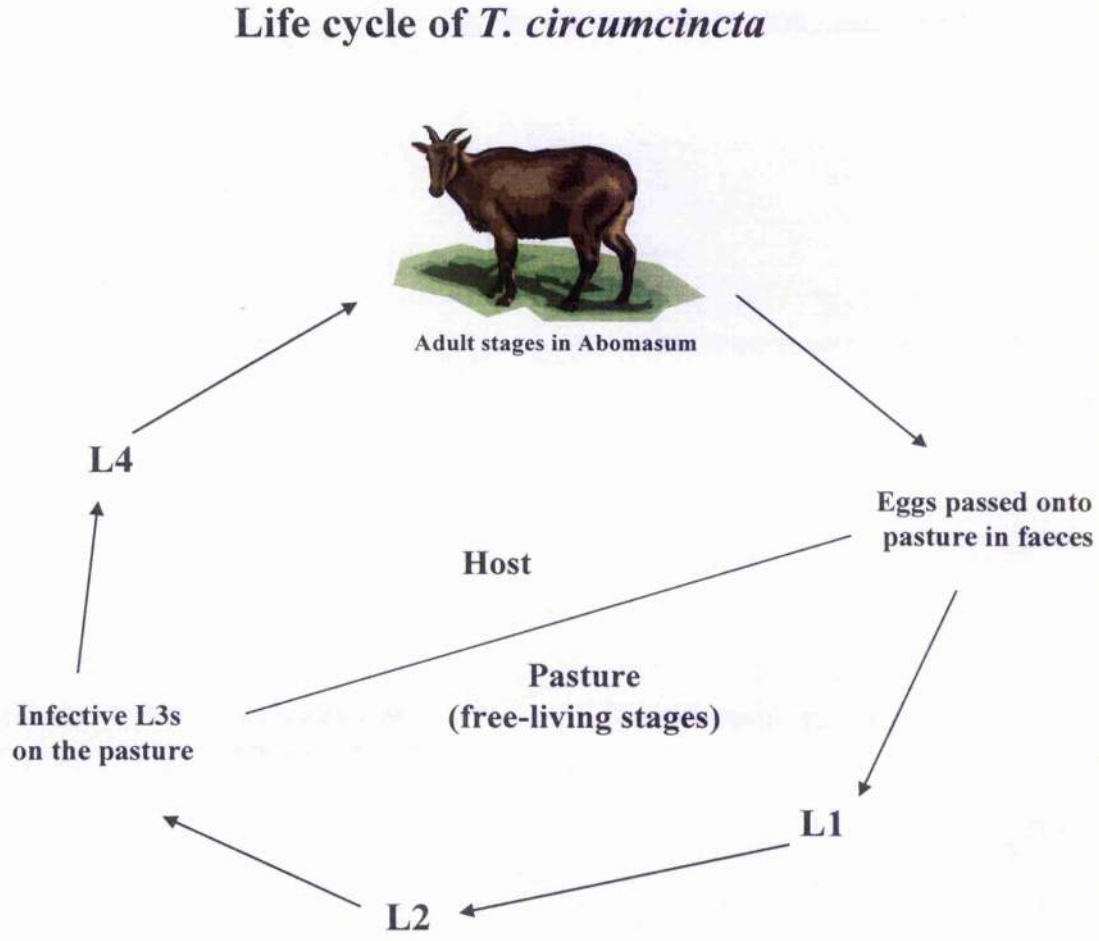


Figure 1.2b: Comparison of adult trichostronglid sizes

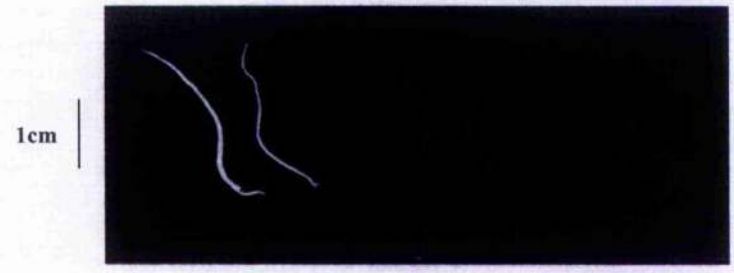


Table 1.1: Prevalence of anthelmintic resistance worldwide

The prevalence of resistance is indicated by the percentage of sheep farms tested. Data are shown for the three main classes of anthelmintics; Benzimidazoles, Levamisoles, and Ivermectins. Table adapted from review by McKellar and Jackson (2004) to include prevalence of resistance in Scottish sheep farms. a: Bartley *et al.* 2003. b: Sargison *et al.* 2001, SAC Report, 2000; Jackson *et al.* 1992.

Country	Benzimidazoles	Levamisoles	Ivermectin
Australia	90 %	80 %	60 %
South Africa	79 %	73 %	73 %
Argentina	40 %	22 %	6 %
Brazil	90 %	84 %	13 %
Paraquay	73 %	68 %	73 %
Uraguay	80 %	71 %	1 %
Scotland	64 %[a]	Sporadic[b]	Sporadic[b]

Chapter 2: Materials and Methods

2.1 Anthelmintic Resistance

2.1.1 Anthelmintic Survey Strategy and Kit

A survey of goat farms was conducted in order to assess the general situation in the U.K. regarding husbandry, use of anthelmintics and the efficacy of anthelmintic treatments. The details of as many goat farms as possible were gathered and each was sent a letter requesting them to participate in the survey. An initial letter was followed up by a phone call to confirm involvement and to obtain details of their veterinary surgeon, who was contacted, to gain agreement for inclusion of their client in the survey. All farms were asked to fill in a questionnaire including general information about the farm in addition to details regarding anthelmintic dosing strategy (Appendix A).

Each farmer was sent two sample collection boxes; the first box was used to collect a pre-dosing sample, taken at time of anthelmintic dosing, which included a 'dry' faecal sample and an anaerobic faecal sample. As the *in vitro* egg hatch assay requires undeveloped eggs, the anaerobic storage of faecal samples as described by Hunt and Taylor (1989) was utilised to prevent the development of nematode eggs for up to seven days after collection. The second box was used to collect a post dosing 'dry' faecal sample and anaerobic sample, to be taken approximately 7 - 10 days post dosing with the anthelmintic. All collection boxes included directions for collection of both aerobic 'dry' and anaerobic faecal samples (Appendix B), in addition to a submission form including details regarding the anthelmintic treatment. Submission details included i) the anthelmintic used and ii) method used to calculate dosage. Guidelines for dosing were included in each box, recommending dosages for goats and best technique to avoid oesophageal reflex (Appendix B).

2.1.2 Faecal Egg Counts

A modified McMaster flotation technique (MAFF 1986) was used to detect the presence of trichostrongylid eggs in the 'dry' faecal samples sent by post from goat farmers participating in the survey. In this technique, 3 g of faeces was homogenised with 42 ml tap water, which was then passed through a coarse mesh sieve of aperture size 250 μm (Endecotts Ltd.). The sieve allows the passage of nematode eggs (size range 70 – 165 microns), while retaining larger particles of debris. The filtrate was then thoroughly mixed prior to decanting into two 15 ml round bottomed centrifuge tubes and centrifuged at

2000 rpm for two minutes. The supernatant from both tubes was discarded and the remaining faecal pellet was broken up by rotary agitation. Each tube was filled to its former level with a saturated salt solution and inverted six times, followed immediately by transferring a sufficient volume of the suspension to fill two chambers of two corresponding McMaster slides using a pipette held horizontally. The number of eggs, under both etched areas, was counted per McMaster slide. The number of egg per gram was calculated by multiplying the total number of eggs under both etched areas by 50, therefore the sensitivity being 1 egg equating to 50 eggs per gram of faeces. (The volume under each etched chamber on the McMaster slide is equal to 0.15 ml. Three grams of faeces were added to 42 ml water giving 45 ml suspension, or 1 g faeces per 15 ml, which is 100 times the volume under each etched square). In order to increase the sensitivity of the test, two McMaster slides were processed per sample and the arithmetic mean of the two faecal egg counts calculated, therefore increasing the sensitivity to 1 egg recovered equally to 25 eggs per gram. Faecal egg counts were conducted by myself, James McGolderick or Stevie Robertson.

2.1.3 Mass Egg Extraction

The anaerobically stored faecal samples sent in by each farmer, were used for mass egg extraction, for the eggs to be used in the egg hatch assay. Mass egg extraction was only performed for anaerobically stored samples, which were no more than 7 days old. The contents of two anaerobically stored pots were poured into a container and tap water added to form a suspension. This suspension was then poured through a 1.0 mm coarse sieve to remove large particulate matter present in the faeces. The remaining suspension was then washed through three sieves in the following order: 210 μm; 63 μm and eggs finally collected in a 38 μm sieve. The material retained by the 38 μm sieve was rinsed for approximately 5 minutes. The retentate was washed off the sieve into a clean beaker using tap water and then poured into 15 ml polyallomer centrifuge tubes (Beckman Coulter Ltd., UK). Tubes were centrifuged at 1000 rpm for 10 minutes and supernatant was carefully removed. The remaining faecal pellet was broken up by rotary agitation and re-suspended by filling the tube ¾ full with saturated salt solution. The tubes were inverted several times to re-suspend the faecal pellet followed by centrifugation at 2000 rpm for 10 minutes. Each tube was clamped just below the meniscus using forceps, and the top layer poured into a 250 ml beaker or directly onto a 38 μm sieve and rinsed with tap water. Further centrifugation washes were performed if the eggs were particularly dirty.

The final retenate was washed off the sieve using tap water and collected in a Falcon tube and used for the egg hatch assay within four hours.

2.1.4 Egg Hatch Assay

Where sufficient numbers of eggs where recovered from anaerobically stored samples, by mass egg extraction, an Egg Hatch Assay (EHA) was conducted. The EHA is based on the ovicidial activity of benzimidazoles and measures the ability of fresh, undeveloped eggs from a nematode population to embryonate and hatch following exposure at various concentrations of anthelmintic (Condor and Campbell 1995). Egg suspensions were adjusted to a final concentration off 1000 strongyle eggs per ml. Each egg hatch assay was set up in a 24 well culture plate (Costar), each assay using 10 wells. The following concentrations of thiabendazole (Sigma; cat. no.: T-5535) were used in duplicate: 0, 0.05, 0.1, 0.3, and 0.5 µg / ml (Figure 2.1). Each well was set up as follows: 1890 µl of distilled water was added to each well followed by 10 µl stock solution of thiabendazole or water (control wells) and immediately followed by the addition of 100 µl of egg solution, containing approximately 100 strongyle eggs. Each plate was incubated at 22°C for 48 hours with 100 % relative humidity. Following incubation, 10 µl of helminthological iodine (Appendix D) was added to each well to stop the assay and prevent further hatching of eggs. The number of eggs and the number of larvae was counted for each well using an inverted microscope. The percent hatch was calculated using the following formula:

Percentage hatch = [No. of larvae / (No. of eggs + No. of larvae)] x 100

The percentage of eggs that hatch at each concentration was determined (using the arithmetic mean of the duplicate wells), corrected for natural mortality from control wells, and an ED_{50} estimated using logit model (logistic regression model: Collett (1991), as referenced by Bartley *et al.*, (2003). Transformation of the data using logit model is required to obtain a logistic regression from which an ED_{50} could be estimated. This analysis was carried out by Dave Bartley at the Moredun Institute, Edinburgh using Minitab version 12. The ED_{50} value refers to the concentration of thiabendazole required to kill 50 % of the eggs (or the concentration of thiabendazole at which 50 % of the eggs still hatch). Each sample was classified as resistant (R) to thiabendazole if the estimated ED_{50} value was greater than 0.1 µg / ml or susceptible (S) if it was less than 0.1 µg / ml as recommended by the WAAVP guidelines (Coles *et al.* 1992). An egg hatch assay was performed on the Moredun

susceptible strain Tci1 (MOSI) as a control at regular intervals throughout the sampling period.

2.1.5 Larval Cultures

Larval cultures were performed to allow the development of trichostrongylid nematode eggs to develop to L3 larval stages to allow morphological identification and to make DNA lysates for PCR genotyping (McMurtry *et al.* 2000). Larval cultures were performed as follows: all faeces remaining after calculating a faecal egg count were placed in one or more small plastic pots with perforated lids to allow for airflow. The pots were incubated at room temperature for 10 - 20 days. Each pot was then filled with warm water and left for two to four hours at room temperature to allow the L3 larvae to pass from the faecal mass into the water. The fluid was then poured through a coarse sieve (1.0 mm) and collected in a clean container. The filtrate was poured into a modified Baermann apparatus (MAFF 1986), consisting of a double layer of ultraflow milk filter paper (MAXA, cat. no. 13606), which will retain third stage larvae from the filtrate. The Baermann apparatus was then suspended in a jar filled high enough with warm tap water to just submerge the filter paper. This allows L3 larvae to migrate through the filter and fall to the bottom of the jar by gravity. The resulting larvae were stored at 4°C.

2.2 Nematode Populations and Preparation

2.2.1 Nematode Populations

Population genetic analysis of *T. circumcincta* has never previously been undertaken using microsatellite markers and so there was insufficient information available to allow a rational detailed sampling regime to be designed. Therefore, *T. circumcincta* populations that were readily available, or of specific interest, were used in this work. Consequently the samples consisted of parasite populations from a number of different countries, including both laboratory and field isolates from goats and sheep. All *T. circumcincta* populations consisted of approximately 22 - 48 single adult male *T. circumcincta* (Table 2.1 - 2.3).

It should be noted that the following four Motri populations, MotriPT, MotriPB, MotriPI, and MotriPL, were derived from a multiple resistant population isolated from sheep in Scotland by Sargison *et al.* (2001), classified as multiple resistant by Bartley *et al.* (2004), and have been individually selected for using three separate classes of anthelmintic in a number of experimental infections. These populations were genotyped by L. Stenhouse with

my supervision and assistance, the results of which have been included within Chapter 5 for completeness as additional U.K. isolates. However, there is very little discussion regarding these populations as it is not appropriate in this thesis, as these populations have been genotyped and analysed elsewhere for Lindsay Stenhouse's Thesis (Moredun Institute, Edinburgh).

2.2.2 Recovery of nematodes from the host

All *T. circumcincta* populations from the U.K. were recovered in the following way at the Moredun Institute, Edinburgh, Scotland. The host animal, sheep or goat, was slaughtered and a ligature placed at the pyloric / duodenal sphincter to prevent loss of abomasal contents and reflux from the small intestine. The abomasum was separated from the small intestine, cut along the greater curvature and the contents washed into bucket 1, using approximately 1 litre of warm water. The abomasum was then transferred to bucket 2 containing approximately 500 - 700 ml 0.85 % (w/v) NaCl. This encourages nematodes within the mucosal lining of the abomasum to move out into the solution. The contents of bucket 1 were sieved through a coarse sieve and the majority of worms collected with the fibrous abomasal retenate. These were then put into a clean container with 250 ml of 0.85 % (w/v) NaCl at 37°C for 1 hour. Clumps of worms were picked out of the bottom of buckets 1 and 2 using a fine needle and placed into petri dishes containing 0.85 % (w/v) NaCl solution. Adult male *T. circumcincta* were identified according Manual of Veterinary Parasitology Laboratory Techniques (MAFF 1986), using male tail spicule morphology, picked into cryotubes and stored in either liquid nitrogen or at -80°C. This procedure was followed at Moredun for the following populations; Tci1 (Mosi), Tci5 (MotriF, MotriPT, MotriPB, MotriPI, MotriPL), ScSo529, ScSo210, and ScS0507.

2.2.3 Genomic DNA (gDNA) Extraction

In order to isolate genomic DNA for small insert library construction, a population of adult Moredun Susceptible Isolate *T. circumcincta,* Tci1 (MOSI), was recovered from the abomasum of a single sheep and concentrated into a pellet. A modification of the protocol described by Johnstone (1999) was used. A pestle and mortar were pre-cooled using liquid nitrogen and the pellet of worms was added to the mortar and ground to a fine powder. Liquid nitrogen was allowed to evaporate before scraping the powdered worms into a 50 ml Falcon tube containing 10 volumes (as compared to the initial volume of pelleted worms) of pre-warmed (60°C) DNA lysis buffer (Williams 1995) (Appendix D) with 200 mg / ml

Proteinase K (Invitrogen; cat. no.: 25530-015), and incubated at 60°C for 1 hour, with occasional mixing. The resulting lysate was transferred to a 1.5 ml eppendorf tube and phenol, phenol / chlorophorm and chloroform extraction was performed (Sambrook *et al.* 1989). DNA was ethanol precipitated using standard methods and resuspended in 50 µl sterile dH_2O (Sambrook *et al.* 1989).

2.2.4 Production of single worm lysates

In order to undertake population genetic analysis of *T. circumcincta* populations, DNA was lysed from a number of single worms per population used (Table 2.1). Adult male, female and L3 *T. circumcincta* were prepared prior to use as follows:

2.2.4.1 Adult lysates

Adult worms which had been stored in liquid nitrogen were thawed in a petri dish of dH_2O. All adult male *T. circumcincta* were identified according Manual of Veterinary Parasitology Laboratory Techniques (MAFF 1986), using male tail and spicule morphology, and picked into a fresh petri dish of M9 (Appendix D). Individual males were picked into single 0.2 µl tubes containing 20 µl or 50 µl of DNA lysis buffer (Appendix D) containing proteinase K at a final concentration of 200 µg / ml.

In the case of adult female worms, DNA was prepared from heads, severed using 23 gauge needles, to avoid contamination with DNA from progeny in the uterus. Each head was picked into single 0.2 µl tubes containing 20 µl of lysis buffer containing proteinase K at a final concentration of 200 µg / ml.

2.2.4.2 Single and pooled L3 lysates

A suspension of L3s were mixed and approximately 100 µl of the solution pipetted, using low retention pipette tips, into an 1.5 ml eppindorf tube. Ten µl sodium hypochlorite (10 % - 13 % v/v) (Sigma-Aldrich, cat. no. 42,504-04) was added in order to exsheath the worms. A small aliquot of the solution was examined under a microscope to confirm L3s exsheathment after 20 minutes of incubation. The suspension was then centrifuged at 4,000 rpm for 1 minute and the majority of the supernatant carefully removed. The L3 larvae were washed twice by centrifugation using 100 µl of M9 solution (Appendix D) and then re-suspended in 50 µl M9. This was then pipetted onto NGM agar plates and the excess liquid allowed to evaporate. Single L3 were picked, using a platinum wire pick, into single

0.2 µl tubes containing 20 µl of lysis buffer containing proteinase K at 200 µg/ml. Pooled L3 lysates were made by adding to 50 µl lysis buffer, with protinase K at 200 µg/ml, to a 0.2 µl tube containing approximately 100 L3s.

All lysates were placed at -80°C for minimum of 10 minutes before incubation at 60°C for 98 minutes followed by 20 minutes at 94°C to denature the proteinase K. Lysates were stored at -80°C. Several tubes containing lysate buffer only were also prepared in parallel as negative lysate controls each time a worm population was prepared. For use as template in PCR reactions, initially individual lysates were used at 1:10 dilutions and 2.5 µl used in a 20 µL PCR reaction. Further optimisation allowed the amount of template used in each PCR reaction to be adjusted to 1 µl of diluted lysate, ranging from 1:5 to 1:40 dilutions (Table 2.1). When dilutions were made from neat lysates several aliquots of dilution dH_2O were made in parallel and used as negative controls.

2.3 Isolation of Microsatellite Loci

2.3.1 Construction of a *T. circumcincta* genomic DNA small insert plasmid library

2.3.1.1 Preparation of genomic and vector DNA for ligations

Genomic DNA (gDNA) was isolated from adult Moredun Susceptible Isolate *T. circumcincta,* Tci1 (MOSI), using standard methods described above.Approximately 5 - 10 µg (10 µl) of genomic *T. circumcincta* DNA was double digested using 20 units of *EcoRI* (BDH; cat. no.: 432072R) and *HindIII* (BDH; cat. no.: 432102G) restriction enzymes and at 37°C for 3 hours. The digested DNA was purified using the QIAquick PCR Purification Kit (Qiagen; 28104) following the manufacturer's instructions and then ethanol precipitated and resuspended in a final volume of 5 µl of sterile dH_2O. A volume of 1 µl digested gDNA was run on a 1.2 % (w/v) agarose gel in 1 x TAE buffer to establish whether DNA digestion was complete and to access the amount of DNA present. Digested *T. circumcincta* gDNA was stored at -20°C.

Approximately 1 - 2 µg of the plasmid pBluescript II SK+ was double digested using 10 units of *EcoRI* and *HindIII* in a 50 µl reaction at 37°C for 3 hours. Digested vector DNA was purified using a QIAquick PCR Purification Kit following the manufacturer's instructions and then ethanol precipitated and resuspended in a final volume of 5 µl. A

volume of 1 µl digested was run on a 1.2 % (w/v) agarose gel in 1 x TAE to establish completion of digestion and the quantity of DNA present. Digested pBluescript II SK+ DNA product was stored at -20°C.

2.3.1.2 Ligation of digested pBluescript II SK+ and *T. circumcincta* gDNA

Ligation reactions were set up as follows: 2µl digested *T. circumcincta* gDNA (5 - 10 µg, as estimated from 1 µl of digested gDNA), 0.5 µl (~50 ng) digested pBluescript II SK+, 10 units of T4 DNA ligase (ABgene; cat. no.: AB-0324), 2 µl 5 x ligase buffer and 4.5 µl dH_2O. Ligation reactions were incubated overnight at room temperature.

2.3.2 Transformation using XL Gold Competent cells

A volume of 0.5 µl ligation reaction was transformed into 50 µl XL10-gold Ultracompetent Cells (Stratagene; cat no.: 200314). Each transformation was plated out in such a way as to produce approximately 400 - 600 colonies per 145 mm agar plate.

Agar plates were prepared with 0.5 mM isopropyl-1-thio-β-D-galactopyrano-side (IPTG; Sigma; cat. no.: 15520-1G) and 80 µg/ml 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranisode (X-gal; GibcoBRL; cat. no.: 15520-018) to allow for blue-white colour selection of colonies (Sambrook *et al.* 1989). Agar plates also contained ampicillin at a concentration of 200 µg/ml allowing only colonies with pBluescript SK+ to grow as this plasmid contains an ampicillin resistant gene.

2.3.3 Hybrisation screening of small insert genomic *T. circumcincta* library

Plates containing transformed colonies were pre-cooled to 4°C and Hybond N nylon membrane (Amersham Life Science) was placed onto the surface, avoiding air bubbles and orientation was marked using a needle and the plate marked with pen in three places. A sandwich technique (Sambrook *et al.* 1989) was followed to make duplicate colony lifts. The nylon membrane was removed from the original transformed plate and placed colony side up on a fresh agar plate. A new nylon membrane was placed on top to make a 'sandwich' and orientation previously marked on first membrane was replicated for duplicate membrane. After colony lift, the original plate containing transformed colonies was stored at 4°C with no further incubation at 37°C. The sandwiched membranes were then removed from the second plate, after being incubated for 20 minutes at 37°C, and the following DNA fixation steps were performed on both sides of sandwiched membranes with

brief blotting on 3 mm Whatmann paper between each step. Membranes were placed on consecutive trays on 2 layers of 3 mm Whatmann paper soaked with the appropriate solution; 10 % (w/v) SDS for 3 minutes, denaturation solution for 15 minutes, neutralisation solution for 15 minutes and finally filter paper soaked in 2 x SSC solution for 10 minutes (Appendix D). Sandwiched membranes were then separated prior to DNA cross linking by exposure to 120 mJ of UV radiation.

Each membrane was placed in a hybridisation tube and pre-hybridised with 15 ml DIG easy Hybrisation solution (Roche; cat. no.: 1796895) for 1 hour at 42°C, followed by incubation overnight at 42°C with 10 ml hybrisation solution. Hybrisation solution consisted of a $(CA)_{25}$ oligonucleotide probe added at a concentration of 1 pmol/l to pre-warmed DIG easy Hybridisation solution. The $(CA)_{25}$ oligonucleotide probe was synthesised to include a 5'-end digoxigenin-11-dUTP label for chemiluminence (MWG-Biotech AG). Following hybridisation the membranes were washed twice at room temperature in 30 ml of 2 x SSC / 0.1 % SDS for 5 minutes and then twice in 30 ml of pre-warmed 2 x SSC / 0.1 % SDS for 10 minutes at 47°C.

Chemiluminescent detection of bound probe was performed using the DIG easy Hybridisation Kit (Roche; cat. no.: 1585614) and autoradiography performed using standard procedures. Only colonies aligning hybrisation signals on both membranes were considered to be potentially positive (Figure 2.2a). The colonies on the original plates were indistinct and could not be reliably picked. Furthermore, incubation of plates at 37°C following colony lifts was unsuccessful with poor re-growth of colonies as were attempts to "Touch duplicate" plates (Sambrook *et al.* 1989). Consequently, putative positive colonies were re-screened in the following manner: each indistinct putative positive colony was picked and streaked onto a fresh agar plate containing ampicillin at 200 µg/ml and six single colonies were picked from the streak plate. *EcoRI* / *HindIII* double digested miniprep DNA from each of these colonies was southern blotted and hybridised with the $(CA)_{25}$ oligonucleotide probe to identify plasmids containing inserts with microsatellite repeats (Figure 2.2b).

2.3.4 Southern Blotting

Southern blotting was performed using standard procedures (Sambrook *et al.* 1989). Following electrophoresis, the agarose gel was submerged in 250 mM HCl for 10 minutes with agitation at room temperature. The gel was then rinsed in dH_2O and submerged in denaturation solution (Appendix D) for 2 x 15 minutes, rinsed in dH_2O and submerged in

southern neutralisation (Appendix D) solution for 2 x 15 minutes and again rinsed in dH_2O. The DNA was transferred to Hybond N nylon membrane (Amersham Life Science) by the standard method (Southern 1992) using 20 x SSC (Appendix D). Prior to removing the blot from the gel, the orientation is marked by cutting one corner and marking each gel well with a pencil. The nylon membrane was then briefly rinsed in 2 x SSC (Appendix C) prior to DNA immobilisation and cross linking onto the nylon membrane by exposure to 120 mJ of UV radiation. Hybridisation of Southern blots were performed exactly as for colony lift filters.

2.3.4.1 Production and optimisation of a TecRep probe for hybridisation of Southern Blots

A DNA probe was PCR amplified, using plasmid MTG 12 as template, and labelled with digoxigenin for hybridisation experiments. MTG 12 was chosen because it had the highest number of TecRep repeat units. The primers used were the primer 12 RepProbe 1 (5'-CTT ATC AGC CCT TAT CAG CTG TTA-3') corresponding to sequence within the first TecRep immediately downstream of the GT microsatellite and primer 12 RepProbe 2 (5'-AGC GCC TCT TAT CAG CCC TTA TC-3') corresponding to sequence 897 bp downstream (Figure 2.3a). This primer pair amplified a product of 897 bp containing six entire TecRep repeat units. This probe was 5'-end labelled with digoxigenin-11-dUTP using the DIG High Prime labelling and detection starter kit II (Roche), following the manufacturer's instructions.

Prior to use in screening plasmids for the TecRep repeat, the specificity of the probe was tested in a number of dot blot experiments. Four plasmids were dot plotted at a series of dilutions (neat, 1/10, 1/100, 1/1000 and 1/10,000) and hybridised with the digoxigenin labelled TecRep probe under a variety of conditions. The four plasmids used were MTG 12, containing the insert from which the probe was amplified; MTG 3, containing an insert with a single TecRep repeat; MTG 13, containing an insert with five TecRep repeat units and of the repeats sequenced from the first screening were the most divergent from those of MTG12 and MTG 1b containing insert with no TecRep repeats. The probe hybridised consistently with MTG 3, MTG 12 and MTG 13 but not MTG 1b under the following hybridisation conditions: pre-hybridisation for 1 hour at 42°C with DIG-easy hybridisation solution, followed by incubation overnight at 37°C with 30 ml pre-warmed probed hybridisation liquid at a concentration of 1 pmol/l. First the membranes were washed twice

at room temperature in 6 x SSC / 0.1 % SDS for 5 minutes and subsequently washed twice in pre-warmed 6 x SSC / 0.1 % SDS for 10 minutes at 42°C (Figure 2.3b).

2.4 General Molecular Biology Techniques

2.4.1 DNA preparation and storage

2.4.1.1 DNA preparation

The miniprep protocol used, is based on the alkaline lysis method used for DNA purification (Sambrook *et al.* 1989) and was used for routine plasmid preparations to screen transformation plates for plasmid clones containing the correct insert. For high quality sequencing template, a QIAprep Spin Miniprep Kit (Qiagen; cat. no.: 27106) was used for inserts sized less than 1 kb or a QIAfilter Plasmid Midi Kit (Qiagen; cat. no.:12243) was used for inserts greater than 1 kb.

The concentration of the plasmid solutions were calculated by comparison of 1 µl of preparation on a 1.2 % (w/v) agarose gel alongside a known concentration of 1 kb ladder (Invitrogen; cat. no.: 15615-024) and also by measuring the absorption of the solution in a spectrophotometer at 260 and 280 nm wavelengths.

2.4.1.2 Glycerol Stocks

For long term storage of plasmids, 500 µl of 30 % glycerol / LB broth solution was added to 500 µl of overnight bacterial culture and stored at -80°C.

2.4.2 Sequencing ABI 3100

Double stranded sequencing reactions were carried out by a method modified from that of Sanger *et al.* (1977). Automated sequencing was performed on an ABI Prism 3100 capillary DNA sequencer (Applied Biosystems) using the Big Dye Terminator Sequencing kit version 3.0 (Applied Biosystems). Each sample contained 2 µl of Ready Mix ®, 200 – 500 ng of double-stranded plasmid DNA, or 3 - 10 ng PCR product DNA, and 0.32 µM of the appropriate oligonucleotide primers, made up to a final volume of 10 µl with dH_2O. Thermocycling was performed using a 9600 ABI thermocycler using the standard conditions outlined in the Big Dye Terminator Sequencing kit version 3.0 protocol (Applied Biosystems). On completion of thermocycling, unincorporated nucleotides were removed from samples using DTR Gel Filteration Cartridges (Edge Biosytems, cat. no.:42453) and freeze-dried. Samples could be stored at -20°C in a dry form. Samples were re-suspended in

20 µl Hi-Di formamide (Applied Biosystems) and transferred into a 96 well plate (Applied Biosystems) which was then loaded into the ABI prism 3100 capillary DNA sequencer. Sequence chromatograms were visualised using Chromas version 2.23 software (Technelysium PTY, Ltd) and analysed using Vector NTI software version 8 (InfoMax).

2.4.2.1 Direct sequencing of PCR products

PCR reactions were performed in 50 µl reactions and products purified either directly by the QIAquick PCR Purification Kit or specific amplicons were excised from 1.2 % (w/v) agarose gel and purified using the QIAquick gel extraction kit (Qiagen; cat. no.: 28704). Routine sequencing was carried out using a primer designed approximately 5 - 10 bp downstream to the original primers used to amplify the PCR product.

2.4.2.2 TA cloning and sequencing

Routine cloning of PCR products was performed using the TA vector system (pCR 2.1, Invitrogen; cat. no.: K2040-01). This vector is linearised at the polylinker with overhanging thymidine residues that act as complementary ends for the PCR product with added adenosine residues added by Taq polymerase. Ligation and transformation were performed as described by the manufacturer's protocol. Generally, 50 ng vector and molar ratio of 1:1 insert to vector were used. Ligations were transformed into TOP 10 competent cells (Invitrogen; cat. no.: C4040-03). Each batch of transformation was plated out onto LB plates containing 100 mM IPTG, 40 mg/ml X-Gal and 100 µg/ml ampicillin and incubated overnight at 37°C.

2.5 Polymerase Chain Reactions (PCR)

All of the following PCR reactions were performed in 20 µl reactions containing 45 mM Tris HCl (pH8.8), 11 mM $(NH4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptethanol, 4.4 µM EDTA, 113 µg/ml BSA, 2 % Tween, 1 mM each deoxyribonucleotide triphosphates, 0.5 µM of each oligonucleotide primer and 0.05 U/µl of Taq polymerase (Applied Biosystems; cat. no.: N808-0166). All oligonucleotides were synthesied by either MWG-Biotech AG or TAGN (VH Bio). PCR reactions were performed using a 9600 ABI thermocycler (Applied Biosystems) unless otherwise stated.

2.5.1 Microsatellite PCR

The following thermocycling conditions were used: 94°C for 3 minutes followed by 40 cycles of 94°C, 15 seconds, A°C, 30 seconds and 72°C for 1minute, followed by one cycle at 72°C for 15 minutes, where A is the annealing temperature optimised for each individual primer pair using a gradient PCR thermocycler (TECHE, Touchgene Gradient). The temperature gradient included the following temperatures: 45.5°C, 47.6°C, 49.7°C, 55.6°C, 58°C, 60.4°C and 64.5°C. Template for all PCR reactions was either gDNA or single worm lysates prepared and used as described in section 2.2.3 and 2.2.4. All sets of PCR reactions had negative controls as described in section 2.5.6. All primers designed for microsatellite amplification can be found in appendix C.

2.5.2 Speciation PCR

Wimmer *et al.* (2004) described a PCR-based assay using ITS-2 species-specific primer pairs which are sensitive and specific for *Teladorsagia spp.* with respect to other gastrointestinal parasitic nematodes found in sheep and goats. This PCR was conducted for all populations to confirm morphological identification of *Teladorsagia spp.*, including *T. circumcincta, T. davtiani* and *T. trifurcata.* Templates for all PCR reactions were single worm lysates prepared and used as described in section 2.2.4. Primers used were ITS T.circ A (5'- TCA CAT TTT TGT GTC ACA ATT ATC- 3') and ITS T.circ B (5'- CAG GAA CGT TAC GAC GGT AAT-3') used at 10 μM concentration (Wimmer *et al.* 2004). The PCR conditions were: 94°C for 2 minutes followed by 40 cycles of 92°C, 30 seconds; 52°C, 30 seconds and 72°C for 30 seconds; followed by one cycle at 72°C for 10 minutes. The predicted size of the PCR product was 320 bp.

In addition to controls used for all PCR reactions, a panel of genomic DNA templates prepared from individual worms lysates from a number of different trichostrongylid species was included to ensure specificity; *T. axei, T. colubriformis, T. vitrinus, Capillaria longipes* and *Nematodirus spp.* provided by the Soay Sheep Project, Edinburgh University.

2.5.3 β-tubulin

Leignel *et al.* (2002) has shown evidence of molecular markers that show polymorphisms between the *T. circumcincta* 'goat', previously described by Gasnier *et al.*(1997) and other *T. circumcincta* isolates. One of these markers, the β- tubulin isotype 1 gene, was used to investigate the four French populations in this study. The Scottish population ScSo507 was

also investigated with this markers to test for consistency. The fragment was amplified using the primers M2 (5'–GAT CAG CAT TCA GCT GTC CA-3') and D7 (5'-GAA CAA TGG ACT CTG TTC G-3') at 10 μM. The PCR conditions were 94°C for 2 minutes, followed by 35 cycles of 94°C, 55 seconds, 55°C, 55 seconds and 72°C for 1minute 30 seconds, followed by one cycle at 72°C for 10 minutes.

2.5.4 rRNA Intergenic Transcribed Spacer (ITS-2) PCR

Another marker previously shown to be polymorphic between the *T. circumcincta* 'goat' (Gasnier *et al.* 1997), and other *T. circumcincta* isolates was the ITS-2. This was also used to investigate the four French populations in this study as well as the Scottish population ScSo507, NzWs, and all Soay populations (ScKiTT, ScKiTD, ScKiTC). An ITS-2 fragment was amplified using the primers designed from the 5.8S and 28S sequences of *C. elegans*; NC1 (5'-ACG TCT GGT TCA GGG TTG TT-3'), 5' labelled with 6-FAM and NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') at 10 μM concentration (Gasser *et al.* 1993). The PCR conditions were 94°C for 5 minutes followed by 35 cycles of 94°C, 30 seconds, 54°C, 30 seconds and 72°C for 30 seconds, followed by one cycle at 72°C for 10 minutes. PCR products were analysed on an ABI Prism 3100 capillary DNA sequencer using Genescan as described in section 2.5.7. The predicted size of the PCR products was either 336 bp or 342 bp.

2.5.5 rRNA Non Transcribed Spacer (NTS) PCR

For most organisms, the non-transcribed intergenic spacer (NTS) of the rRNA cistron is more polymorphic than the ITS-2, and is a potentially useful marker to differentiate closely related species (Dorris *et al.* 1999). Primers were designed using a sequence alignment of 18S and 28S rRNA genes from *C. elegans, Drosophila melanogaster, Saccaromyces pombe* and *Xenopus laevis* to select highly conserved regions of sequence (Dr J. Gilleard, pers. comm.): NTS 1 (5'-GAG CTG GGT TTA GAC CGT CGT GAG-3') and NTS 3 (5'-AGC TCC AGT ATT TCC GCA GTT ATC C-3'). These were used at 10 μM concentration and the PCR conditions were 94°C for 3 minutes followed by 40 cycles of 94°C, 30 seconds, 55°C, 1 minute and 72°C for 2:30 minutes, followed by one cycle at 72°C for 5 minutes.

2.5.6 Prevention of PCR contamination

Due to very low quantities of DNA template being used in all of the above PCR reactions, and the large number of PCR reactions performed, a number of precautions were made to monitor and prevent contamination occurring with unknown DNA template. PCR reactions

and all template manipulations were performed in a PCR UV cabinet (200-275 nm; Scieplas) in a specified "PCR" room. Designated pipettes and disposables were used for making up PCR mixes and all solutions required for PCR reactions or template preparation, i.e. 11.1 x PCR buffer and lysis buffer. All solutions and reagents were divided into aliquots to minimise repeated use of each solution. Designated lab coats were worn and gloves changed frequently, at least when entering or leaving the designated room. UV light has been shown to quickly damage any DNA in solutions or on surfaces, making it unsuitable for amplification template (Kwok and Higuchi 1989;Pao *et al.* 1993;Rys and Persing 1993). A PCR "master mix" was made up including all constituents except Taq polymerase and template. Subsequently, all items to be used, including 96-well plates, pipettes and PCR mix, were left under UV light for 20 minutes in the UV cabinet. Taq polymerase was added after UV irradiation. Template was always added last to each tube. For single worm lysate preparation, lysis buffer was thawed and left under UV light for 20 minutes, prior to adding protinase K at 200 µg/ml. Single worms were picked into individual tubes containing the lysis buffer mix in a designated room free from PCR amplified products. All PCR reactions performed included the following negative controls: 2 x lysis buffer aliquotted at time of single worm lysate preparation for each population, 2 x dH_2O aliquotted at time of single worm dilutions for each population and 4 x dH_2O used in PCR mix. In cases where there was any suspicion of contamination, old aliquots of all PCR constituents were disposed of and fresh aliquots used and experiment repeated. This process was continued until no contamination was present.

2.5.6 Agarose gel electrophoresis

Agarose gels (2 %, w/v) were used for electrophoresis analysis of PCR product sizes predicted to be between 150 and 800 bp, 1.5 % (w/v) agarose gels were used for analysis of PCR products predicted to be between 800 and 1500 bp and 1.2 % (w/v) agarose gels were used for the analysis of digested products or for gel extraction of PCR amplicons. Agarose gels were prepared by dissolving LE analytical grade agarose (Promega) in 1 x TAE and adding ethidium bromide to a final concentration of 0.5 µg/ml. Gels were cast and run in 1 x TAE using Anachem Horizontal Agarose system. DNA samples and markers,1 kb ladder (Invitrogen) which encompassed a quantifying band or 1 kb ladder (VWR / BDH), were diluted in loading buffer (Appendix C) and gels were electrophoresed at 60 - 120 V until appropriate separation of amplicons was achieved. PCR products were visualised following ethidium staining using a UV transilluminator (205 - 260 nm wavelength).

2.5.7 Genotyping using the ABI3100 capillary sequencer

An ABI Prism 3100 genetic analyser (Applied Biosystems) was used for accurate sizing of microsatellite PCR products using capillary electrophoresis. The forward primer, for each microsatellite primer pair, was 5' end labelled with FAM or HEX fluorescent dyes (MWG) and electrophoresed along side GeneScan ROX 400 (Applied Biosystems) internal size standard on an ABI Prism 3100 genetic analyser. Individual chromatograms were analysed using ABI Prism Genotyper 3.7 NT software (Applied Biosystems) to determine the genotype of each sample. Prior to analysis by capillary electrophoresis using the ABI 3100 genetic analyser, all or a subset of the PCR products were visualised by agarose gel electrophoresis to confirm yield of each product. A volume of 1 µl of appropriately diluted PCR product was transferred to 20 µl Hi-Di formamide (Applied Biosystems) containing 0.25 µl of the fluorescent internal size standard GeneScan Rox 400 in a 96-well microtitre plate (Applied Biosystems or Elkay). The plates were transferred to a 9600 ABI thermocycler (Applied Biosystems) to denature the samples for 5 minutes at 94°C, subsequently loaded into the ABI Prism 3100 capillary DNA sequencer and run using the following settings required for genescan: Dye set: D, Run module (polymer): 36cm pop4 and analysis module 400HD (this corresponds to the ROX 400 size standard).

2.6 Population Genetic Analysis

2.6.1 Genetic Diversity

Expected heterozygosity, unbiased for sample size, was calculated using Microsatellite Toolkit (Park 2001) based on Nei (1987) as referenced by Park (2001). The average number of alleles per locus was calculated by GDA (Genetic Data Analysis) version 1.1 (Lewis 2001).

2.6.2 Hardy-Weinberg Equilibrium

Expected heterozygosity, unbiased for sample size, was calculated in Excel Microsatellite Toolkit based on Nei (1987) as referenced by Park (2001). Observed heterozygosity was calculated in Microsatellite Toolkit based on Hendrik (1983) as referenced by Park (2001). P-values were calculated in GDA version 1.1 (Lewis 2001) using Fisher's Exact Test and P-values < 0.05 were taken as evidence of a statistically significant departure from Hardy-Weinberg Equilibrium (HWE). Probabilities shown are estimates of the exact significance levels using 10,000 runs. Null homozygote genotypes (individual worms which failed to

amplify) were removed from data analysis (Zaykin *et al.* 1995 as referenced by Lewis (2001)).

2.6.3 Linkage Disequilibruim

Linkage Disequilibrium (LD) was analysed using P-values calculated in GDA version 1.1 (Lewis 2001) using Fisher's Exact Test. Probabilities shown are estimates of the exact significance levels using 10000 runs. P-values < 0.05 were taken as evidence of a statistically significant linkage between loci. Null homozygote genotypes were removed from data analysis (Zaykin *et al.* 1995) as referenced by Lewis (2001). Linkage Disequilibrium analysis was calculated preserving genotypes, to prevent within locus disequilibrium (i.e. departure from HWE) affecting P-value significance (Zaykin *et al.* 1995) as referenced by Lewis (2001).

2.6.4 Genetic differentiation

2.6.4.1 Fst

Pairwise Fst values were calculated using GDA version 1.1 (Lewis 2001) based on Weir (1996). Pairwise Fst calculations were also conducted in Arelquin version 2.0 (Schneider *et al.* 2000), GenAlEx version 5.1 (Peakall and Smouse 2001), FSTAT version 2.9.3.2 (Goudet 1995) and Excel Microsatellite Toolkit (Park 2001). Nei's genetic distance was also calculated using GenAlEx version 5.1 as described by Weir (1990) which references Nei (1972). All calculations were compared. GDA version 1.1 was chosen for analysis as it was the least complicated to use for both input and output of data files.

2.6.4.2 AMOVA

Analysis of Molecular Variance (AMOVA) was conducted to test for population differentiation of samples at various levels, locus by locus, using Arelquin version 2.0. (Excoffier *et al.* 1992;Schneider *et al.* 2000;Weir 1996;Weir and Cockerman 1984). Data were defined as 'standard' rather than 'microsatellite', as loci did not necessarily adhere to the stepwise mutation model. Similar analysis was calculated using GenAlEx version 5.1 (Peakall and Smouse 2001) for comparison only.

2.6.4.3 PCA

Principle component analysis was conducted using GenAlEx version 5.1 (Peakall and Smouse 2001). The procedure is based on an algorithm published by Orloci (1978) as

referenced by Peakall and Smouse (2001). PCA analysis was conducted using Fst values calculated by GDA version 1.1 to plot individual populations. PCA analysis was conducted preserving individual worm genotypes by GenAlEx version 5.1 to plot individuals.

2.6.5 Genotyping errors

Micro-checker version 2.2.1 (Van Oosterhout *et al.* 2005) is a windows®based software program that was used to investigate data for a number of potential genotyping errors including short allele dominance (large allele dropout), non-amplifying alleles (null alleles), and scoring of stutter peaks as well as detection of typographical errors. The program works by detecting significant deviations from Hardy-Weinberg equilibrium and indicating whether these are consistent with short allele dominance, null alleles, or scoring errors associated with stuttering (Van Oosterhout *et al.* 2004). The program constructs random genotypes by randomising the observed alleles for each loci within each sample, from which two graphs are produced. The first shows the frequency of allele specific homozygotes. The second graph shows the frequency of genotypes categorised by the difference in base pairs between both alleles. Using the first graph, short allele dominance is suggested when there is an excess of homozygotes in the shorter allele sizes and null alleles are suggested when the majority of allele size classes show an excess of homozygotes. In loci with many alleles, excess of homozygotes tends to occur due to segregation of common alleles with null alleles. This will result in the erroneous scoring of homozygotes. Rarer allele classes, on the other hand, are generally not expected to show a homozygote excess, because the probability of segregating with a null allele is dependent on the allele frequency. The Micro-Checker application therefore calculates a cut-off frequency for which allelic classes are analysed to show a homozygote excess (and are therefore expected to segregate with a null allele). This cut-off frequency equals $p > (2Nq\text{-null})^{-1}$, in which p is the allele frequency, N is the total number of samples analysed, and q-null the estimated null allele frequency (Van Oosterhout *et al.* 2004). When most allelic classes, with an allele frequency: $p > (2Nq\text{-null})^{-1}$, show a homozygote excess the application warns of a potential null allele. Stuttering is indicated by a deficiency of heterozygotes with alleles differing by one allele size class, suggesting that these have been erroneously called as homozygotes rather than heterozygotes.

2.6.5.1 Statistical analysis

Statistical analysis was performed using SigmaStat software for Windows version 3.0.1. Statistical comparisons between two different groups were made using an unpaired t-test

and are presented as mean ± standard error of the mean (SEM). A statistical significance is defined as a p-value ≤ 0.05. Correlation of data was calculated using the Spearman Rank Correlation using GraphPad Instat® for Windows version 3.05.

2.6.6 Estimates of null alleles

Estimates of null allele frequency were calculated using GENEPOP version 3.3 (Raymond and Rousset 1995). In order calculate null allele frequency, GENEPOP requires at least one null homozygote per population. Therefore, as not all populations contained a null homozygotes, one extra individual was added to each population and given a null homozygote genotype for all loci. By doing this, null allele frequencies were over estimated. This program calculates the maximum likelihood estimates of gene frequencies when a null allele is present, using the expectation maximisation (EM) algorithm of Dempster *et al.* (1977) as referenced by Raymond and Rousset (1995).

Null allele frequencies were also calculated utilising apparent heterozygote deficiencies using expected and observed heterozygosities. Calculations were first described by Chakraborty *et al.* (1992) referenced as equation 3 by Brookfield (1996) and then modified by Brookfield (1996) as equation 4. When no null homozygotes are found in the sample population, then null allele frequency can be calculated using $N_f = (H_e - H_o) / (H_e + H_o)$ (Chakraborty *et al.* 1992 as referenced by Brookfield (1996), whereas when null homozygotes are present in the population then null allele frequency can be calculated as follows: $N_f = (H_e - H_o) / (1 + H_e)$, (Brookfield 1996). Where N_f = null allele frequency and H_e = expected heterozygosity and H_o = observed heterozygosity. Both methods assume populations are in panmixia and heterozygote deficiencies are due to null alleles only and not due to population subdivision (Brookfield 1996).

Figure 2.1: Dilution plate for Egg Hatch Assay (EHA)

Schematic representation of dilution setup for egg hatch assay in 24-well plate, demonstrating the concentration of thiabendazole in each well, done in duplicate.

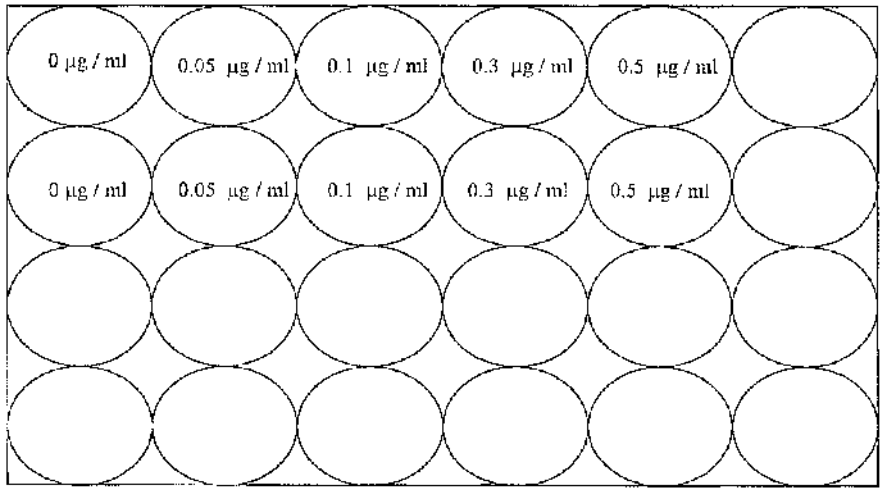


Table 2.1: *T. circumcncta* populations used in study

List of populations used in project and details corresponding to each. M = male, F = female, * = head only used in lysate, ** = see Table 2.3 for further detail, [1] = Lysates were made in lab of origin. [2] = Lysates made and genotyped by L. Stenhouse (Moredun Institute). All other lysates at a final concentration of of individual worms were made in this lab.

Population	Country of Origin	Geographical Location / Lab Strain	Lab / Field	Host Species	Host ID	Parasite stage	Parasite Sex	No. of individuals genotyped	Dilution of lysates used
FrMe	France	Meunier	Field	Goat	98	Adult	M	33	1:20
FrGa	France	Galopin	Field	Goat	5852	Adult	M	40	1:20
FrSo	France	Sorin	Field	Goat	9051	Adult	M	23	1:40
FrMn	France	Meneau Espagnol	Field	Goat	U	Adult	M	40	1:40
Tci1 (MOSI)	Scotland	Moredun Tci 1	Lab	Sheep	U	Adult	M	25	1:20
Tci5 (MotriF)	Scotland	Moredun Tci 5	Lab	Sheep	62P	Adult	F*	22	1:5
Tci5 (MotriPT)	Scotland	Moredun Tci 5	Lab	Sheep	62P	Adult	M	29	1:20[2]
Tci5 (MotriPB)	Scotland	Moredun Tci 5	Lab	Sheep	8P	Adult	M	29	1:20[2]
Tci5 (MotriPI)	Scotland	Moredun Tci 5	Lab	Sheep	61P	Adult	M	29	1:20[2]
Tci5 (MotriPL)	Scotland	Moredun Tci 5	Lab	Sheep	69P	Adult	M	29	1:20[2]
ScSo529	Scotland	Sourhope	Field	Goat	529	Adult	M	30	1:20
ScSo210	Scotland	Sourhope	Field	Goat	210	Adult	M	30	1:20
ScSo507	Scotland	Sourhope	Field	Goat	507	Adult	M	21	1:20
ScKiTD	Scotland	St. Kilda	Field	Sheep	Various**	Adult	M	31	1:30[1]
ScKiTT	Scotland	St. Kilda	Field	Sheep	Various**	Adult	M	30	1:30[1]
ScKiTC	Scotland	St. Kilda	Field	Sheep	Various**	Adult	M	31	1:30[1]
WeCa	Wales	Carmarthen	Field	Sheep	U	Adult	M	44	1:40
NzWs	New Zealand	Wallaceville	Lab	Goat	3049	Adult	M	48	1:40[1]

Table 2.2: *T. circumcncta* populations used in study (continued)

List of populations used in project and further details corresponding to each. Background information detail was dependent on information provided from the lab of origin.

Population	Date of recovery from host	Background Information
FrMe	Feb 1999	Population from the goat Ch098 from the Meunier farm in Touraine, France on 11/02/99. Known to have high prevalence of *Teladorsagia circumcincta* 'goat' (Dr. J. Cabaret, pers. comm.). Farm 'closed' for over 20 years. Provided by Dr. J. Cabaret.
FrGa	Dec 1998	Population isolated from goat 5852 on the Galopin farm in Touraine, France on 28/12/98. Known to have low prevalence of *Teladorsagia circumcincta* 'goat' (Dr. J. Cabaret, pers. comm.). Farm 'closed' for over 25 years. Provided by Dr. J. Cabaret
FrSo	April 1999	Population isolated from goat 9051 from the Sorin farm in Quercy, France. Known to have *Teladorsagia circumcincta* 'standard' (Dr. J. Cabaret, pers. comm.). Farm 'closed' for over 20 years. Provided by Dr. J. Cabaret
FrMn	Unknown	Population from one goat from the Meuneau farm in Touraine, France. The farm has been 'closed' for less than 10 years. Goats on the farm originated from both Pyrenees and Touraine. Provided by Dr. J. Cabaret
Tci1 (MOSI)	Oct 2002	Susceptible to all major classes of anthelmintics, field population originally isolated from sheep on Firth Mains farm in 1983, passage 4-5 times per year through parasite naïve sheep with an infecting dose of 5-10,000 larvae. Provided by Dr. F.Jackson.
Tci5 (MotriF)	July 2003	Resistant to three major classes of anthelminitc (Bartley *et al.* 2004), field population originally isolated from sheep farm in Scotland (Sargison *et al.* 2001), passaged 2-3times per year in parasite naïve sheep with an infecting dose of 5-10,000 larvae. Provided by Dr. F.Jackson.
Tci5 (MotriPT)	2004	Tci5 strain as described above.
Tci5 (MotriPB)	2004	Tci5 strain as described above followed by passage through parasite naïve sheep which were dosed at 28 days post infection with Benzimidazole (using the recommended dose rate) and worms collected at 41 days post infection.
Tci5 (MotriPI)	2004	Tci5 strain as described above followed by passage through parasite naïve sheep which were dosed at 28 days post infection with Ivermectin (using the recommended dose rate) and worms collected at 41 days post infection.
Tci5 (MotriPL)	2004	Tci5 strain as described above followed by passage through parasite naïve sheep which were dosed at 28 days post infection with Levamisole (using the recommended dose rate) and worms collected at 41 days post infection.
ScSo529	Feb 2001	Resistant to benzimidazoles and ivermectin (Jackson *et al.* 1992). All three populations from male goats born on 16/02/2001; culled at 20 month old, main reason for culling was poor fibre, from a group of 30 goats all grazed together since birth. Last dosed with anthelmintic on 14/12/00 then grazed on E2 (11.08 hectares) then moved on 16/01/02 to E1 (16.53 hectares). Farm 'closed' for at least 15 years. Collected with the help of Dr. F. Jackson and his laboratory.
ScSo210	Feb 2001	
ScSo507	Feb 2001	
ScKiTD	Spring 2002	Population isolated from Soay sheep being post-mortem as part of the Soay Sheep Project. Provided by B. Craig, Dr. B.Wimmer and Dr. J.Pemberton. See Table 2.3
ScKiTT	Spring 2002	Population isolated from Soay sheep being post-mortem as part of the Soay Sheep Project. Provided by B. Craig, Dr. B.Wimmer and Dr. J.Pemberton. See Table 2.3
ScKiTC	Spring 2002	Population isolated from Soay sheep being post-mortem as part of the Soay Sheep Project. Provided by B. Craig, Dr. B.Wimmer and Dr. J.Pemberton. See Table 2.3
WeCa	2003/2004	Population recovered from one unknown sheep in Camarthen area of Wales by the Veterinary Investigation Centre. Provided by Dr. Sian Mitchell.
NzWs	Aug 2004	Susceptible to all broad spectrum anthelmintics. Field strain originally isolated from Wallaceville, NZ in 1950's prior to the widespread use of thiabendazole and levamisole, passaged through both parasite naïve sheep and goats with an infecting dose of 10-15,000 larvae. This sample was from a pen-raised Saanen Wether goat in 2004. Provided by Dr. Warwick Grant.

Table 2.3: Specific details for *T. circumcincta* collected from Hirta

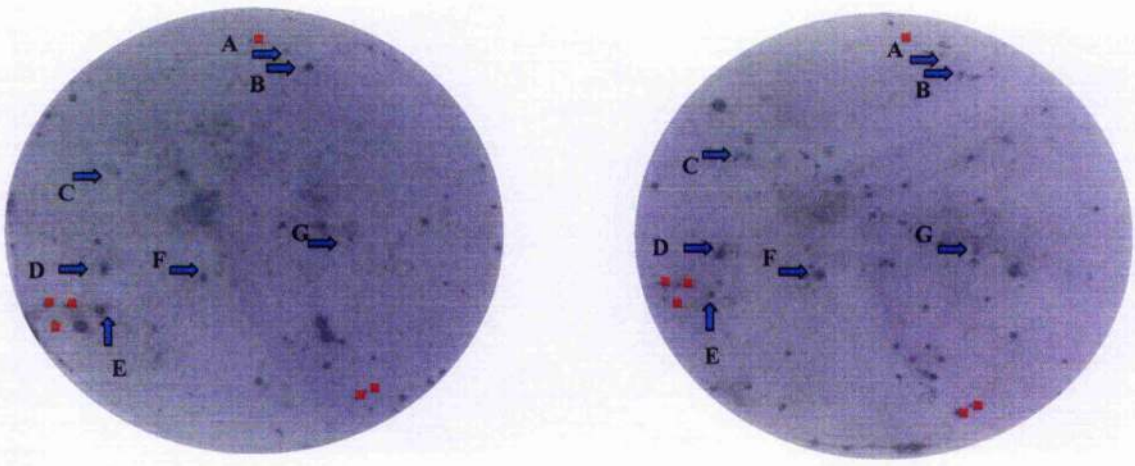
Three populations were isolated from soay sheep on the remote Scottish island of Hirta, St. Kilda as part of the Soay Sheep Project, by B. Craig, Dr. B. Wimmer and Dr. J. Pemberton (University of Edinburgh, Scotland). Nematodes were collect from soay sheep and morphologically typed into three populations consisting of 31 adult male *Teladorsagia davtiani*, 30 adult male *Teladorsagia trifurcata*, and 31 adult male *Teladorsagia circumcincta* by B. Craig. As is was not possible to isolate these numbers of each type from a single host, an equal number of each *Teladorsagia* species was taken from twelve hosts and are listed in the table. The following is a brief description of the history of Soay sheep: St. Kilda is an archipelago of islands including, Soay, Hirta and Boreray, which lies 41 miles (66 kilometers) west of Benbencula in Scotland's Outer Hebrides. The people evacuated Hirta in 1930 along with the Lewis blackface sheep, which are still found on Boreray. Two years later a flock of 107 pure-bred soay sheep including 20 rams, 44 ewes, 22 ram lambs and 21 ewe lambs were brought over to Hirta, from the island of Soay.

Host ID	*Teladorsagia davtiani*	*Teladorsagia trifurcata*	*Teladorsagia circumcincta*
YR514	3	3	3
YO517	7	7	7
NP089	1	1	1
YY557	6	5	5
NW538	2	3	3
YB160	2	2	2
AY058	1	1	1
AY069	1	1	1
NG528	1	1	1
OP553	6	6	6
YL072	-	1	1
AY151	1	-	-

Figure 2.2a: Example of duplicate colony lift to identify putative positive colonies
Red dots show the three key positions for lining up the original and duplicate radiographs of the original and duplicate colony lifts. Blue arrows correspond to positive hybrisation, representing a putative positive colony, present on both the original and duplicate lift. The location of each colony was corresponded back to original transformation plate, using key positions, to be picked and streaked onto fresh plate. Six single colonies picked from each streak plate and minipreps prepared. See Figure 2.2b

Figure 2.2b: Example of Southern blots used to identify plasmids containing microsatellites
EcoRI / HindIII double digested miniprep DNA from each of the six colonies, per putative positive located on the original and duplicate colony lifts, was Southern blotted and hybridised with the $(CA)_{25}$ oligonucleotide probe to identify plasmids containing inserts with microsatellite repeats. A-G corresponds to A-G on the colony lifts in Figure 2.2a, H and I would have come from another screening.

Figure 2.2a: Example of duplicate colony lift to identify putative positive colonies



Original Duplicate

Figure 2.2b: Example of Southern blots used to identify plasmids containing microsatellites

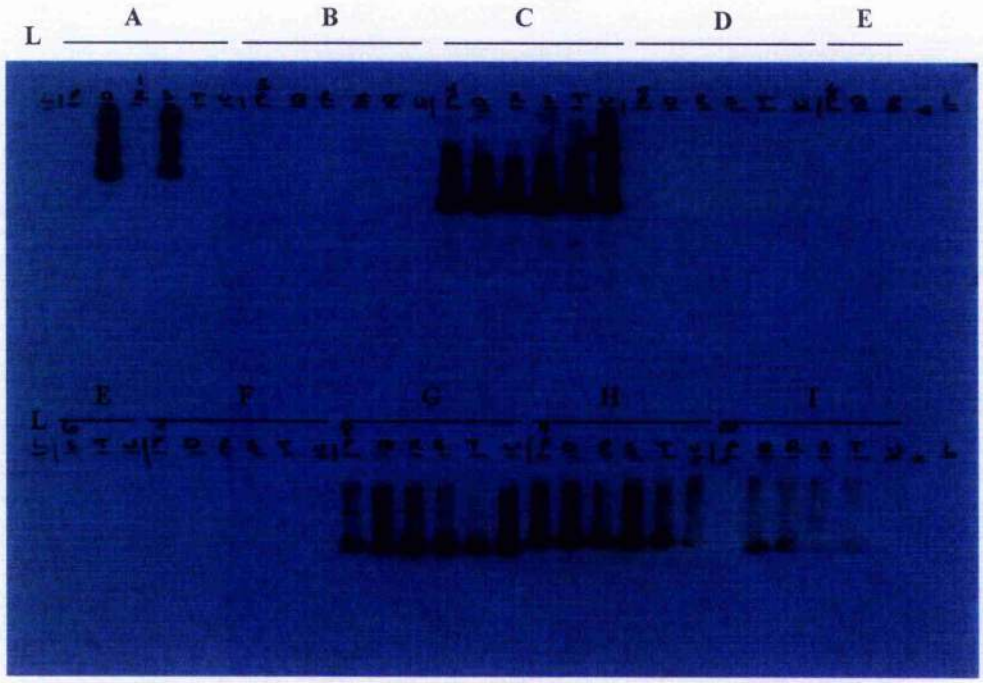


Figure 2.3a: Schematic representation of TecRep probe amplification

Schematic representation of plasmid containing microsatellite MTG 12 and six TecRep repeats. A and B show the position of primers used to amplify TecRep probe, used for subsequent screening.

Figure 2.3b: Testing specificity of TecRep probe using Dot Blot Hybridisation

TecRep probe specificity assayed using Dot Blot hybridisation with the MTG 1b (1.5 μg/μl), MTG 3 (1.8 μg/μl), MTG 12 (150 ng/μl) and MTG 13 (250 ng/μl) using 1 μl of the following dilutions: neat, 1/10, 1/100 and 1/1000 dilutions.

Figure 2.3a: Schematic representation of TecRep probe amplification

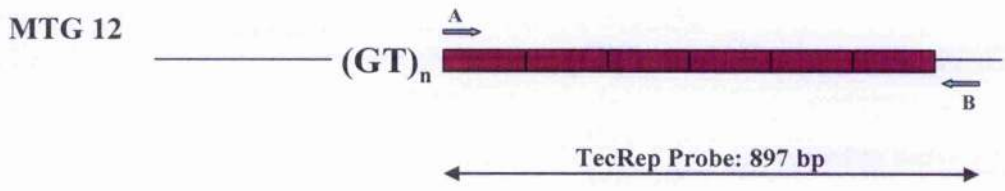
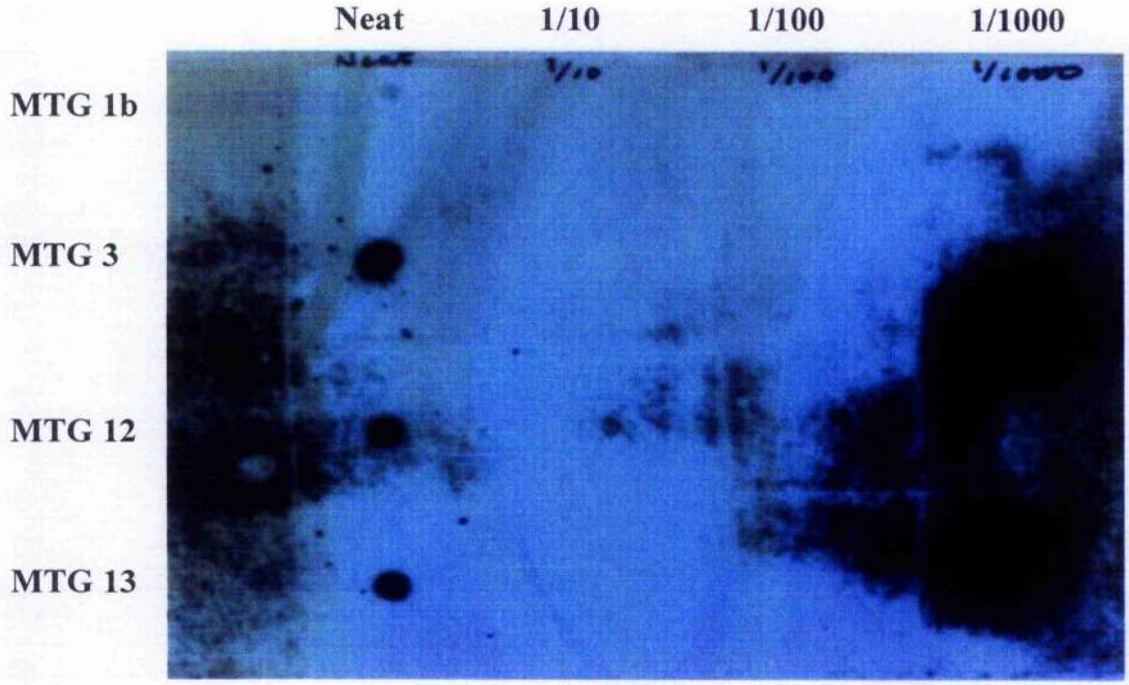


Figure 2.3b: Testing specificity of TecRep probe using Dot Blot Hybridisation



Chapter 3: Anthelmintic Efficacy on U.K. Goat Farms

3.1 Introduction

3.1.1 Anthelmintic resistance in goat parasites

Gastrointestinal parasitic nematodes of sheep and goats are of major economic importance worldwide, including the U.K. Although multiple species of nematode occur as mixed infections, *Teladorsagia circumcincta* is the single most important nematode parasite of sheep and goats in temperate regions such as the U.K. Control of these parasites is reliant on the use of anthelmintics in addition to grazing management. However, the increasing prevalence of resistance to anthelmintics is presenting a serious problem to the sustainability of small ruminant farming, particularly in the southern hemisphere but increasingly in the U.K. (Table 1.1) (Kaplan 2004;McKellar and Jackson 2004). There are three main classes of broad spectrum anthelmintic used to control gastrointestinal parasitic nematodes; the benzimidazoles (BZ), imidazothiazoles / tetrahydropyrimidines (e.g. levamisole) (LEV), and the avermectins (IVM). Resistance of sheep nematodes in the U.K. is predominantly to the benzimidazoles and mainly found in *T. circumcincta* with some reports in *H. contortus* (Hong *et al.* 1992;Hong *et al.* 1996;Jackson and Coop 2000). A recent survey of sheep farms in Scotland, showed that 64 % of farms had evidence of resistance to the BZ anthelmintics (Bartley *et al.* 2003). Furthermore, *T. circumcincta* which show triple resistance have also now been reported and confirmed on two Scottish sheep farms (Bartley *et al.* 2004;Sargison *et al.* 2001) and one sheep farm in England (Yue *et al.* 2003). The results from the surveys which have been conducted in the U.K. suggest resistance is on the rise, most importantly to more than one major class of anthelmintic.

In many countries around the world, drug resistance in sheep nematodes has been preceded by resistance in the same nematode species in goats suggesting that goats may be a source of drug resistant parasites for sheep (Jackson 1993). One survey has shown that the prevalence of benzimidazole resistance in nematodes of goats in the U.K. is higher than in nematodes of sheep (Hong *et al.* 1996). In addition, the first British case of multiple resistance, to benzimidazoles and ivermectin, was reported on a cashmere goat farm in Scotland (Jackson *et al.* 1992a) and triple resistance to ivermectin, benzimidazoles and levamisole was first reported in two commercial angora goat herds in England in 1996 (Coles *et al.* 1996), with the predominant species detected being *Teladorsagia circumcincta*.

There are a number of reasons suggested to explain why nematode parasites of goats develop anthelmintic resistance more rapidly than those of sheep. Firstly, high incidence of rumen by-pass (Sangster *et al.* 1991) and rapid anthelmintic metabolism (Andrews and Fletcher 2001) means the therapeutic dose for goats is higher than that for sheep. This has resulted in goats being commonly under dosed since the majority of anthelmintics are only licensed for sheep and consequently sheep dose rates are often used. Under-dosing reduces the amount of drug available to the internal parasite populations which is believed to allow the greater survival of parasites that are heterozygous for resistance alleles, increasing the rate at which resistance develops. Although there is debate as to the exact interaction of under-dosing and survival of resistant nematodes, there is evidence that under-dosing can have a positive effect on the selection and maintenance of resistant genotypes (Silvestre *et al.* 2001;Smith *et al.* 1999). Specific anthelmintic dosages for goats have been clarified, with recommendations stating that BZ should be used at twice the recommended sheep dosages (Sangster *et al.* 1991) and LEV given at 1.5 times the sheep dose (Jackson 2000). Studies using fibre goats in comparison to sheep, suggest that goats may lack the ability to regulate establishment and rejection of established populations of gastrointestinal parasites by mounting an effective immune response (Huntley *et al.* 1995). This greater susceptibility to nematodes means that goats tend to be dosed more frequently, especially on intensive farms where goats are required to graze rather than browse (Jackson 2000). This management factor leads to higher selection pressure for resistant alleles in goats via increased and more frequent exposure to anthelmintics. The increased risk of anthelmintic resistance in parasite of goats is of practical importance to sheep because many of the same parasite species infect these two ruminant species. Hence there is a significant risk of goats being a source of resistant parasites for sheep if they are co-grazed on the same pasture.

3.1.2 Detection of Anthelmintic Resistance

Populations of parasitic nematodes will harbour both resistant and susceptible individuals. Current anthelmintic resistance detection methods are relativity insensitive. Resistant parasite populations are only detected when they make up more than 25 % of the parasite population being screened (Martin *et al.* 1989). This makes surveillance and diagnosis difficult and inaccurate. The methods used to detect anthelmintic resistance can be divided into *in vivo* and *in vitro* tests.

3.1.2.1 *In vivo* tests

The faecal egg count reduction tests (FECRT) is the "gold standard". It is based on performing a faecal egg count from a group of animals both prior to anthelmintic dosing and at 10 - 14 days post dosing then calculating the percentage reduction in eggs to an untreated control group (Coles *et al.* 1992). Each animal is individually weighed to ensure accurate dosing. This test can be used for all classes of drug, but there is the potential to overestimate drug efficacy due to suppression of egg production by the anthelmintic (Jackson 1993;Martin *et al.* 1985). In addition, this test is labour intensive and not really suitable for field surveys. A less accurate but easier test is the Drench Efficacy Test (DET) which involves comparison of the faecal egg count in eggs per gram (EPG) taken from a group of animals before dosing with an anthelmintic to a second faecal sample taken at approximately 10 - 14 days after the anthelmintic dosing. However unlike the Faecal Egg Count Reduction Test a control group for comparison is not included. Drench efficacy is calculated from pre and post dosing samples using the following equation: 1 - (post dosing EPG / pre dosing EPG) x 100.

3.1.2.2 *In vitro* tests

There are two *in vitro* tests which are most commonly used. These are the egg hatch assay (EHA) and the larval development assay (LDA). The EHA is considered to be a reasonably reliable test for resistance to the benzimidazoles, but not to the other classes of anthelmintic. The egg hatch assay (EHA) is based on the ovicidial activity of benzimidazoles and examines the ability of fresh, undeveloped eggs from a parasite population to embryonate and hatch *in vitro* following exposure to various concentrations of anthelmintic (Condor and Campbell 1995). The larval development assay (LDA) is less reliable but has been used to demonstrate resistance to all three drug classes, although its use for the diagnosis of resistance in field populations has been questioned (Grimshaw *et al.* 1994). This method uses hatched first stage larva, removing the requirement for fresh undeveloped eggs. The proportion of L1 larvae that develop to L3, in the population to be tested, is compared to that of a susceptible population over a range of drug concentrations.

The sensitivity of both of the *in vitro* methods is poor, and resistance can only be detected when at least 25 % of individuals in the parasite population are phenotypically resistant (Martin *et al.* 1989). Consequently there is an urgent need to develop improved diagnostic tests using molecular biology techniques. For example, the ability to study the dynamics of the drug resistance evolution depends on the measurement of allele frequencies which

would require molecular techniques (Anderson *et al.* 1998). However, further research into the underlying mechanisms of resistance is necessary before assays which can detect the frequency of resistance alleles in a parasite population can be developed, which in turn is necessary in order to detect resistance at an early stage. This is crucial to allow interventions and changes in control strategies to slow down the rate at which resistance develops. An understanding of the genetics of drug resistance together with the genetic structure of parasite populations is a prerequisite to understanding the impact of different parasite control strategies on the development of anthelmintic resistance.

3.1.3 Determining the efficacy of anthelmintic usage on U.K. goat farms

As discussed above anthelmintic resistance appears to develop in parasite populations of goats more rapidly than those of sheep (Jackson and Coop 2000). Hence goat parasite populations represent a potential source of resistant parasite genotypes for sheep. A survey of goat farms in the U.K. was carried out in order to assess the general situation in U.K. regarding husbandry, use of anthelmintics and anthelmintic efficacy. The approach was to undertake a small postal survey and obtain faecal samples on which to carry out drench efficacy tests and, where possible, egg hatch assays. It is important to be clear that the data generated from this type of approach is a test of the effectiveness of particular anthelmintic treatments and is not a reliable test of anthelmintic resistance in particular parasite populations. This is because it is impossible to determine whether anthelmintics were administered appropriately and at an accurate dose. At present there are no reliable *in vitro* tests for levamisole or ivermectin resistance from faecal samples collected from the field, but the egg hatch assay is considered to be a reasonably reliable test for benzimidazole resistance. Consequently, where adequate material was available, an egg hatch assay was performed to test for BZ resistance.

In summary the objectives of the work presented in this chapter were:

1. To obtain preliminary data on patterns of anthelmintic usage and of anthelmintic treatment efficacy on U.K. goat farms.

2. Identify farms with potential anthelmintic resistance problems for further investigation.

3. Obtain field samples of *T. circumcincta* for population genetic analysis.

3.2 Results

Letters were sent to members of the SPCA (Scottish Cashmere Producers Association) and several goat clubs, asking for goat farmers willing to participate in the survey. Twenty-eight of the forty-five farms contacted agreed to be involved in the survey. Two farms returned questionnaires but did not send samples, 21 farms sent samples and returned questionnaires and 4 farms sent samples but no questionnaire. Two farms participating in the survey were wildlife parks, the rest were private farms. Twelve farms took part in both 2002 and 2003.

3.2.1 Questionnaire

Twenty-three questionnaires were returned (Appendix A). The majority of goat herds in the U.K. are small and predominantly consist of dairy breeds, as is reflected in this survey. There were also a small number of farms with large goat herds for commercial dairy or fibre production. Not all questionnaires were completed fully and some contained inconsistencies. Therefore the results of the questionnaire were used to obtain a general overview (Figure 3.1).

3.2.2 Drench Efficacy Test and Egg Hatch Assay

Generally, samples where sent in from March to September for 2002 and 2003 depending on the farm's anthelmintic usage regime. A faecal egg count was performed using the McMaster technique performed at a sensitivity of one egg representing 25 eggs per gram by counting two McMaster slides per sample, in order to increase accuracy (See Chapter 2, section 2.1.2). Faecal egg counts were carried out on fresh pre and post dosing samples sent by farmers in the post. Drench efficacy was calculated from pre and post dosing samples using the following equation: 1 - (post dosing EPG / pre dosing EPG) x 100. A faecal egg count reduction of > 95 % is expected if the drug is fully effective, whereas a reduction of < 95 % indicates a lack of drug efficacy. The Drench Efficacy Test only gives an indication of the possible presence of resistant parasites and is not a definitive test, since the accuracy of anthelmintic administration cannot be objectively assessed. Guidelines for dosing including recommended dosages for goats and best technique to avoid oesophageal reflex were sent to each farmer (Appendix B). On follow-up phone conversations, it was noted that some farmers had not read the directions sufficiently carefully and were unaware that goat dosages differed from sheep dosages. The results of the Drench Efficacy Tests are shown in table 3.1 with a simplified table of anthelmintic usage and efficacy in table 3.2a. Three farms were not included in the data, as both the pre and post dosing samples were found to

have zero eggs per gram of faecal matter. In 2002, eight of the fourteen farms for which a drench efficacy test was carried out were found to have poor anthelmintic efficacies ranging from 0 - 95 %. The main anthelmintics being used in 2002 were BZ, LEV and IVM (Table 3.2a). In 2003, ten out of 19 farms showed evidence of poor drench efficacy. Due to evidence of poor drench efficacy, farmers were encouraged to discuss the results with their veterinary surgeon and send pre and post dosing samples for their subsequent anthelmintic treatment, hence multiple Drench Efficacy Tests were carried out for eight farms. More farmers were using moxidectin and eprinomectin in 2003 compared to 2002 (Table 3.2a).

In the first year of the survey (2002), egg extractions (Chapter 2, section 2.1.3) were carried out on anaerobically stored pre and post dosing faecal samples, sent by farmers along with the fresh faecal samples used for the Drench Efficacy Test. An EHA was carried out for eleven farms, where a sufficient number of eggs could be extracted from faecal samples, to test for resistance to benzimidazoles. The aim of the EHA is to incubate the undeveloped nematode eggs in serial concentrations of thiabendazole (a benzimidazole anthelmintic). The percentage of eggs that hatch at each concentration is determined (using the arithmetic mean of the duplicate wells), corrected for natural mortality from control wells, and an ED_{50} estimated using logit model (logistic regression model). The ED_{50} value refers to the concentration of thiabendazole required to prevent 50 % of the eggs hatching. Each sample was classified as resistant (R) to thiabendazole if the estimated ED_{50} value was greater than 0.1 μg/ml or susceptible (S) if it was less than 0.1 μg / ml as recommended by the WAAVP guidelines (Coles *et al.* 1992). The Moredun laboratory strain (Tci1 (MOSI)), which is susceptible to benzimidazoles, was used as a control at regular intervals throughout the sampling period. Examples of graphs showing the corrected percentage hatch plotted against the drug concentration are shown to illustrate a susceptible isolate (Figure 3.2a) and a population from a farm with apparent resistance (Figure 3.2b). Based on the EHA threshold ED_{50} of 0.1 μg/ml, eight of the eleven farms showed evidence of BZ resistance on pre-dosing samples whereas BZ resistance was found on all five samples tested post dosing (Table 3.2b). The concentration of thiabendazole calculated as the ED_{50} for a number of these farms was high (Farm 2 pre-dosing ED_{50} was 0.577). A number of the EHA failed as indicated by failure of eggs in control samples (i.e. incubated without thiabendazole) to hatch after 72 hrs incubation at 22°C. Failure to hatch may be caused be egg exposure to frost (while in the post), owner refrigeration (despite 'Do not refrigerate' instructions), failure of the anaerobic conditions (i.e. pots not closed properly) or a high proportion of

H. contortus eggs in the sample. *H. contortus* eggs are particularly cold sensitive (Dr F. Jackson, pers. comm.).

3.2.3 Molecular identification of *T. circumcincta* in faecal samples

One of the problems that confounds the accuracy of both drench efficacy tests and egg hatch assays is the fact that gastrointestinal parasitic nematode burdens of ruminants are generally comprised of a number of different species. Indeed, a large number of different trichostrongylid nematodes routinely co-infect sheep and goats and the eggs present in faeces are morphologically indistinguishable for each of these species (Urquhart *et al.* 1996). Each of the different nematode species have differing inherent sensitivities to different anthelmintics which can distort the results of both *in vitro* and *in vivo* tests of anthelmintic resistance. It is also worth noting that different nematode species may have different mechanisms conferring resistance (Kohler 2001;Silvestre and Cabaret 2002). At present the standard method of identification of nematode species in faecal samples is to culture eggs to L3 which allows differentiation of species on the basis of morphology. This is a time consuming process and requires an experienced operator for reliable results. Hence there is a need for improved methods for species identification of trichostrongylid nematodes in faecal samples.

In addition one of the aims of the survey described above was to identify *T. circumcincta* populations in order to isolate potential anthelmintic resistant strains and to obtain material for population genetic analysis. Consequently, a test was required to identify *T. circumcincta* larvae within faecal samples containing mixed populations of parasites. Consequently an attempt was made to develop a simple molecular test to identify *T. circumcincta* larvae from samples containing mixed species.

The rDNA cistron has been commonly used for both phylogenetic studies and for developing diagnostic markers in parasitic nematodes (Blaxter *et al.* 1998;Dorris *et al.* 2002;Hoste *et al.* 1998;Stevenson *et al.* 1995). The three rRNA genes - 18S, 5.8S and 28S - are highly conserved between organisms and have been used for phylogenetic studies from the species to the Phylum level. In *C. elegans* there are approximately 55 copies of the rRNA cistron organised in a single tandem array (Ellis *et al.* 1986) and it is presumed a similar situation exists in parasitic nematodes. The different regions of the rRNA cistron evolve at different rates and the more rapidly evolving regions can be used to examine organisms that are more closely phylogenetically related. The transcribed spacer regions ITS-1 and ITS-2 (Figure 3.3a) have been used to discriminate different species of parasitic

nematode including different members of the Trichostronglyus genus (Gasser *et al.* 1994;Stevenson *et al.* 1995;Wimmer *et al.* 2004). The non-transcribed spacer (NTS) is the most rapidly evolving region and the possibility of indels in this region gives the potential for a simple PCR assay to discriminate between species based on the size of a PCR product. For this reason the non-transcribed spacer (NTS) of the rDNA cistron was investigated as a potential target for a PCR amplification to distinguish *T. circumcincta* from other trichostrongylid nematodes.

Primers were designed in order amplify the NTS region of the rRNA cistron from a wide range of species; a sense primer corresponding to sequence at the 3' end of the 28S rRNA gene (primer NTS1) and an anti-sense primer corresponding to sequence at the 5' end of the 18S rRNA (primer NTS3) (Figure 3.3a). These were designed using a sequence alignment of 18S and 28S rRNA genes from *C. elegans, Drosophila melanogaster, Saccaromyces pombe* and *Xenopus laevis* to select highly conserved regions of sequence (Dr J. Gilleard, pers. comm.).

Initial experiments were conducted to investigate the amplification of the NTS region from single adult male worms of *T. circumcincta* and *H. contortus* that had been previously identified by male tail morphology. A consistent banding pattern was produced from all of the initial 20 *T. circumcincta* and 15 *H. contortus* male adult worms tested. Three amplicons (~1500 bp, ~ 800 bp - 700 bp) were consistently amplified from all the single *T. circumcincta* adults (Figure 3.2b) and two amplicons (both between ~ 1400 – 1500 bp) are consistently produced from *H. contortus* (data not shown). No intra-species variation in the banding pattern was seen. A number of monospecific L3 cultures, well characterised for each species morphologically, were obtained from Dr F. Jackson at the Moredun Institute, including *Ostertagi ostertagi, Nematodirus battus, Trichostrongylus colubriformis, Trichostrongylus vitrinus* and *Cooperia oncophora*. Initial experiments were conducted using pooled larval samples, using approximately 100 L3s, to test the NTS primer pair for each species. This experiment was repeated using 5 single L3s for each species. Consistent and repeatable distinguishing products could be produced from *N. battus, T. circumcincta* and *H. contortus* L3s (Figure 3.3c). No consistent PCR products were obtained with *O. ostertagi, T. colubriformis, T. vitrinus* and *C. oncophora*. Subsequent experiments found that the NTS PCR also amplified consistent PCR products with *Haemonchus placei* which were distinct from those of *H. contortus*, (Dr E. Packard and Dr J. Gilleard, pers. comm.). These experiments suggested that the NTS PCR could be used to positively identify individual *T. circumcincta, H. contortus, H. placei* or *N. battus* L3s and adults.

The utility of the NTS PCR was tested on larvae harvested from field samples obtained from the goat farm survey. The faecal samples sent in by participating farms were cultured to allow development of eggs to L3s, and sufficient numbers of larvae for further analysis were obtained from three farms. The guidelines for the identification of larvae advise initial identification using 100 ensheathed larvae into three main groups, 'short' tails, 'medium' tails and 'long' tails. Individuals identified as 'short' tailed could be either *Teladorsagia* or *Trichostrongylus* species and 'medium' tails classified as *Haemonchus* or *Cooperia* species (MAFF 1986). 'Long' tails could be either *Chabertia* or *Oesophagostomum* (MAFF 1986). A further 100 exsheathed larvae are identified to determine the specific species make up of the short and medium tails only. For two of the farms (Farms 5 and 12) 100 ensheathed and 100 exsheathed larvae were examined. However for the other farm (Farm 6) there was only sufficient larvae harvested to examine 100 ensheathed larvae. Hence in this case the individual species could not be determined but the larvae just categorised into 'short', 'medium' or 'long' tails. The relative proportions of the different species present in the larvae samples based on morphological identification are shown in figure 3.4.

Following morphological examination, a small number of larva from the three farms were analysed using the NTS PCR. A total of 11, 20 and 12 individual L3s were lysed from Farm 5, Farm 6 and Farm 12, respectively, to produce DNA templates for molecular analysis using the NTS primer set. A sample of the PCR results and the morphological speciation results are shown in figure 3.4. Generally, the results from the NTS PCR correspond to the results from the larval morphology results. For example, *H. contortus* and *T. circumcincta* are the main species identified using both methods for Farm 5. Due to the low number of larvae typed with the NTS PCR, true comparisons cannot really be made. Interestingly, of the 10 L3s amplified with the NTS PCR, seven seemed to show the same pattern (U^1 - Figure 3.4), suggesting these could represent the same species potentially corresponding to either *T. colubriformis* or *T. vitrinus*, which can not be distinguished at the L3 larval stage. Further work could optimise this PCR for additional species and could make this marker very useful for speciation work. However, it was not possible to explore this in the given time frame.

Figure 3.1: Results from questionnaire

The figure shows the results which could be taken from the questionnaire, as not all questionnaires were completed fully and some contained inconsistencies. The results are based on twenty-three farms. * Percentages do not add up to 100 % because some farms use a combination of anthelmintics (more than one anthelmintic class) for one treatment.

Figure 3.1: Results from questionnaire

Farm Type

68 % Dairy Farms

- 66 % British Saanen
- 33 % British Alpine
- 26 % British Toggenburg

27 % Cashmere Farms
5 % Mixed breeds

Farms with co-grazing

26 %	co-graze with sheep
9 %	co-graze with cattle
13 %	co-graze with Horses/ ponies

Number of goats per farm

10 %	< 3 animals
55 %	3-10 animals
18 %	11-30 animals
10 %	> 30 animals

Drenching Strategy:

50 %	Follow a set anthelmintic routine
27 %	At housing
23 %	At turn out
10 %	When docking / hoof trimming
10 %	Pre-tupping
45 %	Post kidding

Most recent anthelmintic used*

43 %	Class I: Benzimidazoles
35 %	Class II: Levamisoles
39 %	Class III: Avermectins
9 %	Moxidectin

Length of time using present anthelmintic

39 %	1-2 years
22 %	3-5 years
30 %	> 5 years

Anthelmintic previously used on farm

61 %	Class I: Benzimidazoles
48 %	Class II: Levamisoles
69 %	Class III: Avermectins
13 %	Moxidectin

* 1 farm using homeopathic treatment

Number of Anthelmintic treatments per year

14 %	1 x per year
38 %	2-3 x per year
24 %	4-5 x per year
24 %	≥ 6 x per year

Calculation of Anthelminitic Dosage

82 %	Estimate weight of individuals and dose accordingly
5 %	Weigh individuals and dose accordingly
5 %	Weigh individuals and dose according to heaviest animal
8 %	Dose to average estimated weight of group

Pasture Management used for parasite control

67 %	Do not move to clean pasture after dosing as clean pasture often unavailable
24 %	Drench and move to clean pasture
9 %	Drench and move only when possible

Rotation of Anthelmintic Drug Class

14 %	Never rotate
24 %	Rotate with no strategy
24 %	Rotate annually
33 %	Rotate on a > annual basis
5 %	Unsure what to do

Quarantine Drenching:

27 %	Closed herd
68 %	Dose animals when brought onto the farm
5 %	Do not dose animals when brought onto farm
45 %	Keep animals separate with quarantine period (Length of time varied from 1 week to 2 months)
46 %	Specific policy on drug choice for quarantine (57 % IVM, 15 % MOX, 28 % BZ)
53 %	No specific policy

•56 % bring in a billy for breeding

•86 % administer anthelmintics in drenches

•Two farms have confirmed evidence of resistance to Class I (benzimidazole) anthelmintic

Table 3.1: Results for the Drench Efficacy Test for Goat Farms in 2002 and 2003

Summary table of results obtained for the Drench Efficacy Test from goat farms surveyed in 2002 and 2003. First and last column refers to individual farm identity. For each farm the number of goats is shown. The following data shows the faecal egg count (FEC) for the pre and post dosing sample sent in by farmer. Anthelmintic refers to the drug used for dosing and the % DET (drench efficacy percentage) indicated the percent reduction in egg count calculated from pre and post dosing faecal egg counts. A DET < 95 % is considered evidence of poor drench efficacy. Anthelmintic classes were: BZ = Benzimidazoles, LEV = Levamisoles, IVM = Ivermectins. The use of moxidectin and eprinomectin were indicated separately. Eprinomectin is an anthelmintic, licensed for cattle, which is recommended to be used at twice the cattle dosage for goats; CD - cattle dose used, 2 x CD – twice the cattle used. * Farm 6 had a mix of sheep and goats which was tested as one group for the results.

Table 3.1: Results of the Drench Efficacy Test for goat farms in 2002 and 2003

Farm	Number of goats	2002				2003					Farm
		Pre dose FEC	Post dose FEC	Anthelmintic	% DET	Sample	Pre dose FEC	Post dose FEC	Anthelmintic	% DET	
1	22	3000	75	LEV	98 %	Sample 1	400	150	LEV	63%	1
						Sample 2	525	42	IVM	98 %	
2	7	425	125	BZ	72 %		/	/	/	/	2
3	18	550	25	IVM	96 %		100	0	IVM	100 %	3
4	26	300	1800	Homeopathic	Rise		75	950	Homeopathic	0	4
5	8	650	125	LEV	81 %	Sample 1	700	325	LEV	54 %	5
						Sample 2	800	0	Eprinectin	100 %	
6	10*	450	25	BZ	95 %	Sample 1	275	225	IVM	19 %	6
						Sample 2	2850	400	IVM	86 %	
7	37	800	0	IVM	100 %		/	/	/	/	7
8	22	50	0	Moxidectin	100 %		100	0	LEV	100 %	8
9	18	150	50	LEV	75 %	Sample 1	25	750	BZ	0	9
						Sample 2	500	0	Moxidectin	100 %	
10	9	3025	4075	BZ	0		750	0	IVM	100 %	10
11	12	2000	2700	LEV	0		1675	280	LEV	86 %	11
12	60	2000	1850	BZ / LEV	8 %	Sample 1	875	725	Eprinectin (CD)	17 %	12
						Sample 2	725	25	Eprinectin (2 x CD)	97 %	
13	100	1100	675	IVM	39 %	Sample 1	3700	1150	IVM	69 %	13
						Sample 2	2300	800	IVM	66 %	
						Sample 3	500	200	IVM	60 %	
						Sample 4	800	400	IVM	50 %	
						Sample 5	1450	50	Moxidectin	97 %	
14	12	500	0	IVM	100 %	Sample 1	225	375	BZ	0	14
						Sample 2	900	0	IVM	100 %	
15	6	No samples in 2002					150	100	BZ	66 %	15
16	40	No samples in 2002					800	0	IVM	100 %	16
17	9	No samples in 2002					950	25	IVM	99 %	17
18	17	No samples in 2002					125	0	Moxidectin	100 %	18
19	162	No samples in 2002					475	0	Moxidectin	100 %	19
20	10	No samples in 2002					200	0	Moxidectin	100 %	20
21	300	No samples in 2002				Sample 1	350	175	Eprinectin (CD)	50 %	21
						Sample 2	225	0	Moxidectin	100 %	

Figure 3.2a: Egg Hatch Assay (EHA) graph for the susceptible Tci1 (MOSI) isolate

The graph shows the mean percentage hatch taken from duplicate wells plotted against the thiabendazole concentration. Mean percentage hatch of each data point was corrected for natural mortality from the control wells (no thiabendazole). The results from the Moredun Susceptible isolate Tci1 (MOSI) control are shown, demonstrating an $ED_{50} = 0.067$ μg/ml, which is less than the threshold level for detection of resistance of $ED_{50} > 0.1$ μg/ml. Therefore this isolate is susceptible, based on the EHA result.

Figure 3.2b: Egg Hatch Assay (EHA) graph for a sample of eggs from Farm 5 in the survey

The graph shows the mean percentage hatch taken from duplicate wells plotted against the thiabendazole concentration. Mean percentage hatch of each data point was corrected for natural mortality from the control wells (no thiabendazole). The results from Farm 5 are shown, demonstrating an $ED_{50} = 0.304$ μg/ml, which is greater than the threshold level for detection of resistance of $ED_{50} > 0.1$ μg/ml. Therefore Farm 5 parasites are benzimidazole resistant based on the EHA result.

Figure 3.2a: Egg Hatch Assay (EHA) graph for the susceptible Tci1 (MOSI) isolate

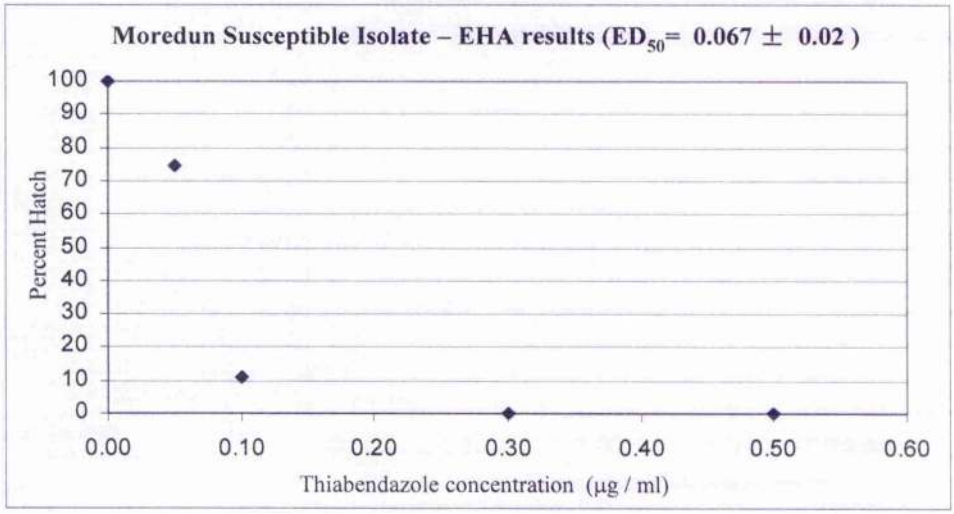


Figure 3.2b: Egg Hatch Assay (EHA) graph for a sample of eggs from Farm 5 in the survey

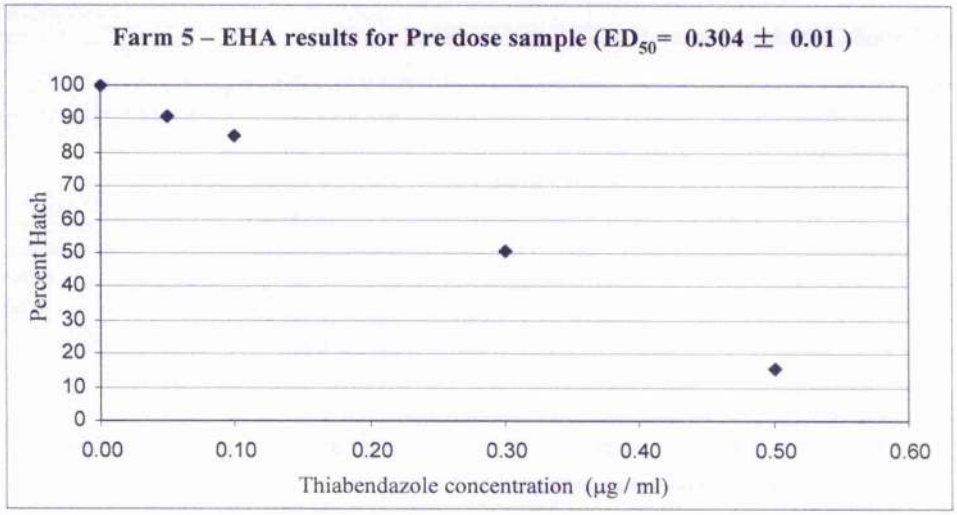


Table 3.2a: Summary of anthelmintic usage and efficacy on U.K. goat farms

This table shows the total number of farms which have used each anthelmintic drug class during the calendar years 2002 and 2003 and the number of farms with anthelmintic efficacy of < 95 % as detected using the Drench Efficacy Test. BZ = Benzimidazoles, LEV = Levamisoles, IVM = Ivermectins. Moxidectin and Eprinomectin are classed separated as discussed in Chapter 3. Other = homeopathic drug treatments. * Some farms used more than one class of anthelmintic per year, however multiple treatments with the same class were not counted. ** Two farms used eprinomectin originally at the cattle dose and found efficacies to be 17 % and 50 % respectively, suggesting poor drug efficacy in these cases to be the result of incorrect dosages.

Table 3.2b: Summary of EHA results

BZ = Benzimidazoles, LEV = Levamisoles, IVM = Ivermectins. N/D = not done. % DET = Drench Efficacy Test: percent reduction in faecal egg count. R = resistance detected to BZ with EHA, S = no resistance detected. ED_{50} ± se (standard error) indicates the concentration of thiabendazole at which 50 % of the eggs failed to hatch (50 % still survive). * Farm 25 only sent one sample and therefore DET could not be estimated.

Table 3.2a: Summary of anthelmintic usage and efficacy on U.K. goat farms

Anthelmintic	2002		2003	
	Number of farms using anthelmintic*	Number of farms with low efficacy	Number of farms using anthelmintic*	Number of farms with low efficacy
BZ	4	4	2	2
LEV	5	4	4	3
IVM	4	1	8	2
Moxidectin	1	0	6	0
Eprinomectin	0	0	2	0**
Other	1	1	1	1
No. of farmers surveyed / No. of farms with low drench efficacy to at least 1 anthelmintic class	14	8	19	10

Table 3.2b: Summary of EHA results

Farm	Pre dose sample		Post dose sample		Anthelmintic	DET %
	Result	ED_{50} ± se (µg/ml)	Result	ED_{50} ± se (µg/ml)		
1	R	0.247 ± 0.01	N/D		LEV	98 %
2	R	0.577 ± 0.05	R	0.332 ± 0.01	BZ	72 %
4	R	0.295 ± 0.01	N/D		homeopathic	0 %
5	R	0.304 ± 0.01	R	0.148 ± 0.01	LEV	81 %
6	R	0.300 ± 0.01	R	0.591 ± 0.04	BZ	95 %
8	S	-0.062 ± 0.07	N/D		MOX	100 %
10	R	0. 277 ± 0.01	N/D		BZ	0 %
12	N/D		R	0.293 ± 0.01	BZ/LEV	8 %
13	S	0.089 ± 0.02	R	0.134 ± 0.01	IVM	39 %
14	R	0.334 ± 0.01	N/D		IVM	100 %
25*	R	0.132 ± 0.01	N/D		N/D	N/D

Figure 3.3a: Schematic diagram of rDNA cistron

Schematic diagram of the ribosomal DNA cistron adapted from Dorris *et al.* 1999, sizes are not to scale. The rDNA cistron is present in approximately 55 copies of the repeat unit per genome in *C. elegans*. The repeat unit consists of the small subunit gene (SSU; 18S), the internal transcribed spacer 1 (ITS-1), the 5.8S, internal transcribed spacer 2 (ITS-2), and the large subunit gene (LSU; 28S). The NTS (non-transcribed spacer) separates each cistron. The figure shows the position of the primers NC1 and NC3, used to amplify the NTS region.

Figure 3.3b: Consistency of products amplified using NTS primers from *T. circumcincta*

The agarose gel shows the consistent banding pattern for *Teladorsagia circumcincta* amplified using the NTS PCR from six individual adult males.

Figure 3.3c: NTS products amplified from three nematodes species

The agarose gel shows the consistent banding pattern amplified using the NTS PCR for the three nematode species: *Teladorsagia circumcincta*, *Haemonchus contortus* and *Nematodirus battus*.

Figure 3.3a: Schematic diagram of rDNA Cistron

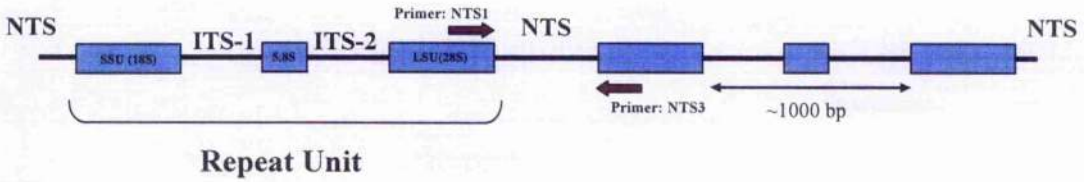


Figure 3.3b: Consistency of products amplified using NTS primers from *T. circumcincta*

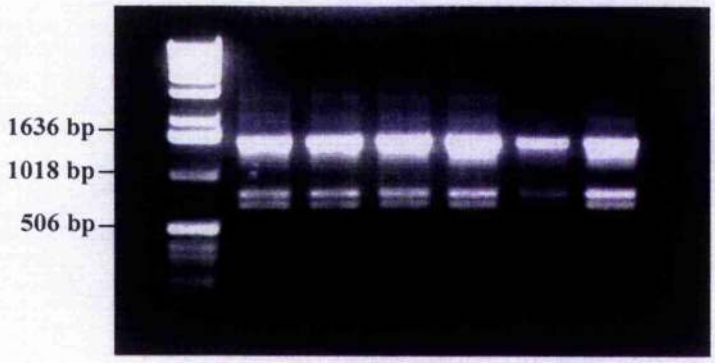


Figure 3.3c: NTS products amplified from three nematode species

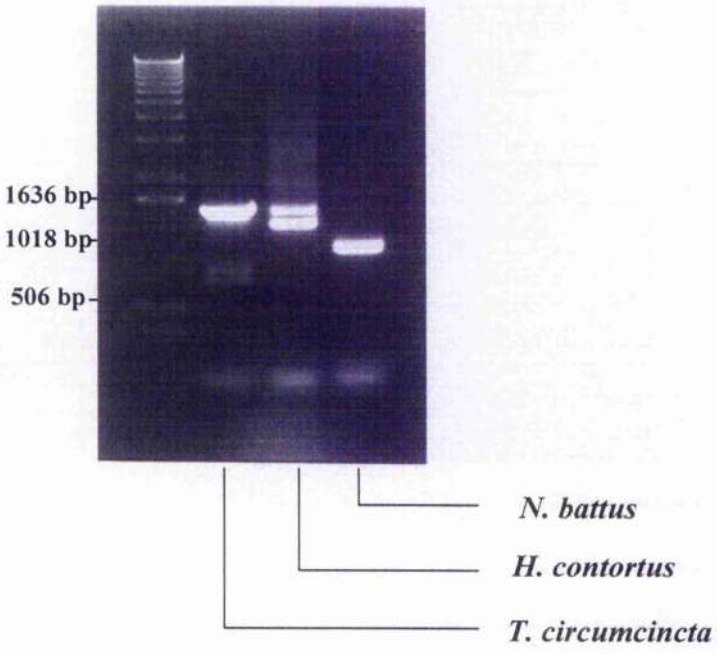


Figure 3.4: Utility of NTS PCR versus larval morphology

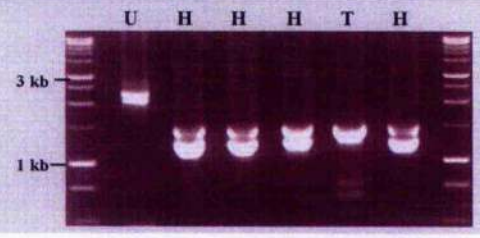
The utility of the NTS PCR was tested on larvae obtained on Farm 5, Farm 6 and Farm 12 and compared to the speciation results found using larval morphology. The actual number of larvae amplified using the NTS PCR is found at the top of the NTS PCR column. The guidelines for the identification of larvae advise initial identification using 100 ensheathed larvae into three main groups, 'short' tails, 'medium' tails and 'long' tails. Individuals identified as 'short' tailed could be either *Teladorsagia* or *Trichostrongylus* species and 'medium' tails classified as *Haemonchus* or *Cooperia* species (MAFF 1986). 'Long' tails could be either *Chabertia* or *Oesophagostomum* (MAFF 1986). A further 100 exsheathed larvae are identified to determine the specific species make up of the short and medium tails only. * larval speciation results based on only 100 ensheathed larvae for Farm 6. ***T. vitrinus* and *T. colubriformis* cannot be distinguished from each other. The agarose gels show a subset of 6 of the 11 larvae amplified for Farm 5, 11 of the 20 larvae for Farm 6 and all 12 amplified for Farm 12 for illustration. T= *T. circumcincta,* H= *H. contortus,* N= *N. battus,* U= unknown species, U^1 = unknown species 1 (all showing the same pattern).

Figure 3.4: Utility of NTS PCR versus larval morphology

Farm 5

NTS PCR 11 individuals typed in total	Larval Morphology 200 individuals examined
5 *H. contortus* 5 *T. circumcincta* 1 Unknown	76 % *Haemonchus contortus* 12 % *Teladorsagia circumcincta* 6 % *Cooperia* 3 % *Chabertia/ Oesphagostum* 2 % *T. colubriformis / T. vitrinus*** 1 % Unknown

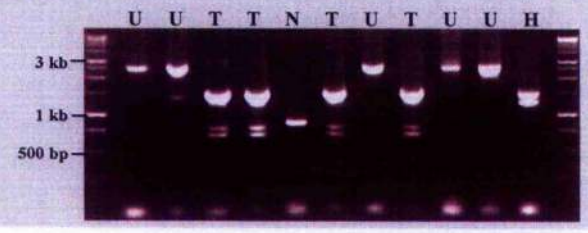
Example of NTS PCR results



Farm 6

NTS PCR 20 individuals typed in total	Larval Morphology* 100 individuals examined*
8 Unknown 9 *T. circumcincta* 2 *H. contortus* 1 *N.battus*	64 % *Chabertia / Oesphagostum* 33 % *Trichostrongylus / Teladorsagia* 3 % *Haemochus / Cooperia*

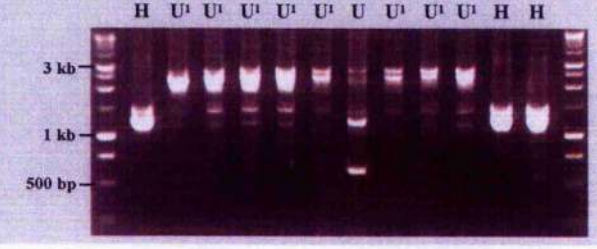
Example of NTS PCR results



Farm 12

NTS PCR 12 individuals typed in total	Larval Morphology 200 individuals examined
9 Unknown species 3 *H. contortus*	89 % *T. colubriformis / T. vitrinus*** 3 % *Teladorsagia circumcincta* 8 % *Haemonchus contortus*

Example of NTS PCR results



3.4 Discussion

3.4.1 Reliability of the drench efficacy and egg hatch assays

This small survey of anthelmintic efficacy of treatments used on U.K. goat farms was performed using the most widely used techniques for this purpose; the Drench Efficacy Test (DET) and the EHA. These were chosen because they are the most practical approaches that are considered to be of any value and the only methods possible for a postal survey. In spite of their widespread use these diagnostic methods have severe limitations of both sensitivity and accuracy and this small survey highlights these. The limitations of each of these techniques will now be considered in turn.

3.4.1.1 Drench Efficacy Test

The faecal egg count reduction tests (FECRT) is the gold standard, based on performing a faecal egg count from a group of animals prior to anthelmintic dosing and at 10 - 14 days post dosing, then comparing the percentage reduction in eggs to an untreated control group (Coles *et al.* 1992). A minimum of 10 days is recommended because samples collected earlier may overestimate drug efficacy due to suppression of egg production by the anthelmintic (Jackson 1993;Martin *et al.* 1985). Each animal is individually weighed to ensure accurate dosing. However, as the FECRT is relatively labour and personnel intensive, a Drench Efficacy Test (DET) was carried out for this survey to allow a larger number of farms to be surveyed by post. The DET involves comparison of the faecal egg count in eggs per gram (EPG) from a group of animals taken before dosing to a second faecal sample at approximately 10 - 14 days after the anthelmintic dosing, without any comparison to a control group. The nature of this testing strategy means that there are a number of variables which are in the hands of the farmer and therefore can not be controlled. Hence, the Drench Efficacy Test is not a definitive test for anthelmintic resistance, but rather a test of drug efficacy, mainly because the accuracy of anthelmintic administration cannot be objectively assessed. Anthelmintic administration includes not only physically dosing the animal but also accurate dosage calculations based on the weight of the animal. In many cases, farmers do not accurately weigh animals to calculate dosages and therefore evidence of poor efficacy could be due to the use of dosages lower than that recommended in the product data sheets. These factors would be avoided in the FECRT, as all animals are weighed and dosed accurately. There are also other factors that may cause the DET to be inaccurate: poor adherence to the details of the instructions for faecal sample collection (e.g. not collecting

samples exactly 10 - 14 days post dosing), or differences in the parasite species on the farm at time of test. In addition, in cases where the mean faecal egg count for the group is lower than 150 epg (eggs per gram) objective assessment of efficacy is not reliable (Coles *et al.* 1992). Also it is worth noting that the Drench Efficacy Test is less sensitive than the Faecal Egg Count Reduction Test which also has an influence on accuracy on the results. Overall, these factors limit the accuracy of quantitative comparison of results among farms.

3.4.1.2 The Egg Hatch Assay

The EHA is the main *in vitro* test used for BZ resistance and can be used to confirm results found using the Drench Efficacy Test when benzimidazoles have been used for dosing. This assay is able to detect resistance only if greater than 25 % of the population is resistant (Martin *et al.* 1989). In order to obtain meaningful results the EHA assay has be to performed on eggs within three hours of being shed from the host or after mass egg extraction from anaerobically stored samples (Coles *et al.* 1992). Therefore, it is difficult to repeat an experiment in a field survey such as this unless additional samples are obtained. In addition, egg mortality increases to greater than 5 % if anaerobic faecal samples are stored for longer than 7 days (Hunt and Taylor 1989). Consequently all samples sent in were processed as soon as possible and always within 7 days of the sampling date, although it was not possible know how long the samples had been stored prior to arriving at the laboratory. Delays between collection and posting of the sample may be responsible for some of the EHA that failed due to poor hatching rates in the control wells. As for the Drench Efficacy Test, the fact that field samples typically consist of mixed species is a major limiting factor to the reliability of the assay. Different species have different inherent sensitivities to benzimidazoles and so the ED_{50} will inevitably vary between individual species. Similarly results obtained from samples taken during different seasons or years may vary because the proportions of the different nematode species will vary. An indication of the potential problems of the EHA was highlighted in a recent study, in which a "ring test" was conducted to investigate inter-laboratory variation of EHA results (von Samson-Himmelstjerna *et al.* 2005). Twelve laboratories from different parts of Europe performed the EHA on the eggs from two isolates of *Haemonchus contortus*; one isolate was benzimidazole susceptible and the other benzimidazole resistant. The eggs were distributed to the laboratories from a central source as anaerobically stored samples. Using a standard protocol, the laboratories tested the two samples blind. The results showed conflicting data, as some labs identified the correct strain as resistant and the others did not. Some conflicting

results were even found when the same thiabendazole stock solution was distributed between laboratories. A number of variables could account for the difference between labs. For example the solvent used to the dilute thiabendazole stock, the source of the water used to fill each well and the subtle variations in operating procedure. This work highlighted the need to standardise the EHA, including ED_{50} threshold levels for resistance. It also highlights the needs to treat EHA results with caution. The results from the EHA and Drench Efficacy Test could only be compared on the three farms where a benzimidazole alone had been used for the treatment. In two of these cases (Farms 2 and 10) the results of the pre-drench EHA and drench efficacy test were in agreement in terms of diagnosing resistance. These had ED_{50} values of 0.577 and 0.277 and Drench efficacy values of 72 % and 0 %, respectively (Table 3.2b). Hence it can be seen that the samples with the highest EHA did not have the lowest drench efficacy as might be expected. Furthermore for one farm (Farm 6) the EHA indicated benzimidazole resistance ($ED_{50} = 0.300$) but the drench efficacy was 95 % (Table 3.2b). This could be argued to be the greater sensitivity of the EHA but may just reflect the general inaccuracies of the two tests. Also for the two farms that had EHA performed on a pre-dose sample and then on a post dose sample following benzimidazole treatment one might expect an increase in the ED_{50} the post-dose sample. This is due to selection for survival of resistant genotypes by the anthelmintic dosing. However whilst this was true for one farm (Farm 6) the opposite was true for the other (Farm 2). In summary, it is important to use the EHA results, particularly when performed on single samples, as a cautious indication of benzimidazole resistance rather than a definitive diagnostic test or an accurate quantitative assessment of a resistance phenotype.

3.4.2 Efficacy of anthelmintic treatments on UK goat farms.

For the reasons discussed above, the results of a survey of the sort presented in this chapter must be interpreted with caution. Nevertheless the results highlight a number of issues worthy of discussion.

3.4.2.1 General observations

The results of the Drench efficacy tests and EHAs were communicated back to the farmers in writing and this often led to follow up telephone discussions. It was apparent that although farmers were generally aware of parasites being a major problem, and were keen to learn more, most were less aware about anthelmintic resistance and the appropriate use of anthelmintics to minimise this problem. Confusion over particular strategies i.e. dose and

move, rotation of pasture, rotation of anthelmintic and frequency of and appropriate timing of dosing, were also apparent. However, in general, those farms with poor understanding of parasite control did not necessarily correspond to farms with poor drench efficacies. The results from the survey showed that only 50 % followed a set anthelmintic dosing routine. This is similar to results from Denmark, where 49 % of farms did not follow a set drenching routine (Maingi *et al.* 1996). It is also noteworthy that the high faecal egg counts generally obtained from the pre-dose faecal samples on many farms suggest parasite control schemes are not very effective on most of the goat farms examined (Table 3.1).

3.4.2.2 Why is anthelmintic treatment efficacy so poor?

Drench Efficacy Tests demonstrated that anthelmintic treatments used were often of poor efficacy, in particular for BZs and LEVs, which is of major concern. There are two possible reasons for the poor efficacies seen; it is either due to the presence of anthelmintic resistant parasites and / or alternatively inappropriate administration or dosing with anthelmintic. The presence of benzimidazole resistant strains is supported by the results from the egg hatch assays, albeit with the caveats outlined above, which detected benzimidazole resistance on eight of the ten farms tested. Hence it appears that benzimidazole resistance is well established in the U.K. goat populations. This supports both anecdotal evidence from farmers and veterinarians and is consistent with findings from a survey of six fibre goat farms in Scotland, of which five had evidence of BZ resistance based on Faecal Egg Count Reduction Test and EHAs (Little 1990). However due to insufficient *in vitro* tests for the macrocyclic lactones and levamisole, it is not possible to distinguish between poor drenching practice and anthelmintic resistance as a cause of poor drench efficacy for these drugs. Even if the poor efficacy is due to poor dosing procedures this is still a major concern. Under-dosing is thought to be a predisposing factor for the development of anthelmintic resistance (Silvestre *et al.* 2001;Smith *et al.* 1999), by allowing the survival of heterozygous resistant individuals which in turn allows the accumulation of resistant alleles in the population (Jackson and Coop 2000). Under-dosing can occur in a number of ways. Firstly, this can occur due to inaccurate weighting of animals or poor estimations of individual animal weights. Secondly, this might be due to farmers using sheep dosage rates that are insufficient for goats. Thirdly, unintentional under-dosing could occur due to the physiological occurrence of the oesophageal reflex, causing the drug to by-pass the rumen and rendering it less effective. It is interesting to note that 90 % of farmers in the survey did not weigh any animals prior to dosing (Figure 3.1). This is similar to results found in

Demark where 69 % of 89 goats farms surveyed, estimated weights were used for anthelmintic dosage calculations (Maingi *et al.* 1996). In an Australian survey, 86 % of farmers under estimated the weight of sheep when tested (Condor and Campbell 1995). From conversations with farmers, the main reasons for estimating rather than weighing were both the time involved and the cost implications of having a weighing machine, especially on farms with very few animals. Furthermore, although not asked in the questionnaire, it was apparent from correspondence that many farmers did not know that dose rates for some anthelmintics differed between sheep and goats. In a New Zealand postal survey, it was reported that 41 % of goat farmers were under-dosing by using sheep dose rates (Pearson and MacKenzie 1986). An additional issue was that farmers often did not know which anthelmintic class the particular product they were using belonged. This confusion about drug classification has been previously noted by Sangster (1999) and proposed that drug classes should have internationally recognisable labelling (e.g. all avermectins labelled 'A' ; all BZ label 'B' etc).

3.4.2.3 Anthelmintic practices potentially selecting for resistance

Frequent dosing with anthelmintics can promote resistance (Condor and Campbell 1995), providing intense selection pressure on parasite populations. The results from the survey revealed that many farms (86 %) dose more frequently than twice a year with 48 % dosing greater than four times a year. The mean annual frequency was 3.7 which is higher than the mean annual frequency for sheep in Scotland of 2.7 (Bartley *et al.* 2003) and of goats in Denmark of 3.0 (Maingi *et al.* 1996). Although not directly asked in the questionnaire, some farmers stated that they dosed all their goats as regularly as every 6 weeks throughout the year. This is an extremely high frequency of dosing with no strategy and would provide an extreme selection pressure for anthelmintic resistant alleles.

Comparison of the anthelmintics used in 2002 to 2003, show a marked increase in the number of farms using Ivermectin, Eprinomectin and Moxidectin. Although three of the six farms that used moxidectin in 2003 did so in response to feedback of the results of poor efficacy from the survey in 2002, the other three farms were new to the survey in 2003. Although this is a small survey this might suggest some shift in favour of macocyclic lactone use. This again is of concern since moxidectin in particular is the last broad spectrum anthelmintic to which there are no confirmed reports of resistance in the U.K.

An additional observation causing concern was that 53 % of farmers had no set quarantine policy with 32 % reporting not to have dosed animals brought onto the farm. These results are similar to a survey of sheep farms in Scotland, in which 34 % of farms did not dose animals on arrival to the farm (Sargison and Scott 2003). Appropriate quarantine methods, involving dosing with ivermectin, moxidectin or a sequential combination of two different classes followed by withholding sheep from pastures for 24 - 48 hrs is considered to be an important precaution to stop the spread of anthelmintic resistance (Abbott *et al.* 2004).

3.4.3 Molecular identification of nematode species

3.4.3.1 Need for speciation PCR

Accurate identification of gastro-intestinal nematode species is essential for diagnosis, treatment and control of infections. A large number of different trichostrongylid nematodes routinely infect sheep and goats. The eggs present in faeces are morphologically indistinguishable for each of these species, except in the case of *Nematodirus battus*, the eggs of which are significantly larger than eggs from the other trichostrongylid species. Different nematode species show variation in the level of pathogenecity. For example, *H. contortus* is a particularly pathogenetic parasitic nematode, causing severe anaemia in infected animals and so warrants early specific diagnosis. Furthermore, each of the different species have differing inherent sensitivities to different anthelmintics which can distort the results of both *in vitro* and *in vivo* tests of anthelmintic resistance. This is a major limitation to the sensitivity and accuracy of these tests. A speciation technique applicable to eggs extracted from faecal samples could also be used to minimise the number of anthelmintic treatments by targeting only those animals with the most pathogenic species (Zarlenga *et al.* 2001). The ability to use drug treatments more strategically would directly decrease the selective pressure of frequent anthelmintic treatment of the entire herd and therefore decrease the pressures which inherently lead to the development of resistance. In addition, identification methods could be used in conjunction with *in vitro* or *in vivo* assays of anthelmintic resistance, to specifically identify which species are resistant in the population. At present the standard method of identification of nematode species in faecal samples is to culture eggs to L3 which allows differentiation of species on the basis of morphology. This is a time consuming process and requires an experienced operator for reliable results. In addition to these diagnostic applications, a molecular test to reliably identify individual species from eggs and larvae extracted from faecal samples would be an extremely valuable tool for population genetic studies on these parasites.

3.4.3.2 Development of species-specific PCR for *T. circumcincta*

As discussed above, there are many different applications for molecular based methods of species identification of trichostrongylid nematode larvae. However, the primary motivation in this project was for use in population genetic studies. The specific objective was to develop a simple PCR to identify *T. circumcincta* larvae from faecal samples which contain multiple trichostrongylid nematode species. Primers designed to the non-transcribed region (NTS) of the rDNA cistron were found to be consistent and robust in identifying not only *T. circumcincta,* but also *H. contortus, H. placei* and *N. battus,* based on unique banding patterns for each of these species. The identification of *N. battus* is not particularly useful as the eggs and larval stages of this parasite are easily distinguished based on morphological characteristics. However, this PCR is very practical for the identification of *T. circumcincta* and *H. contortus*, from sheep or goat faecal samples. This PCR is rapid and specific, with increased sensitivity due to amplification from a repetitive region of DNA. The utility of this PCR was demonstrated by a small pilot investigation of samples obtained from three farms in the survey. The relative numbers of these two species identified by the PCR albeit on small numbers tested, broadly corresponded (Spearman Rank Correlation; $r = 0.79$, p-value $= 0.048$) to the proportions of each identified using morphological criteria. Although, this needs to be investigated further by NTS-PCR analysis of at least 100 L3s. This PCR was also found to be useful in distinguishing between *H. contortus* and *H. placei* in laboratory studies, and is more convenient than the two step RFLP-PCR techniques described by Stevenson *et al.* (1995) (Dr J. Gilleard and Dr E. Packard, pers. comm.). It is interesting to note that this result is consistent with earlier work from Zarlenga *et al.* (2001), which when amplifying a 176 bp product from the NTS rDNA region found *H. contortus* to have a doublet band and *H. placei* to have a single band, distinguishing these species. The main advantage of this PCR stems from the use of one primer set and visualising size differences easily seen on agarose gel, making this a simple, sensitive, rapid and relatively cheap technique. Being able to differentiate between species using one primer set is a major advantage over multiple step assays. However, the use of this PCR is restricted to four species since a number of other trichostrongylid nematode larvae failed to amplify with the primers. It seems unlikely that this amplification failure is due to sequence polymorphisms in the primer sites since the primers were designed to well conserved regions of the 18S and 28S rDNA sub unit genes. Instead it seems more likely that the failure of amplification could be due to size polymorphisms of the NTS region or differences in the efficiency of DNA lysis between species. If this is the case, further optimisation might be possible to

enable these additional species to be identified which would make this assay extremely valuable for identification of individual species in faecal samples.

3.4.3.2 Other Molecular methods available for identification of trichostrongylid nematode species in faecal samples.

A number of investigations have been made into finding a simple and efficient method of speciation for gastro-intestinal nematode eggs and L3s from sheep, goats and cattle (Colditz *et al.* 2002;Gasser and Hoste 1995;Newton *et al.* 1998;Silvestre and Humbert 2000;Wimmer *et al.* 2004;Zarlenga *et al.* 2001). The majority of these studies have made use of the abundant ribosomal genes (Newton *et al.* 1998;Wimmer *et al.* 2004;Zarlenga *et al.* 2001) whereas others have investigated the use of species specific lectin binding characteristics of eggs (Colditz *et al.* 2002). There are numerous studies concentrating on distinguishing between species of the same genus (Gasser and Hoste 1995;Leignel *et al.* 1997;Stevenson *et al.* 1995). However in order to have a useful assay for diagnostic purposes, these techniques have been broadened to include a number of genus groups, moving towards the identification of all parasitic nematode species likely to be present in sheep and goats (Newton *et al.* 1998;Silvestre and Humbert 2000;Wimmer *et al.* 2004). Newton *et al.* (1998) were able to differentiate between 12 sheep parasitic nematode species (*H. contortus, T. circumcincta, T. vitrinus, C. curticei, N. battus, N. filicollis, N. spathiger, Chabertia ovina, Oesphagostumum columbianum* and *O. venulosum*) using a PCR-RFLP technique, amplifying the rDNA region spanning the ITS-1, 5.8S rRNA gene and ITS-2 followed by digestion using either of the restriction enzymes, *NlaIII* or *RsaI*. This technique is specific and simple, distinguishing between eggs of the main pathogenic species infecting sheep and goats. Silvestre and Humbert (2000) used the same technique of PCR-RFLP, using a set of primers, common to *T. circumcincta, H. contortus* and *T. colubriformis*, to amplify the isotype 1 β-tubulin gene followed by digestion with the restriction enzyme *RsaI*. The three species could be easily distinguished by their restriction patterns on agarose gel electrophoresis. The PCR-RFLP techniques used by both authors offer the advantage of a common set of primers for species being distinguished; however the multiple steps make this technique relatively cumbersome and slow. A recent study was published during the work described in this thesis, subsequent to the development of the NTS markers described above (Wimmer *et al.* 2004). Species specific primer pairs, amplifying a fragment of the ITS-2 rDNA region, were designed for the following nine species; *Bunostomum trigonocephalum, Dictyocaulus filaria, Trichostrongylus axei, Trichostrongylus vitrinus,*

Trichuris ovis, *Nematodirus battus*, *Nematodirus filicollis*, *Chabertia ovina* and *Teladorsagia circumcincta*. One primer of each pair encompassed a single nucleotide polymorphism (SNP) that was specific to a single species. The resulting ITS-2 PCRs were found to be sensitive and specific, with no cross-amplification between species. This method is very useful for identifying individual species present in an infected host particularly since the primer sets cover most of the major nematode species commonly found in sheep in temperate regions. The only disadvantage is that the technique requires nine individual PCR reactions to be performed on the same sample to identify the nine species covered. Nevertheless this set of primers provides the most comprehensive molecular diagnostic test to speciate trichostrongylid larvae to date. The primers described by Wimmer *et al.* (2004) and the NTS PCR developed in this chapter have both been invaluable in confirming the identity of individual *T. circumcincta* worms and parasite lysates in the population genetic studies presented in Chapters 4 and 5.

Chapter 4: Development of microsatellite markers for *T. circumcincta* population genetics.

4.1 Introduction

Microsatellites are repetitive sequences consisting of 2 - 3 base pair tandem repeats. They are found in both prokaryotes and eukaryotes, including humans, and appear to be scattered throughout the genome (Bennett 2000). These repetitive sequences are highly polymorphic, due to variation in the number of repeats present (Tautz 1989). Due to their high variability, they have been well documented as powerful population genetic markers (Zane *et al.* 2002). This polymorphism is thought to originate from slippage events during DNA replication (Zane *et al.* 2002). Due to the nature of the sequence, unpaired DNA strands may loop out, resulting in the loss or gain of repeat units. In most cases, the cell's DNA mismatch repair system corrects any mutations caused by slippage and so the observed rate of microsatellite mutation represents the net result of slippage and mismatch repair (Bennett 2000). Single microsatellite loci can be amplified by PCR using primers designed to flanking sequence and the resulting amplicons can be visualised by acrylamide gel or capillary electrophoresis allowing the variation in repeat number between different alleles to be detected (Schlotterer 1998). Microsatellites are found both in coding and non-coding sequence throughout the genome and are generally considered to be neutral markers. There have been relatively few studies on microsatellites in nematodes. The most detailed information comes from studies in the free-living nematode *Caenorhabditis elegans* due to the availability of its full genome sequence. A study by Toth *et al.* (2000), which studied the abundance of microsatellites in several eukaryotic groups found that in *C. elegans*, CA and GA were the most abundant dinucleotide repeats (7.5 per Mb and 10 per Mb, respectively) and, overall, dinucleotide repeats were more abundant than other repeats. From the relatively small number of studies on parasitic nematodes, the majority of microsatellites used as population genetic markers are dinucleotide repeats (Anderson *et al.* 2003;Hoekstra *et al.* 1997;Otsen *et al.* 2000). Three of the five microsatellites available for *Ascaris lumbricoides*, a parasitic nematode of humans, are CA repeats (Anderson *et al.* 2003). Attempts to isolate microsatellites from *H. contortus*, a nematode closely related to *T. circumcincta*, resulted in 59 CA / GT dinucleotide repeats being isolated, despite libraries being probed with $(CT)_{15}CC$, $(CT)_{25}$, and $(TA)_{25}$ oligonucleotide probes (Hoekstra *et al.* 1997;Otsen *et al.* 2000). For organisms where significant amounts of genome sequence are available, microsatellites can be identified using bioinformatics. This provides large numbers of microsatellites from which

to choose population genetic markers. The criteria used to select potential markers include the level of perfection of the microsatellite repeat and repeat length. More perfect and larger repeats are generally considered most likely to be polymorphic. However, since there is little genome sequence available for *T. circumcincta*, molecular biology approaches are needed to isolate microsatellites.

One common molecular approach to microsatellite isolation is hybridisation screening of small insert genomic libraries. This is appropriate for isolating a limited number of dinucleotide repeats from genomes in which repeats are abundant (Schlotterer 1998). For organisms with genomes in which microsatellites are less abundant, it is possible to enrich small insert libraries for repetitive sequences. However previous work from the trichostrongylid nematode *H. contortus* found dinucleotide repeats to be relatively abundant which suggested that library enrichment might not be necessary for the related nematode *T. circumcincta* (Hoekstra *et al.* 1997;Otsen *et al.* 2000). Another approach is to isolate orthologous microsatellites using primers designed to sequences flanking microsatellites already identified from a closely related species. For example, 127 out of 173 (73.4 %) and 129 out of 174 (74.1 %) primer pairs corresponding to sequence flanking bovine microsatellites could amplify products from sheep and deer, respectively (Slate *et al.* 1998). Similarly, it has been reported that approximately 25 % of human primer pairs detect polymorphic microsatellites in baboons (Rogers *et al.* 2000). Consequently, since microsatellites have already been identified from *H. contortus,* this approach has potential for isolating microsatellites from *T. circumcincta.* A final approach to microsatellite identification is to search EST databases. Various studies have found polymorphic microsatellite markers suitable for population genetic studies using available EST sequences; including plants (Thiel *et al.* 2003;Yu *et al.* 2004), invertebrates (Li *et al.* 2004;Walton *et al.* 2004) and vertebrates (DeSilva *et al.* 2003;Rohrer *et al.* 2002). Since there are approximately 4,000 EST's available for *T. circumcincta* this represents another potential approach.

This chapter presents the results of three approaches used to isolate microsatellites from *T. circumcincta.* The first approach used hybridisation screening of small insert genomic libraries; the second approach involved using sequence flanking microsatellites already isolated from *H. contortus* to amplify orthologues from *T. circumcincta,* and the third approach involved screening a *T. circumcincta* EST dataset for repetitive sequences. This

chapter also describes the characterisation of the microsatellites isolated and the determination of their suitability as population genetic markers.

4.2 Results

4.2.1 Isolation of microsatellites from *T. circumcincta* genomic libraries

4.2.1.1 Screening a small insert genomic library

A short insert *T. circumcincta* genomic library was made by ligating *EcoRI* and *HindIII* digested genomic DNA into pBluescript II SK(+). An initial 3 - 4,000 colonies were screened from duplicate colony lifts and hybridised with a $(CA)_{25}$ oligonucleotide probe using a final wash stringency of 2 X SSC / 47°C. Miniprep DNA from twenty putative positives was digested with *EcoRI* and *HindIII* (BDH), Southern blotted and probed with the original $(CA)_{25}$ oligonucleotide probe using the same stringency in order to confirm the presence of a CA repeat sequence. Nineteen of the twenty clones remained positive on the Southern blot (Figure 4.1) and contained inserts ranging from 0.5 kb to 3 kb. The inserts of seventeen of the positive clones were fully sequenced and all were found to include CA / GT dinucleotide repeats (Table 4.1). These ranged from 11 - 40 GT repeats of variable perfection. The inserts of the remaining two clones were only sequenced using vector primers because they had inserts larger than 3 kb; full sequencing was consequently considered too time consuming, relative to isolating additional clones with smaller inserts, in the event that more microsatellites were needed.

All repeats were orientated in GT polarity to facilitate comparative analysis (Table 4.1). All the microsatellite loci that were isolated and sequenced consisted of several stretches of GT nucleotide repeats interspersed with short regions of non-repetitive sequence (up to 11 bp in length). MTG 8 was the only exception being $(GT)_{11}$. The total length of sequence encompassing the GT repeats at each loci varied from 21 bp to 80 bp. The copy number of GT dinucleotide repeats varies from 11 to 40 GT units at each locus with the longest stretch of perfect dinucleotide repeats being $(GT)_{31}$ in MTG 7. Six of the loci also contained the tetranucleotide repeat (GTCT) interspersed with the GT repeats, for example MTG 3 comprises $(GTCT)_5(GT)_{11}$.

Each sequence was aligned against itself using Dot plot analysis to investigate the presence of any additional repeat sequences using Vector NTI (Invitrogen). Dot plot analysis revealed that ten of the seventeen microsatellite loci that were sequenced were associated with an

additional tandem repetitive sequence of 146 bp in length, which we have called TecRep (Figures 4.2, 4.3a and 4.3b). These TecRep tandem repeats were, in all cases, located immediately downstream of the microsatellite when in GT (5'- 3') orientation with the first repeat unit being within 2 bp of the final GT dinucleotide unit (Figure 4.3a). The number of TecRep repeats associated with each individual microsatellite varied from one to six repeats although in several cases the precise copy number cannot be determined since the plasmid inserts do not extend beyond the repetitive region (Figure 4.3b).

4.2.1.2 Investigation of TecRep repeats

Alignment of the repeat units with each other shows there is polymorphism both within and between loci (Figure 4.4). The sequence identity between repeats varies from 96.6 % identity (MTG 14 repeat 2 and MTG 14 repeat 3) to 50.4 % identity (MTG 13 repeat 2 and MTG 14 repeat 4). Repeats in the same locus were generally more similar to each other than repeats between loci although this was not exclusively the case (Figure 4.4) and there tends to be a decrease in similarity between repeats the further downstream they are (data not shown).

The 146 bp TecRep repeat does not appear to have any significant identity to another repetitive element, OcRep, previously isolated from *T. circumcincta* (Callaghan and Beh 1996). However it has clear similarity with repetitive elements found within the genomes of two other trichostrongylid nematodes; a 145 bp element from *Trichostrongylus colubriformis* (TcRep) (Callaghan and Beh 1994) and a 128 bp element from *Haemonchus contortus* (HcRep) (Hoekstra *et al.* 1997). The consensus sequence of TecRep is 64.2 % identical to the *T. colubriformis* TcRep element and 54.6 % identical to the *H. contortus* HcRep element (Figure 4.5a and 4.5b). Both TcRep and HcRep have been shown to be multi-copy and randomly distributed in their respective genomes (Callaghan and Beh 1994;Hoekstra *et al.* 1997). Both HcRep and TcRep are also located immediately downstream of GT microsatellites (Figure 4.5a). HcRep was identified in a similar fashion to that described here for TecRep, i.e. as an incidental finding during isolation of GT microsatellites from *H. contortus*. From 71 microsatellites isolated from *H. contortus*, 34 (48 %) were found to be associated with this tandem repetitive sequence, HcRep (Hoekstra *et al.* 1997;Otsen *et al.* 2000). TcRep was isolated from a screen for repetitive elements in the *T. colubriformis* genome in which genomic libraries were hybridised with radio labelled genomic DNA (Callaghan and Beh 1994).

The sequence of each TecRep repeat was aligned with 16 different HcRep sequences from *H. contortus* (Hoekstra *et al.* 1997) and 5 different TcRep sequences from *T. colubriformis* (Callaghan and Beh 1994) and a bootstrapped Neighbour-Joining tree was constructed using ClustalX (Thompson et al. 1997) (Figure 4.6). The repeat sequences of each species cluster separately, with the *T. circumcincta* sequences being more closely related to the *T. colubriformis* sequences than either are to *H. contortus*. The neighbour-joining tree in addition to alignments between TecRep and HcRep or TcRep, suggest that *T. circumcincta* is more closely related to *T. colubriformis* than it is to *H. contortus*. This conflicts with phylogenetic results based on 22 morphological characteristics (Hoberg and Lichtenfels 1994) and more recent results based on the sequences of D1 and D2 domains of the 28S rRNA (Gouy de Bellocq *et al.* 2001). Yet, the evolutionary relationships within the trichostrongylidae nematodes could not be adequately resolved using ITS-2 rDNA sequences, as none of the trees produced had a topology similar to trees derived using morphological data (Chilton *et al.* 2001). Although neither of these more recent studies could adequately resolve the exact phylogenetic relationships within the trichostrongylidae, the debate is still ongoing as how best to clarify these evolutionary relationships. It can also be seen from the neighbour-joining tree that different repeat units of the same tandem array are often more similar to each other than to repeat units in other tandem arrays although this is not always the case.

The presence of the TecRep repeat could potentially create problems for using associated microsatellites as population genetic markers since its presence could compromise the ability to design primers that are specific to a single locus. In addition, variation in TecRep repeat number at a locus could lead to size polymorphisms in addition to the differences in dinucleotide repeat number. In order to investigate the nature of the size polymorphisms of the microsatellites associated with the TecRep repeat, primer pairs were designed for six of these markers (MTG 1a, MTG 1c, MTG 6, MTG 11, MTG 14 and MTG 18). PCRs were performed with each primer pair using genomic DNA as template prepared from a population of worms from the Tci1 (MOSI) *T. circumcincta* isolate (Table 2.1-2.2) and the optimum annealing temperature was determined using a series of temperatures across a gradient PCR block of 45.5° C, 47.6°C, 49.7°C, 55.6°C, 58°C, 60.4°C and 64.5°C. All markers gave products of the size expected from sequence data and were subsequently tested on a panel of lysates prepared from single adult male *T. circumcincta* from the ScSo529 population (Table 2.1-2.2). Five of the markers showed size polymorphisms that were larger than would be predicted from simple variation of the GT dinucleotide repeat

number and / or amplified more than two bands from single worms (Figure 4.7). Only MTG 11 showed no unexpected size polymorphisms from the five single worms tested. In order to investigate the nature of the size polymorphisms, 5 alleles from MTG 1a, ranging in size from 200 bp to 1,000 bp, were cloned into pCR®2.1 TA cloning vector (Invitrogen) and sequenced (Figure 4.8a and 4.8b). The variation in the sequence between these different alleles involved a combination of polymorphisms within the GT dinucleotide repeats, the sequence and copy number of the TecRep repeat, SNPs within the flanking sequence and the presence of indels immediately upstream of the dinucleotide repeat. The complex nature of this variation and the possibility that individual amplicons are not truly allelic make this locus unsuitable for population genetic analysis. Consequently, it was decided that microsatellites associated with the TecRep repeat would not be used as population genetic markers and that further screens for additional markers should seek to avoid isolating such markers.

A second screening of 6,000 - 8,000 colonies of the small genomic *T. circumcincta* insert library was performed using the same $(CA)_{25}$ oligonucleotide probe and hybridisation conditions as previously described. Southern blotting and hybridisation of miniprep DNA from 26 putative positive clones with the same $(CA)_{25}$ oligonucleotide confirmed the presence of microsatellite sequence in all cases (Figure 4.9a). In order to avoid sequencing microsatellites associated with the TecRep repeat, a duplicate Southern blot was probed with a 897 bp probe containing 6 copies of the TecRep sequence using a final wash stringency of 6 X SSC / 42°C (Figure 4.9b). Eighteen of the twenty six sequences hybridised to the TecRep probe and these were discarded from further analysis. The inserts of five of the remaining plasmids, which ranged in size from 0.6 kb to 6 kb, were sequenced and a GT microsatellite was found in four cases. Dot plot analysis revealed no repeat sequences additional to the dinucleotide repeats confirming the success of the strategy for avoiding the TecRep repeat.

In summary, a total of approximately 9,000 – 12,000 colonies of a short insert *T. circumcincta* genomic library were screened with a $(CA)_{25}$ oligonucleotide probe. This yielded 45 hybridising clones confirmed as positive by Southern blotting; 19 from the first screening and 26 from the second screening. Twenty-eight positive clones were found to be associated with the tandem repetitive sequence TecRep, from sequence analysis or Southern blot, and therefore discarded. From the remaining seventeen putative positives, eleven were

sequenced, analysed and confirmed to have GT / CA microsatellites with no additional associated repetitive sequence.

4.2.1.3 Primer design and PCR amplification

Sufficient flanking sequence was available for ten microsatellites to allow primers to be designed in sequence flanking the repeats which would amplify PCR products in the range of 140 and 350 bp. A single primer pair for each of these loci was tested, and the optimal annealing temperature determined using gradient PCR on bulk genomic DNA as template prepared from adult Tci1 (MOSI) *T. circumcincta* worms. Eight of the ten primer pairs produced a product of the predicted size. Since the small insert genomic library was made from worms isolated from a sheep abomasum there is a possibility of host DNA contamination. Consequently, the primer pairs were tested by PCR using host gDNA (from a soay sheep) as template (provided by Dr J. Pemberton, University of Edinburgh). None of the primer pairs amplified products from host gDNA of a size similar to that predicted from sequence data confirming their identity as *T. circumcincta* microsatellites (Figure 4.10). The eight markers were then tested on panels of single worm DNA lysates prepared from the Tci1 (MOSI), ScSo529, and FrMe populations, using the highest annealing temperature that worked well from the gradient PCR results. If amplification from single worms was poor then lower annealing temperatures were tested down to a minimum for 45°C. Markers were chosen as suitable for population genetic analysis based on the proportion of individuals from which products of the expected size were amplified (Table 4.2 and Figure 4.11). There were problems with inconsistent amplification for seven of the eight markers. However, MTG 15 consistently amplified products of the expected size from both the ScSo529 and Tci1 (MOSI) *T. circumcincta* populations (Table 4.2 and Figure 4.11). A second pair of primers was designed for MTG 1b, MTG 5, MTG 8 and MTG 38 but also produced inconsistent amplification from single worms as for the original primer pairs (data not shown). In order to isolate trinucleotide repeat microsatellites three different oligonucleotide probes corresponding to $(AAC)_{16}$, $(AAG)_{16}$ and $(AGC)_{16}$, were used to screen the *T. circumcincta* short insert library by hybridisation but no convincing positive clones were identified (data not shown). A summary of results for the microsatellites isolated from screening the small insert *T. circumcincta* genomic libraries are summarised in table 4.3. Of the 45 markers isolated from the two screenings only MTG 15 was considered suitable for population genetic analysis.

4.2.2 Isolation of *T. circumcincta* orthologues of *H.contortus* microsatellites

Otsen *et al.* (2000) and Hockstra *et al.* (1997) previously isolated 71 GT / CA microsatellites from *H. contortus* and, of the 40 tested, 33 were found to be polymorphic. Studies have shown that microsatellite loci are often conserved between closely related species (Kayang *et al.* 2002;Mattapallil and Ali 1999;Nijman *et al.* 1998). Consequently the presence of orthologues of known *H. contortus* microsatellites in *T. circumcincta* was tested. Twelve previously characterised *H. contortus* microsatellites were chosen which were polymorphic in different *H. contortus* populations (Hcms 11, 17, 18, 19, 20, 25, 26, 28, 35, 37, 38, and 39) (Otsen *et al.* 2000). The previously published primer pairs (Otsen *et al.* 2000) corresponding to *H. contortus* flanking sequence, were used in PCR amplification reactions with *H. contortus* and *T. circumcincta* genomic DNA as template. Each primer set was tested using the published annealing temperature for *H. contortus* and if no product was obtained from bulk genomic DNA template, prepared from adult Tci1 (MOSI) *T. circumcincta* worms, a second PCR reaction was performed using an annealing temperature of 45°C. Of the 12 microsatellites tested, only Hcms 17, 25 and 28 amplified products from *T. circumcincta* gDNA (all at an annealing temperature of 50°C). These PCR products were of a similar, but not identical, size to the *H. contortus* products which, along with the absence of products in the negative controls, showed they were amplified from *T. circumcincta* DNA and not from contaminating *H. contortus* DNA (Figure 4.12). The three PCR products were cloned and sequenced and were shown to contain GT dinucleotide repeats (Table 4.4), the sequence of which was distinct from their *H. contortus* counterparts as might be predicted for polymorphic loci. Due to the small size of the PCR products (130 bp to 200 bp), only short regions of flanking sequence were available for comparison with the *H. contortus* loci (Figure 4.13). In the case of Hcms 17 and Hcms 25, although the level of sequence identity outside the GT repeat between the *Teladorsagia* and *Haemonchus* sequences is low, it is still sufficient to suggest these may be orthologous loci. In the case of Hcms 28, two blocks of GT repeats are separated by sequence that has a high level of identity between the *Teladorsagia* and *Haemonchus* (81 % identity over 100 bp) consistent with these being orthologous loci in the two species. Hcms 17, 25 and 28 were tested on panels of single adult male *T. circumcincta* worms from the Sourhope population, ScSo529, and only Hcms 28 was found to amplify a product of the predicted size robustly from individual worms suggesting it may be a potentially useful population genetic marker (Figure 4.14).

As the criteria for screening markers was refined, six of the previous twelve *H. contortus* microsatellites primer pairs were revisited (Hcms 11, 18, 19, 20, 37, and 38), along with six new *H. contortus* microsatellite primer pairs (Hcms 7, 15, 22, 23, 27, and 33) selected on the basis of polymorphism and lack of association with HcRep (Hoekstra *et al.* 1997;Otsen *et al.* 2000). In this case, the previously published primer pairs were tested by gradient PCR, using a range of annealing temperatures from 45.5°C to 64.5°C, on Tci1 (MOSI) *T. circumcincta* bulk genomic DNA template. Five sets of *H. contortus* primer pairs, Hcms 19, 22, 23, 27 and 38, amplified products of the approximately expected size from *T. circumcincta* gDNA (data not shown). These were tested against a population panel of 15 individual adult male worms, made up of 5 individuals from each of following populations: Tci1 (MOSI), ScSo529 and FrMe (Table 4.5). All five primer pairs were found to be inconsistent when tested across the population panel and so considered unsuitable for population genetic analysis.

In summary, of the nineteen primer pairs corresponding to sequence flanking *H. contortus* microsatellites that were tested, eight amplified products from *T. circumcincta* gDNA but only one, Hcms 28, amplified products consistently from individual *T. circumcincta* adult male worms from three separate populations.

4.2.3 Isolation of microsatellites from EST sequence data

The third approach to identifying microsatellite sequences from *T. circumcincta* was to search the available expressed sequence tags (EST) sequences. Tandem Repeat Finder (TRF) (Benson, 1999) was used to search the 4,379 *T. circumcincta* EST sequences present in the NEMBASE database (www.nema.cap.ed.ac.uk/nematodeESTs) which have been made from the Tci1 (MOSI) strain. These EST sequences had been placed into 1,670 clusters using the CLOBB.pl program and each of these clusters contains variable number of EST sequences assembled into contigs using the PHRAP program (http://zeldia.cap.ed.ac.uk/nematodeESTs/nembase.html). A Fasta file containing a non-redundant string of all the *T. circumcincta* EST contigs (supplied by Dr J. Parkinson) was searched with Tandem Repeat Finder. The least permissive parameters for alignment and mismatch were used in the first Tandem Repeat Finder search (2,7,7) with a minimum alignment score of 50, for which 8 microsatellites (MTG 61 to MTG 67) were isolated. The second round of searching used the most permissive parameters for mismatches and indels that was allowed (2,3,5) and again a minimum alignment score of 50, for which a further six microsatellites (MTG 68 to MTG 74) were isolated (Table 4.6). Microsatellites were

considered to be unique only when found in separate clusters. Hence a total of fourteen microsatellites were identified in the EST sequence file, including two dinucleotide, ten trinucleotide and three tetranucleotide microsatellite repeats (Table 4.6). The number of repeat units ranged from 7.5 to 49.3 varying in perfection from 62 % - 100 % under TRF criteria.

Primer pairs were designed for the thirteen EST microsatellites for which there was sufficient flanking sequence available to amplify PCR products in the size range of 140 bp - 350 bp. Each primer pair was tested by gradient PCR, using a range of annealing temperatures from 45.5°C to 64.5°C, on bulk Tci1 (MOSI) *T. circumcincta* genomic DNA as template. For those loci where no product was amplified a second set of primers were designed and all possible combinations of the two primer pairs were similarly tested (Table 4.7). Eleven of the microsatellite markers had at least one primer pair that amplified unambiguous products so they were then tested by single worm PCR against a population panel of 5 individual worm lysates from each of the Tci1 (MOSI), ScSo529 and FrMe populations. For six markers, products of a consistent size could be amplified from at least 4 out 5 single worms from each of the three populations (Table 4.7). These were considered to be potentially useful population genetic markers.

Sequence analysis performed on the six microsatellites selected for further analysis, suggested that MTG 65, MTG 68, MTG 69 and MTG 73, were likely to be located in open reading frames due to the lack of evidence of stop codons or a nearby poly-A tail. Blast searches were performed against the non-redundant nucleotide database (GenBank / EMBL) and the non-redundant protein database (SwissProt / TREMBL) using Blastn 2.2.3 and Blastx 2.2.3 (Altschul *et al.* 1997), respectively. Only the EST containing MTG 73 scored a significant match. This showed high identity to the coding sequence for a myosin essential light chain isoform from a number of species including *C. elegans* (85 % identity). The MTG 74 sequence is located 30 bp upstream from a poly-A tail, suggesting this may be in a 3' UTR region. MTG 67 was isolated from the centre of a very long EST sequence (9 kb) however there were numerous stop codons present in all six frames. Further analysis was not performed.

4.2.4 Assessment of microsatellite polymorphism

In total, from the screening procedures outlined above, eight microsatellites were identified that could be robustly and consistently amplified from gDNA derived from a panel of 15

single worms from 3 populations; MTG 15, Hems 28, MTG 65, MTG 67, MTG 68, MTG 69, MTG 73, and MTG 74 (Figure 4.15). The next step in assessing the potential value of these markers as population genetic markers was to assess their levels of polymorphism in a number of different parasite populations. Forward primers flanking these eight microsatellites were synthesised with a FAM or HEX 5' end fluorescent dye label (MWG) to allow Genescan genotyping to be performed (Table 4.8). A panel of single male worms from the Tci1 (MOSI) (25 males), ScSo529 (30 males) and FrMe (33 males) populations were genotyped with each microsatellite. Each of the markers produced clear genotypes on Genescan analysis (Figure 4.16). As expected for a diploid organism, either a single or a double peak was detected for all worms with all of the markers except for MTG 65, which amplified three products from 4 out of 25 Tci1 (MOSI) single worms and 1 out of 30 ScSo529 single worms (Figure 4.16). The fact that all the other seven microsatellite markers amplified a maximum of two products from all worms in the panel, together with an absence of products in the negative controls, suggests that the amplification of three peaks with marker MTG 65 on a small number of worms is unlikely to be due to cross sample contamination. MTG 65 was used to genotype three more populations and three products were also amplified from 6 / 40, 1 / 21 and 1 / 22 single worms from FrGa, ScSo507 and Motri populations, respectively. No one allele could be attributed as the reason for the additional product in these individuals. A new primer pair was designed for MTG 65 but did not successfully amplify products when tested by gradient PCR and MTG 65 was consequently removed from further analysis. Six of the remaining microsatellites were found to be polymorphic in the populations tested with the number of alleles per locus ranging from 4 to 19 (Table 4.8). MTG 69 was considered monomorphic in the populations tested, as the mean allele frequency of one allele (122) was 0.96, based on the three populations (ScSo529, FrMe, and Tci1 (MOSI)) genotyped and so this marker was removed from further analysis in this study.

4.2.5 Allele sequencing

A number of individual alleles were sequenced from each of the six microsatellite loci to be used in population genetic analysis. This was done to confirm the presence of GT repeats and to investigate the nature of allelic variation. In addition, allele sequencing is helpful to confirm that allele binning is appropriate and, in the case of microsatellites in ESTs, to identify the presence or absence of introns. Two approaches to allele sequencing were used. For MTG 15, alleles were PCR amplified from homozygote worms and directly sequenced

using a sequencing primer nested inside the forward or reverse primer. In the case of MTG 67, 68, 73 and 74, alleles were PCR amplified from heterozygote worms, cloned into the pCR®2.1 TA cloning vector (Invitrogen) and inserts sequenced using vector primers. Both approaches were used for Hcms 28. Between one and seven individual alleles were sequenced for each microsatellite and aligned with the original sequence from which primers were designed (Figure 4.17a - f). For the microsatellite markers Hcms 28, MTG 67, MTG 73, MTG 74 the entire allele was sequenced and the allele size was in agreement with that determined by Genescan to within 1 - 3 bp. The allelic variation for Hcms 28, MTG 73 and MTG 74 was due to differing numbers of dinucleotide repeat units and also, for a number of alleles, to single nucleotide polymorphisms (SNPs) present within the repeat units. These markers also showed evidence of sequence variation between alleles of the same sequence length (size homoplasy) or same electromorphic size (electromorph size homoplasy). For example, size homoplasy was seen between the 1A7 allele and original gDNA sequence of Hcms 28 due to different microsatellite arrays (Figure 4.17a). Size homoplasy was also seen between alleles of microsatellites MTG 73 and MTG 74 due to point mutations within the microsatellite array (Figure 4.17c and d). Electromorph size homoplasy was seen in MTG 67, where alleles M36-1 and M42-2 were sized as 178 bp by Genescan analysis, but the sequence data revealed M42-2 to be 1 bp longer (Figure 4.17b). The variation between these two alleles included sequence polymorphism both in the repeat and in sequence flanking the microsatellite. The sizes determined by Genescan data for these alleles, M36-1 and M42-2, were 178.66 and 179.22 respectively.

Direct sequencing of seven MTG 15 alleles was performed using a single nested primer and so one end of the amplicon remained un-sequenced. Consequently, although this was presumed to be approximately 64 bp based on the original genomic clone sequence, Genescan determination of allele size could not be directly compared (Figure 4.17e). Variation in the size of alleles for MTG 15 was mainly due to variation in the number of microsatellite repeat units, with the exception of alleles sequenced from individuals K22 and M25, for which there were indels of 12 bp and 1 bp, respectively (Figure 4.17f). Additional SNPs between individual alleles were found both in the flanking and microsatellite sequences, yet did not affect allele sizes (Figure 4.17f). Only one allele was successfully sequenced for MTG 68 (Figure 4.17g). The primers designed to EST sequence were predicted to amplify a product of 153 bp. However, the amplicons from single worm lysates were consistently 400 bp or greater. The sequence data was consistent with a 400 bp allele and identified the presence of two introns explaining the size discrepancy.

4.2.6 Allele Scoring and binning ranges

Each new microsatellite requires new alleles and boundaries (bins) to be defined, so that individual alleles for each individual genotype can be defined by the same criteria for all populations. As these are new microsatellite markers, individual allele sizes and their binning ranges were assigned for each using the ABI Prism 3100 genetic analyser and the ABI Prism Genotyper 3.7 NT software (Applied Biosystems), which are extremely sensitive and accurate, sizing alleles to the nearest two decimal points. Two approaches were used to define boundaries of each allele for each microsatellite marker, initially using data from the first three populations genotyped (Tci1 (MOSI), ScSo529, and FrMe). Allele scoring and binning ranges were then refined throughout the project and finally based on all fourteen populations genotyped in this project.

The first approach for defining allele sizes and their binning ranges consisted of plotting each decimal point reading for each allele, as defined by the Genotyper 3.7 NT software, on a histogram, with binning intervals set at 0.2 bp or 0.5 bp. Allele scoring and defining of binning ranges using this approach was straightforward for Hcms 28, MTG 73 and MTG 74 (Figure 4.18), but was less clear for markers MTG 15, MTG 67 and MTG 68 (Figure 4.19). The histograms for the later three markers, there was no clear division between allele size classes, with the genescan demical point readings being continous from the smallest allele size to the largest. As a result of this, the second approach involved numerically sorting the data for each microsatellite by decimal point reading in ascending order. The data were analysed to locate natural breaks between allele sizes (Figure 4.20). This second approach confirmed that there was no scoring bias of any one population. For example, individuals from each population genotyped for a particular allele size were randomly spread over the allele binning range (Figure 4.20). Accurate scoring and binning of alleles can also be affected by the non-template addition of an adenosine base by Taq polymerase at the 3' end of the PCR product, resulting in a doublet peak for each allele, referred to as a 'plus-A effect' (Breen *et al.* 1999). This was particularly pronounced for Hcms 28 and MTG 73 (Figure 4.16). Thermocycling conditions during PCR were adjusted to include a long final extension phase of 15 minutes at 72°C to promote adenosine base addition to the amplicon leading to the fewer doublet peaks. In spite of this a double peak was still seen with Hcms 28 and MTG 73 in > 97 % of samples and so for these markers the second peak was always scored.

Allele scoring and binning ranges for each microsatellite marker was based on the above two methods, except for MTG 68. For the marker MTG 68, it was not possible to sensibly bin alleles based on the genotypes from the three populations; Tei1 (MOSI), ScSo529 and FrMe, it was therefore discarded at this point. However, based on the analysis of the other 5 microsatellite markers, it was evident that scoring and binning ranges became more evident and clear cut as more individuals were genotyped and data re-analysed. Consequently, it is possible that allele binning would have improved for MTG 68 had all fourteen populations been genotyped. All individual genotypes were manually scored into appropriate allele bins, and correct assignment was manually checked at least three times. The microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74 and the corresponding binning ranges have successfully been used by two other labs (L. Stenhouse, Moredun Institute and Nicholas Temperley, University of Glasgow, pers. comm.). The range, mean and standard deviation of allele sizes were calculated for each allelic bin of each marker using Excel 2003 (Table 4.9).

4.2.7 Ambiguous genotypes and repeatability of allele binning

In order to ensure all binned alleles were correct, individual genotypes were only accepted as definitive under strict criteria. Of the 448 worms genotyped with the five markers 79.3 %, 86.3 %, 76 %, 93.5 % and 80.4 % were of a quality comparable to the examples in figure 4.16 for MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74, respectively. Where there was any possibility of ambiguity, the individual worm was genotyped again with the same primers and re-analysed using Genescan. Criteria for which PCR and genotyping was repeated included: i) if intensity of peaks on Genescan were below 200 fluorescent units or of poor quality; ii) if any individual worm genotyped with any microsatellite gave rise to a new allele size not previously seen; these were repeated to ensure the new allele was not an artefact; iii) if the size of the allele was either outside or at the outer limits of the binning range; and iv) individuals from which no product was amplified were repeated at least twice before being considered homozygous for potential null alleles. Poor template quantity and / or quality was considered to be the main reason for non-amplification when failure to amplify product occurred in more than three of the five microsatellites. These individual samples were discarded from further analysis.

The number of individuals that were genotyped more than once ranged from 6.4 % for MTG 73 to 24.4 % for MTG 67 from a total 448 individual worms genotyped (Table 4.10). The error rate of these potentially ambiguous genotypes was calculated using the formula

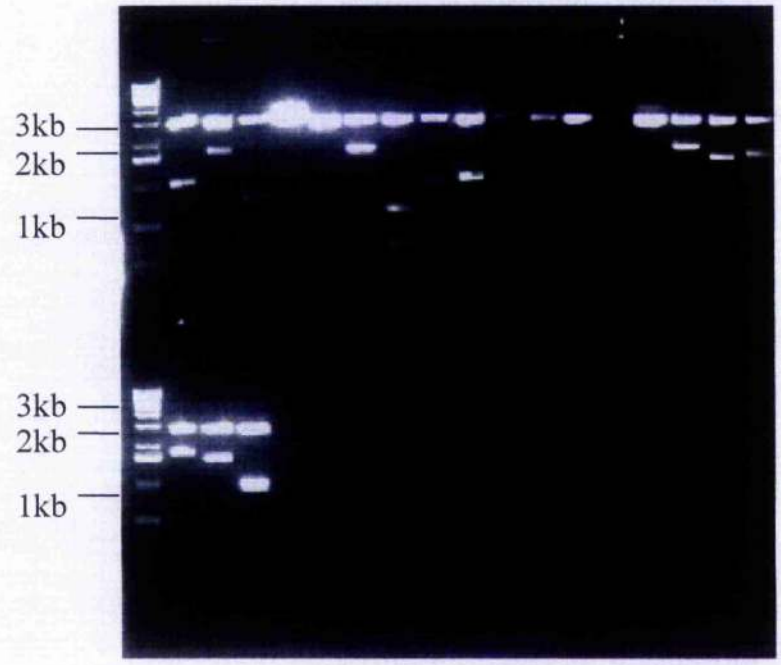
(a / 2b) x 100 where a = the number of alleles which changed and b = the number of genotypes repeated and ranged from 0 % Hcms 28 to 7.4% for MTG 74 (Table 4.10). Assuming these ambiguous genotypes are the only source of error this would equate to error rates of 0.2 %, 0 %, 0.7 %, 0.2 % and 1.5 % for MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74, respectively, for the full data set.

One issue of potential concern is that limitations in the accuracy of allele sizing by Genescan could lead inappropriate binning of alleles. In other words, it is important to be confident that if an individual was genotyped on separate occasions, alleles would be repeatably ascribed to the same bins. Consequently, the repeat genotype data described above was also used to test the repeatability of the allele size scoring by the Genescan / Genotyper software. The precise allele sizes (decimal point readings) between repeat genotypes were compared. The arithmetic mean difference in allele sizes between repeat genotypes of the same individual ranged from 0.14 bp for MTG 15 to 0.29 bp for MTG 73 confirming the high level of accuracy and repeatability of the genotyping data (Table 4.10). In consequence, there was only a single allele out of a total of 776 alleles that were repeat genotyped for the five microsatellites for which there was a change of bin assignment (Table 4.10). This result suggests allelic binning was highly repeatable.

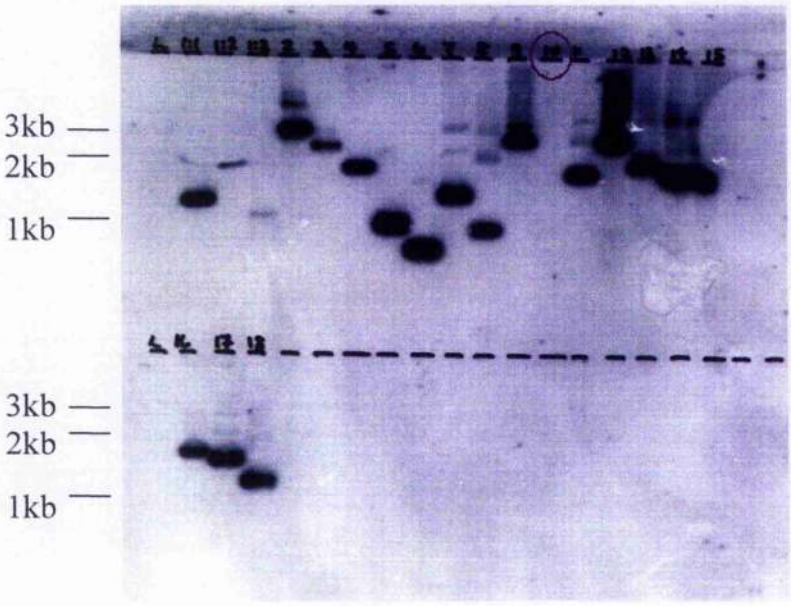
Figure 4.1: Agarose gel and corresponding Southern Blot of putative positive clones

Final twenty putative positive clones from the first screening, double digested with *EcoRI* and *HindIII* run on 2 % agarose gel, and the corresponding Southern blot probed with $(CA)_{25}$ oligonucleotide probe. The putative positive numbered 10 on the Southern blot was the only clone not to hybrise. On the Southern Blot; L – DNA ladder lane.

Figure 4.1: Agarose gel and corresponding Southern Blot of putative positives



AgaroseGel



Southern Blot

Table 4.1: Summary table of microsatellite sequences isolated from small insert genomic library

Microsatellite sequences isolated from screening a small insert genomic *T. circumcincta* library. First and second screenings are shown separately. The microsatellite sequence for MTG 39 was not completely sequenced due to repeated failure of sequencing.

Microsatellite : First screening	Accession Number	Repeat Sequence
MTG 1a	DQ355411	$GTAT(GT)_5AT(GT)_{17}GC(GT)_2GCGT$
MTG 1b	DQ355412	$(GT)_3CGGTTT(GT)_3ATG(GT)_2CTGT$
MTG 1c	DQ355413	$(GTCT)_2(GT)_7(GC)_2GTCCGTTTGT$
MTG 3	DQ355414	$(GTCT)_5(GT)_{11}$
MTG 4	DQ355415	$T(GTCT)_3(GT)_9$
MTG 5	DQ355416	$(GT)_4TGTGG(GT)_2TAT(GT)_2ATGTGGTGTAT(GT)_3AA(GT)_5TT(GT)_8$
MTG 6	DQ355417	$(GTCT)_2(GT)_3AT(GT)_5CT(GT)_9$
MTG 7	DQ355418	$(GT)_{31}CC(GT)_4CC(GT)_3$
MTG 8	DQ355419	$(GT)_{11}$
MTG 11	DQ355420	$(GT)_6AT(GT)_4AT(GT)_5ATGTAT(GT)_4$
MTG 12	DQ355421	$(GT)_3CTGTTG(GT)_{15}$
MTG 13	DQ355422	$(GT)_3CTGTTG(GT)_{10}TTG$
MTG 14	DQ355423	$(GTCT)_4(GT)_{13}$
MTG 15	DQ355424	$(GT)_6GC(GT)_6GGGTTTGT$
MTG 16	DQ355425	$(GT)_4GAGTGG(GT)_2GGGTGCGGAT(GT)_4CCGT$
MTG 17	DQ355426	$(GT)_4(G)_{13}A(GT)_3GG(GT)_3AG(GT)_2$
MTG 18	DQ355427	$(GT)_3\ CTGTTG(GT)_3(CTGT)_3\ (GT)_9$
Microsatellite : Second screening		**Repeat Sequence**
MTG 38	DQ355428	$(GT)_3\ CTGTTG(GT)_3CT(GT)_9\ TT(GT)_3$
MTG 39		Failed to sequence through microsatellite
MTG 41	DQ355429	$(GT)_3T(GT)_3TGTTCG(GT)_3$
MTG 46	DQ355430	$(G)_9(GT)_6$

Figure 4.2: Example of Dot Plot for plasmid containing MTG 14

Dot Plot analysis, using Vector NTI, of the plasmid insert sequence containing the microsatellite MTG 14, showing graphical representation of sequence plotted against itself. M represents the microsatellite downstream from the TecRep. The vertical lines radiating from midline represents the TecRep sequence.

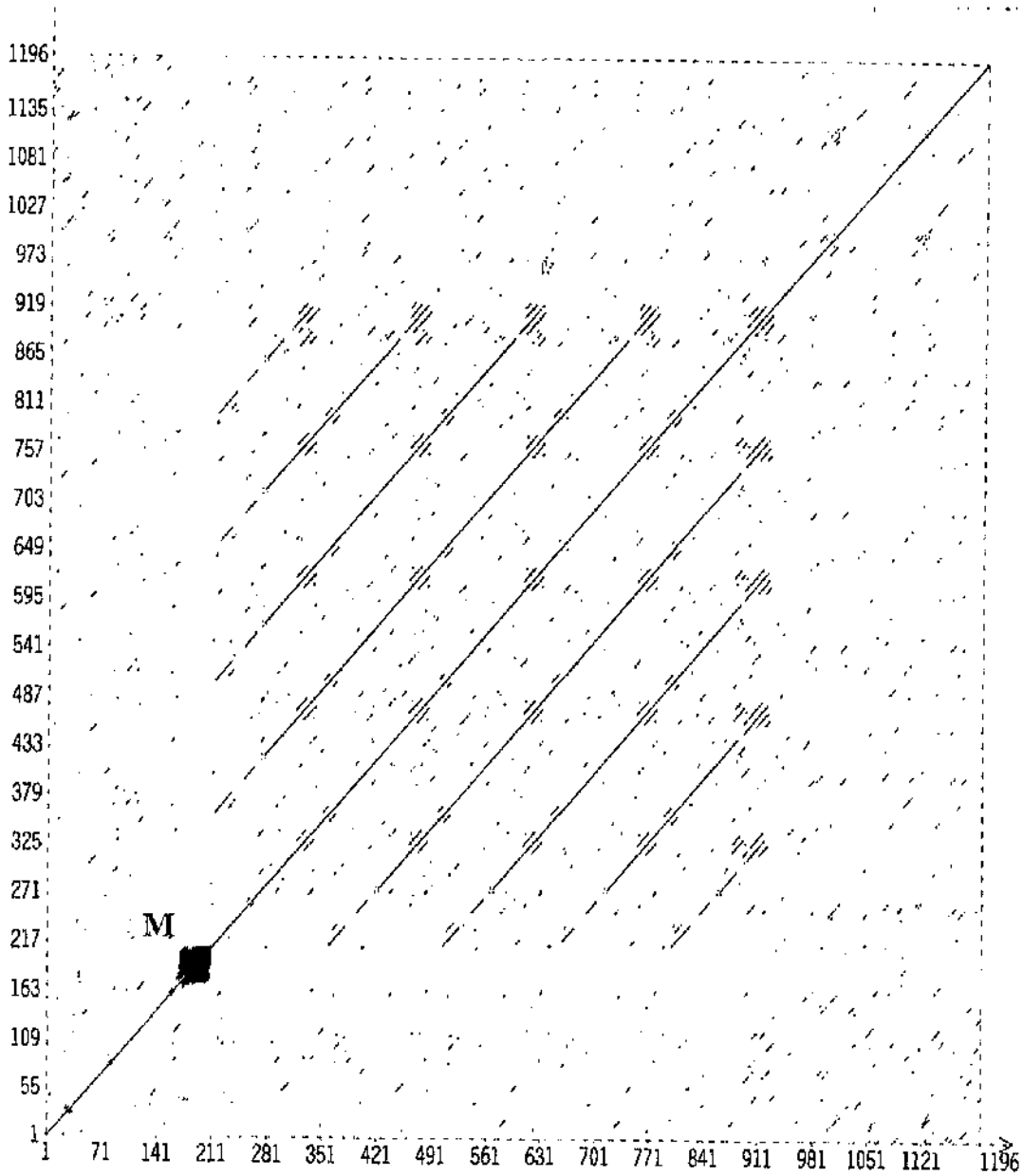


Figure 4.3a: Schematic diagram of TecRep position in relation to $(GT)_n$

Schematic diagram of 146 bp TecRep repeat shown to be downstream from the $(GT)_n$ repeat sequence. The top diagram shows a microsatellite associated with only one TecRep repeat sequence and the bottom diagram shows a microsatellite associated with three TecRep repeat units.

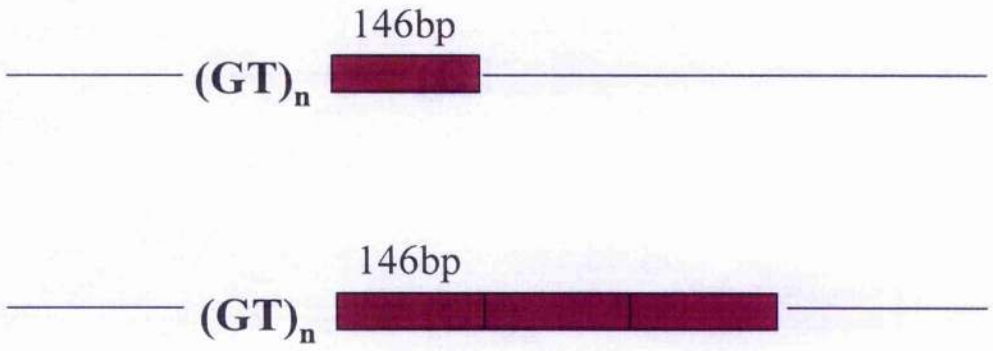


Figure 4.3b: Number of TecRep repeats associated with each microsatellite

Microsatellites isolated from genomic *T. circumcincta* library which are associated with the TecRep repeat, including the number of TecRep repeats units each one is associated with. * indicates sequences for which the true number of TecRep repeats could not be ascertained due to lack of sequence data.

MTG 1a	**1***	**MTG 11**	**1**
MTG 1c	**1***	**MTG 12**	**6**
MTG 3	**1**	**MTG 13**	**4***
MTG 4	**3**	**MTG 14**	**5**
MTG 6	**1**	**MTG 18**	**1***

Figure 4.4: Sequence alignment of TecRep repeats

Each individual TecRep repeat sequence associated with each microsatellite was aligned using Vector NTI. Each TecRep repeat is named according to the corresponding microsatellite (first number) and the position in relation to the $(GT)_n$ microsatellite repeat array (rep1-6), where rep 1 is directly downstream from microsatellite. TecRep repeats associated with MTG 3, MTG 6 and MTG 13 rep4 have not been included in this diagram. Grey shading = conserved nucleotides between all sequences. Light blue shading = the most common nucleotide across all sequences.

Figure 4.4: Sequence alignment of TecRep repeats

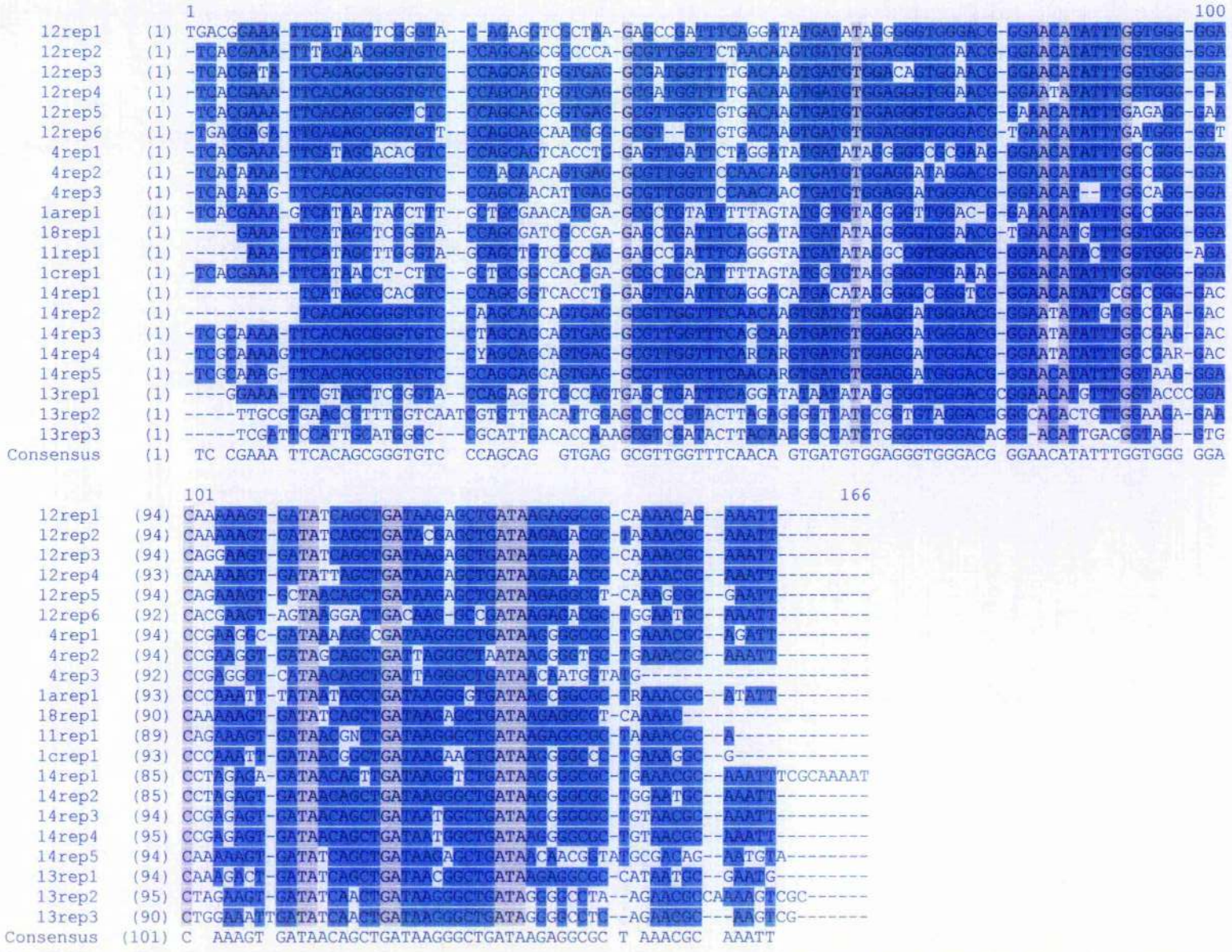


Figure 4.5a: Schematic diagram of HcRep and TcRep

Schematic representation of three HcReps and one TcRep in relation to a $(GT)_n$ microsatellite and the percentage identity between each when compared to TecRep.

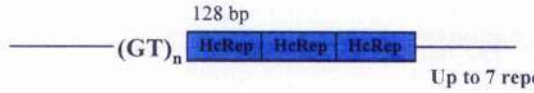
Figure 4.5b: Sequence alignment between TecRep, HcRep and TcRep

Sequence alignment of TecRep, HcRep, and TcRep. Grey shading = consensus for all sequences, Light blue shading= common base pair for two of the three sequences aligned.

Figure 4.5a: Schematic diagram of HcRep and TcRep

***Haemonchus contortus* - HcRep**

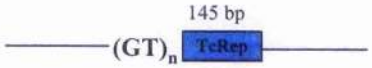
(Hoekstra *et al.*, 1997; Osten *et al.*, 2000)



44% identical with TecRep

***Trichostrongylus colubriformis* - TcRep**

(Callaghan and Beh, 1994)



62% identical with TecRep

Figure 4.5b: Sequence alignment between TecRep, HcRep and TcRep

```
TecRep          -----TCACGAAATTCACAGCGGGTGTCCCAGCAGTGGTGAGGCGTTGGTTTTGACAAG(54)
HcRep           GTTTGACCGGAAATTCATAACTCCTGTCCGCCGTGGACGTAGAGCGTTCAAATTTGGTAG(59)
TcRep           -------------TCATATCTCGCGTCGCAGTAGAGCTGGGACCCTGAATGTTTAAAG(45)
Consensus             C  GAAATTCATA CTCGTGTCCCAGTAGAGCTGGGGCGTTGAATTTT  AAG

TecRep          TGATGTGGAGGGTGGGACGGGAACACATATTTGGTGGGGGACAAAAAGTGATATCAGCTG(112)
HcRep           GAGTACAGGGGGCAGGATGGGAACACTTATGGTGGGTGGCAGGACCGGAC-TCCGCYC(117)
TcRep           TGATAGAGGGGGTGGTAAGGGAACACTCAATGGTGGGGGGAACGGAAGTGATAGCAACTG(103)
Consensus       TGATA AGGGGGTGGGA GGGAACAT TATGGTGGGGGACAGGAAGTGATATCAGCTG

TecRep          ATAAGAGCTGATAAGAGAAGCCAAAACGCAAATT(146)
HcRep           ACAGGGGGCGGG----------------------(129)
TcRep           ATAAGGGCTGATAAGGAGCCTCGAGTGGCTAAT-(136)
Consensus       ATAAGGGCTGATAAG   C  C A   GC AAT-
```

Figure 4.6: Unrooted Neighbour-joining Tree

The sequence of each TecRep repeat were aligned with 16 different HcRep sequences from *H. contortus* (Hoekstra *et al.* 1997) and 5 different TcRep sequences from *T. colubriformis* (Callaghan and Beh 1994) and a bootstrapped Neighbour-Joining tree was constructed using ClustalX (Thompson *et al.* 1997). The repeat sequences of each species cluster separately, with the *T. circumcincta* sequences being more closely related to the *T. colubriformis* sequences than either are to *H. contortus*. c = *T. circumcincta* TecRep repeats, h = *H. contortus* HcRep repeats, Tr = *T. colubriformis* TcRep repeats.

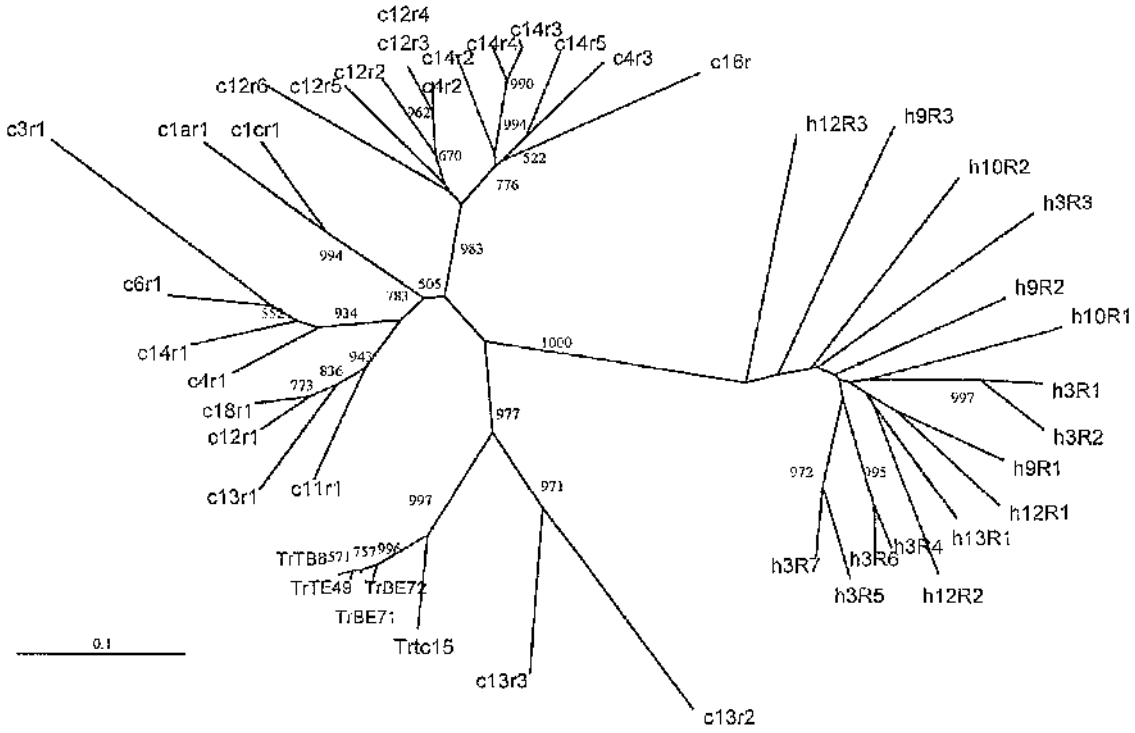


Figure 4.7: Example of unexpected size polymorphisms due to TecRep

PCR products amplified from single adult male worms from the ScSo529 population, using the primer sets corresponding to the microsatellites MTG 1a, MTG 6, MTG 14 and MTG 18, visualised on 2 % agarose gels, demonstrating the extreme variation in amplicon sizes amplified from these microsatellites. Each single worm was PCR amplified in duplicate and duplicate samples run in adjacent wells. C = controls using dH_2O in replacement for template.

Figure 4.7: Example of unexpected size polymorphisms due to TecRep

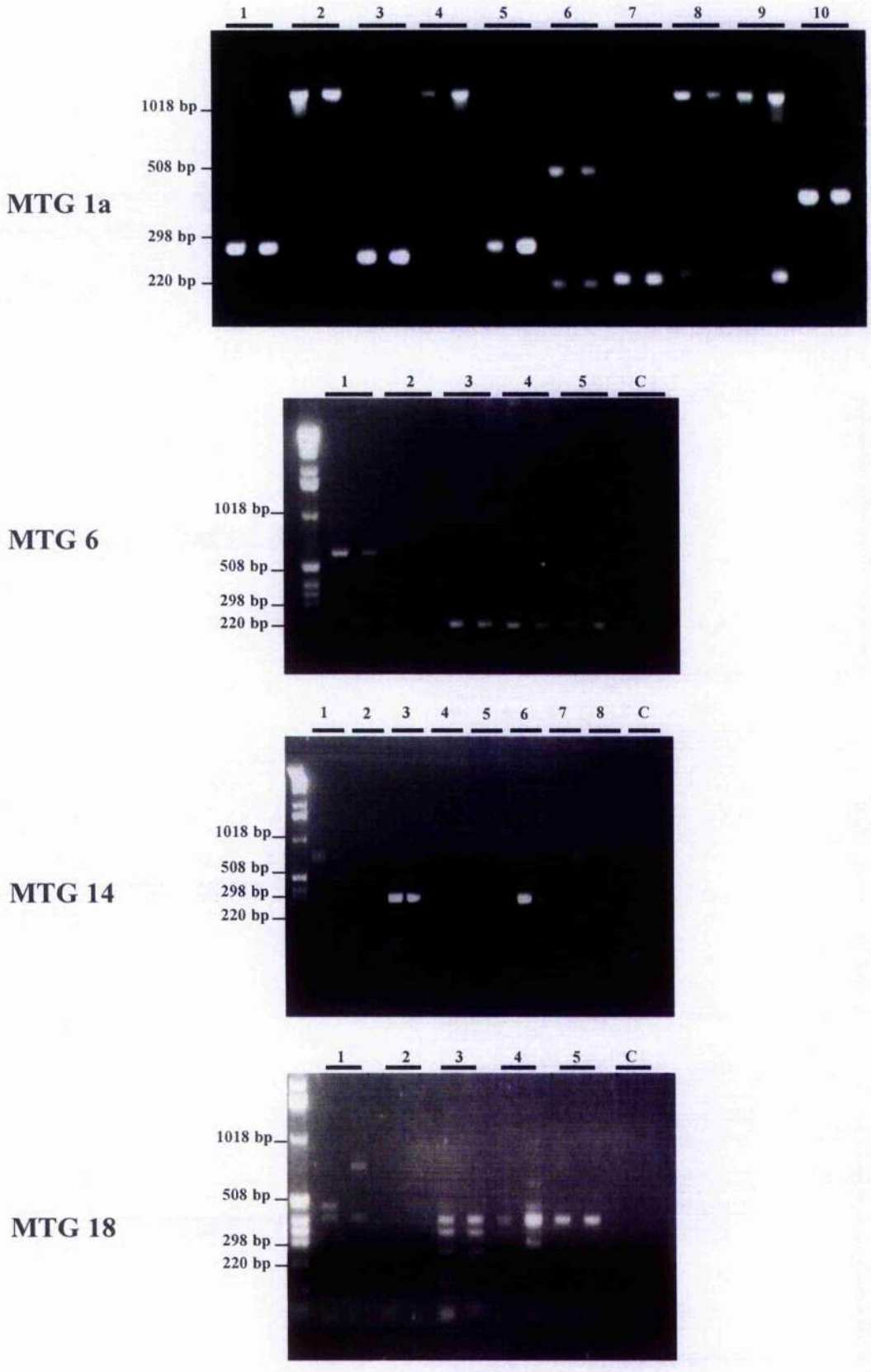


Figure 4.8a: Schematic diagram of MTG 1a allele sequences alignments

Schematic diagram demonstrating alignment of individual alleles amplified from single worms for the microsatellite MTG 1a. A = the forward primer, pr1aa; B = the reverse primer, pr1ab. Alignment of sequences flanking the microsatellite (designated X and Y) is presented in figure 4.8b.

Figure 4.8b: MTG 1a allele sequences alignments

Alignment of sequences flanking the microsatellite repeat shown as X and Y in figure 3.8a. Y1, Y2 and Y3 represent three tandemly arranged TecRep in clone D31 and have been included in the alignment. Y3 is not a complete TecRep. Grey shading = consensus for all sequences, Light blue shading = common base pair for two of the three sequences aligned.

Figure 4.8a: Schematic diagram of MTG 1a allele sequences alignments

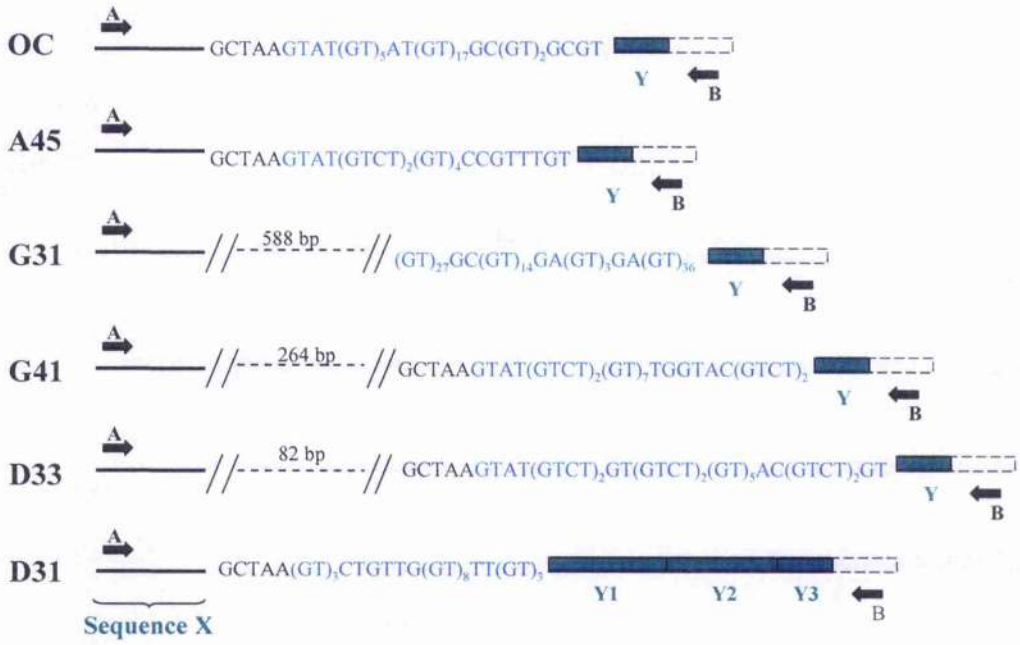


Figure 4.8b: MTG 1a allele sequences alignments

Sequence X

```
OC      ATTGAAAATTGACTGCGGCAACCTCTTGTTACGGTAATTTTTGCCC--CCCTGTA---TAATTC----------(59)
A45     -TTGAAAATTGACTGCGGCAACCTTTTGTTACGTAATTTCTGCCCA-CCCTGTA---TAATTC----------(59)
G31     -TTGAAAATTGACTGCGGCAACCTCTTGTTACGTAATTTTTGCCCA-CCCTGTATAGTAATTG- +588 bp--(62)
G41     ATTGAAAATTGACTGCGGCAACCTCTTGTTACGTAATTTTTGCCCACCCCTGTAATTTA----- +264 bp--(60)
D33     ATTGAAAATTGACTGCGGCAACCTCTTGTTACGTAATTTTTGCCCA-CCCTGTA---TA-----  +82 bp--(56)
D31     ATTGAAAATTGACTGCGGCAACCTCTTGTTACGTAATTTTTGCCCA-CCCTGTA---TAAGCG----------(60)
```

Sequence Y: TecRep Alignments

```
OC  Y   CACGAAAGTCATAACTAGCTTTGCTGCGAACATGGAGCGCTGTATTTTT--AGTATGG(56)
4A5 Y   CACGAAATTCGTAACTCGCTTCGCTGCGGCCATGGAGCGCTGTATTTTT--AGTATGG(56)
G34 Y   CACAAAATTCATAACTCGCTTCGCTGCGGCCATGGAGCGCTGTATTTTT--AGTATGG(56)
G41 Y   CACGAAATTCATAGCTCGCTTCGCTGCAGCCATGGAGCCCTGTATTTTT--GGTATGG(56)
D33 Y   CACGAAATTCATAGCTCGCTTCGCTGCAGCCATGGAGCCCTGTGTTTTT--GGTATGG(56)
D31 Y3  CACAAAATTCACAGCGGGTGTTCCAGCAGCGATGAGGCGTTGGTTCTGACAAGTGATG(58)
D31 Y1  CCGGAAATTCATAGCTCGGGTACCAGCGATGACCAGGAGCTGATTTCAG--GATATGATATAGGGGGTGGGACGG(73)
D31 Y2  CACGAAATTCACAGCGGGTGTTCCAGCAGCGATGAGGCGTTGGTTCTGG--CAAGTGATGT-GGGGGTGGAACGG(72)

D31 Y1  GAACATGTTTAGTGGAGGACAAAAAGTGATAACAGCTGATAAGAGCTGATAAGAGACGCCAGAACGCGAATTTC(147)
D31 Y2  GAACATGTTG-GTTGGGGACAAAAAGTGATAACAACTGATAAGAGCTGATAAGAGGCGCCAAAAGGCGAATT--(143)
D31 Y3
```

Figure 4.9a: Southern Blots of second screening with $(CA)_{25}$ probe

Southern blot of the final twenty-six putative positive clones from the second screening, which were double digested with *EcoRI* and *HindIII* and run on 2 % agarose gel, probed with $(CA)_{25}$ oligonucleotide probe. L – DNA ladder lane.

Figure 4.9b: Southern Blots of second screening with TecRep probe

An identical Southern blot to that in figure 4.9a, probed with the 897 bp probe containing 5 copies of the TecRep sequence from MTG 12. Red arrows indicate the eight clones which did not hybridise with the TecRep probe and went on to be sequenced. In addition, one red arrow indicates the negative control clone, MTG 1b, the insert of which was known not to contain the TecRep from sequence data. Clones which hybrised with the TecRep probe were discarded from further analysis. L – DNA ladder lane.

Figure 4.9a: Southern Blots of second screening with $(CA)_{25}$ probe

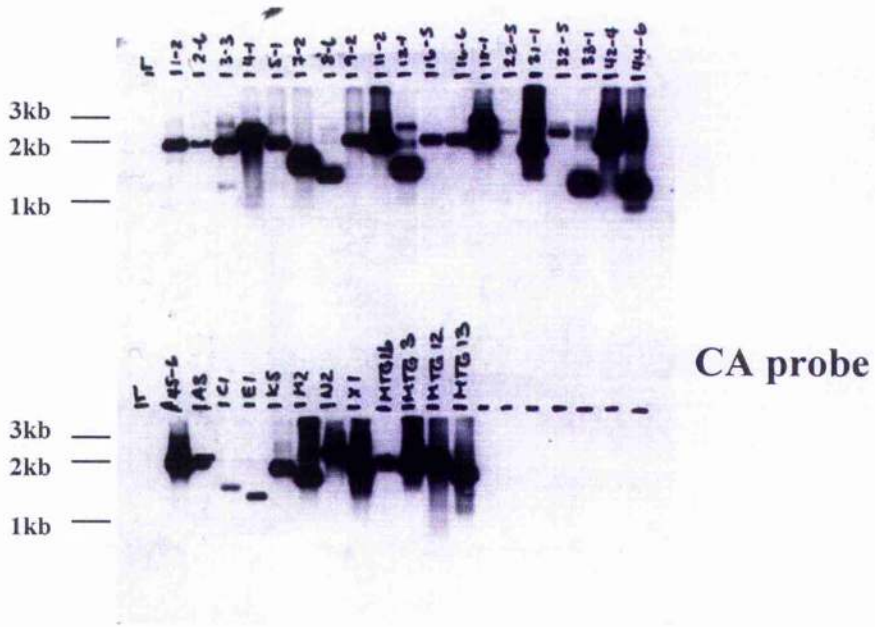


Figure 4.9b: Southern Blots of second screening with TecRep probe

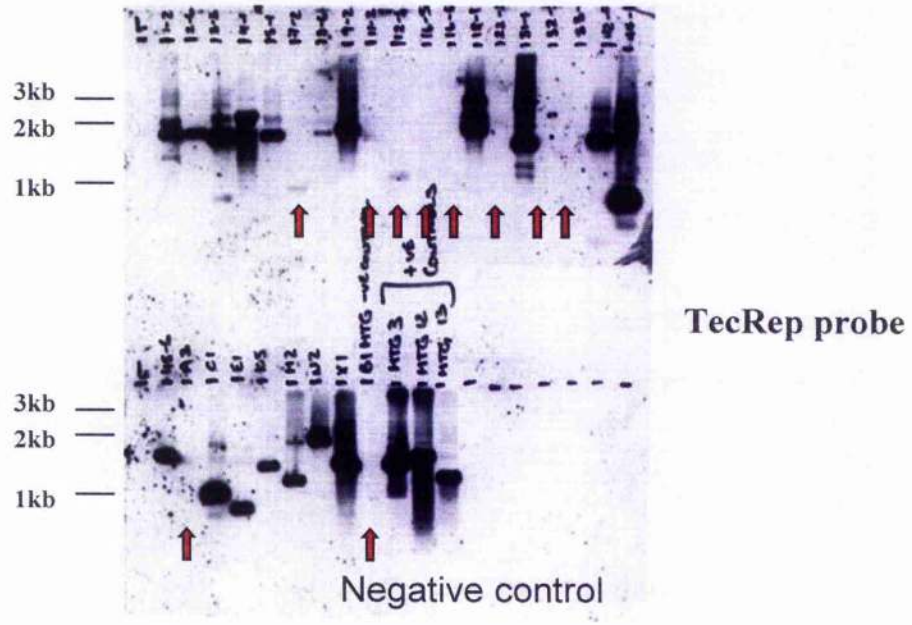


Figure 4.10: PCR amplification from host and parasite genomic DNA

Sheep gDNA was isolated from a Soay sheep and a sheep microsatellite successfully amplified to confirm adequate template prior to use in testing parasite primer pairs (data not shown). PCRs with primer pairs to the parasite microsatellites were performed on three duplicate samples of sheep gDNA (soay sheep), *T. circumcincta* Tci1 (MOSI) gDNA, negative controls (dH_2O) and four duplicate samples using a bulk *T. circumcincta* Tci1 (MOSI) L3s. Microsatellites MTG 1b, MTG 8, MTG 15, MTG 16 and MTG 17 were tested. The results of MTG 15 and MTG 17 are shown as examples below.

Table 4.2: Summary of results from single worm PCR panel – Genomic screening

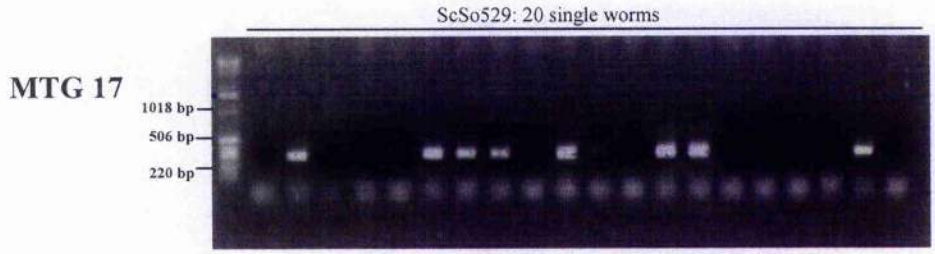
Summary of results from single worm PCR panel for microsatellites isolated from the small insert genomic *T. circumcincta* library. Several criteria were used to decide whether or not the marker was usable for population genetic analysis. The main categories were: a) non-amplification of PCR product and b) inappropriately sized PCR products* compared to sequence data, these included amplification of products at least 200 bp greater than the expected size and amplification of more than two amplicons, which would be inconsistent with amplification from single worms. ScSo529, Tci1 (MOSI) and FrMe indicate the *T. circumcincta* populations from which single worms were used. See table 2.1-2.3 for more information on populations.

Microsatellite: 1st Screening	Single Worm Panel			
	No. of worms tested	Products of expected size	Inappropriately sized products*	Non-amplification
MTG 1b	5 (ScSo529)	1	3	1
	4 (MOSI L3s)	2	1	1
MTG 8	17(ScSo529)	8	-	9
	10 (MOSI L3s)	5	2	3
MTG 15	20 (ScSo529)	18		2
	10 (MOSI L3s)	9		1
MTG 16	20 (ScSo529)	3	1	17
	10 (MOSI L3s)	-	-	10
MTG 17	20 (ScSo529)	9	-	11
	10 (MOSI L3s)	3	-	7
Microsatellites: 2nd Screening				
MTG 38	5 (ScSo529)	-	-	5
	5 (MOSI)	-	-	5
	5 (FrMe)	-	-	5
MTG 39	30 (ScSo529)	11	-	19
	26 (MOSI)	11	-	15
MTG 41	5 (ScSo529)	5	-	-
	5 (MOSI)	3	-	2
	5 (FrMe)	1	-	4

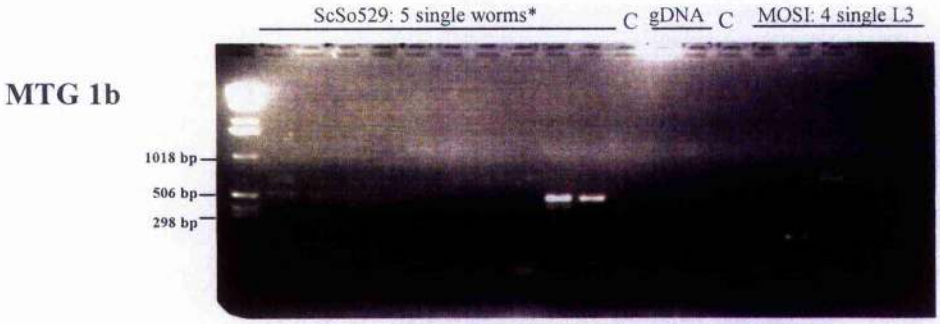
Figure 4.11: PCR products amplified from single worm panels – Genomic Screening

The example 2 % agarose gels are shown here for illustration of the results summarised in table 4.2. PCR products amplified using the microsatellites: MTG 16, MTG 1b and MTG 15, on 2 % agarose gels, demonstrating non-amplification, inappropriate PCR products and consistent PCR products, respectively. Template consisted of single adult male *T. circumcincta* from the populations; ScSo529 and Tci1 (MOSI), or from single L3s from the Tci1 (MOSI) population, as indicated. * for the microsatellite MTG 1b each single worm was amplified in duplicate and run in adjacent wells. C = controls using dH_2O in replacement for template. For population information see tables 2.1-2.3.

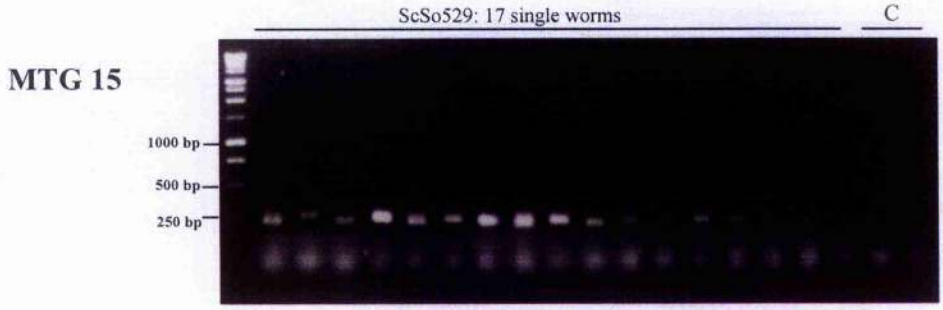
Figure 4.11: PCR products amplified from single worm panels – Genomic Screening



Non- amplifying



Inappropriately sized products



Products of expected size

Table 4.3: Summary of reasons for discarding putative positive clones isolated from genomic library

Reasons for discarding putative positives clones isolated from the genomic library screening. *The putative positives MTG 2, MTG 9, MTG 35, MTG 45 and MTG 50 had inserts greater than 3 kb, therefore were not sequenced.

Reason for discarding Microsatellites	Microsatellites	Total
Associated with TecRep		28
Found by sequence analysis	MTG 1a,1c, 3, 4, 6, 11, 12, 13, 14 , 18	10
Found by Southern Blot	18 putative positive clones	18
Not sequenced*	MTG 2, 9, 35, 45, 50	5
No microsatellite found when sequenced	MTG 43	1
Insufficient flanking sequence for primer design	MTG 46	1
No consistent product amplified using gradient PCR	MTG 5, 7	2
Non-amplification from single worms	MTG 1b, 8, 16, 17, 38, 39, 41	7
Total		**44**

Figure 4.12: PCR products amplified using *Haemonchus contortus* microsatellites

A 2 % agarose gel showing the PCR products amplified from gDNA from *H. contortus* (H) and *T. circumcincta* (T) using primer pairs flanking the *H. contortus* microsatellites Hcms 17, Hcms 25 and Hcms 28. The primer sequences are those described by Otsen *et al.* (2000) and Hoekstra *et al.* (1997).

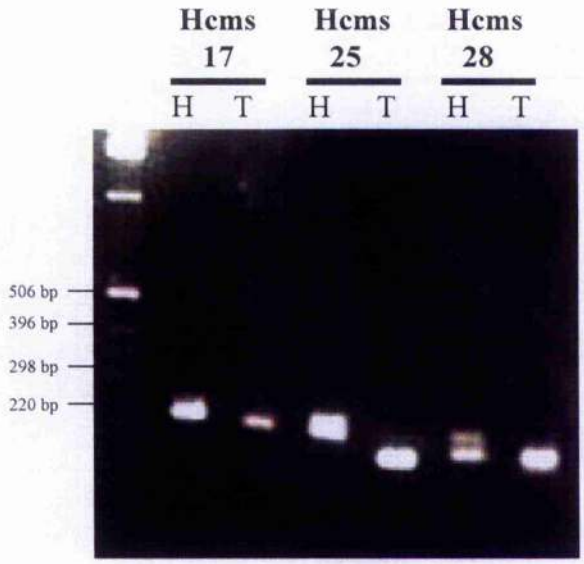


Table 4.4: Comparison of *H. contortus* and *T. circumcincta* microsatellite sequences
Comparison of microsatellite sequences amplified from *H. contortus* and *T. circumcincta* by primers designed to the sequence flanking the *H. contortus* microsatellites Hcms 17, Hcms 25 and Hcms 28. The primer sequences are those described by Otsen *et al.* (2000) and Hoekstra *et al.* (1997). *The Genebank accession numbers corresponding to Hcms 17, Hcms 25 and Hcms 28 are: AF178466, AF178474, and AF178477 respectivily. **The *T. circumcincta* microsatellites (Genebank accession numbers) corresponding to the *H. contortus* microsatellites were named as follows: Hcms 17: MTGHC1 (DQ355144), Hcms 25: MTGHC2 (DQ355409), Hcms 28: MTGHC3 (DQ355410).

Figure 4.13: Alignment of *H. contortus* and *T. circumcincta* microsatellite sequences
Alignment of *H. contortus* sequences from Gene bank with sequences amplified, cloned and sequenced from *T. circumcincta* corresponding to the *H. contortus* microsatellites Hcms 17, Hcms 25 and Hcms 28. Grey shading = consensus for all sequences, Light blue shading = common base pair for two of the three sequences aligned. Bold black type = microsatellite sequence.

Table 4.4: Comparison of *H. contortus* and *T. circumcincta* microsatellite sequences

Microsatellite	*H. contortus**	*T. circumcincta***
Hcms 17	$(GT)_3GCCTGTATT(GT)_5T(GT)_5$	$(GT)_{15}$
Hcms 25	$(GT)_3CTGTTG(GT)_3CG(GT)_3TT(GT)_5TT(GT)_{10}TT(GT)_6CG(GT)_5$	$(GT)_3CTGTTG(GT)_8$
Hcms 28	$(GT)_3$----------113bp-------$(GT)_8$	$(GT)_8$----87bp--$(GT)_6$

Figure 4.13: Alignment of *H. contortus* and *T. circumcincta* microsatellite sequences

Hcms 17

H. contortus Hcms 17 seq: AF178466

```
T.circ  GAGTAGGGGGAAGATGTCTGCATCCCGTCTACGGGATTTTTGCCACAAAGGAAAGCAGTACAGCGGAAGGTGTAACGCAG(80)
H.cont  ----------------------TGTCCCCAAGGAAATGTTTGCGAAGGGCGCGAAC-GTTCAAATGGTGTTTT--CGCCT(56)

T.circ  AGAAGCGCTGTCCCGGCGGTTGACGGAGGACCAATCTGGTCCTACTATATATAATCGGCTAAGCCGGTTGTGTGT---GTG(157)
H.cont  CTTTG---TGTCAAGATAGTA-ACAAGGGAGCAAAGTT-TCCTACTATCTATAACGCACTAAGTGTGTGCCTGTATTGTG(131)

T.circ  TGTGTGT-GTGTGTGTGTGTGTCTGA---------------------------- (183)
H.cont  TGTGTGTTGTGTGTGTGTATATATGTATGTGTCTATATGTGTGTGTGTGTGTTTGA(187)
```

Hcms 25

H. contortus Hcms 25 seq: AF17874

```
T.circ  ---TTCACTATTATCGTTTTCATGACACCGTGCCTTACAACAGTATCACTGTCGGGGAGGTAATAAGAGCGAGCAGAAAG(77)
H.cont  CGAGGGTGDGTAATTGAATGCAACTTCGCGCCACTGAAAAATGAATGGATATATCGTACTTTATATAATTC-GCTCAGTG(79)

T.circ  AATCCTACTATGTATAAGCGGCTAAGTGTGTCTGTTGGTGTGTGTGTGTGTGTTTGA----------------------(134)
H.cont  TGTCTGTTGGTGTGTCCGTGTGTTTGTGTGTGTGTTTGTGTGTGTGTGTGTGTGTTTGTGTGTGTGTGTCGGTGTGTG(159)

T.circ  -------(134)
H.cont  TGTTTGA(166)
```

Hcms 28

H. contortus Hcms 28 seq: AF178477

```
T.circ  TCGTTTTCTGTGTGTGTGTGTGTGTGCTCTGT---TGTGGAAGGCGGTCTCT---GCTATTGGCGGTGGCGTATCCGTC(73)
H.cont  --------------TCGTTGTGTGTGCTCTGTGCTGGTGGAAGGCGGTCTCTCTGGCTGTTGGCGGTGGCGTATCCGTC(65)

T.circ  ----CGT----------------GTGGTGCAGTGCTGCGTCCTCTTTGATTGCTTGCCTGTGTGTGTGTGTTT---(126)
H.cont  CGTCCGTCCGTGTGGTGGTGGTGGTGGTGCAGTGCTGCGTCGTCTTTGATTGCTTGCCTGTGTGTGTGTGTGTTT(141)
```

Figure 4.14: PCR products amplified from single worm panels – *H. contortus* microsatellites

PCR products amplified from single adult male *T. circumcincta* using primers designed to flanking sequence of the *H. contortus* microsatellites Hcms 17, Hcms 25 and Hcms 28, visualised on a 2 % agarose gel. For Hcms 17 each single worm was amplified in duplicate and run in adjacent wells. Template consisted of single adult male *T. circumcincta* from the populations; ScSo529 and Tci1 (MOSI), or from single L3s from the Tci1 (MOSI) population, as indicated. For population information see tables 2.1-2.3.

Figure 4.14: PCR products amplified from single worm panels – *H. contortus* microsatellites

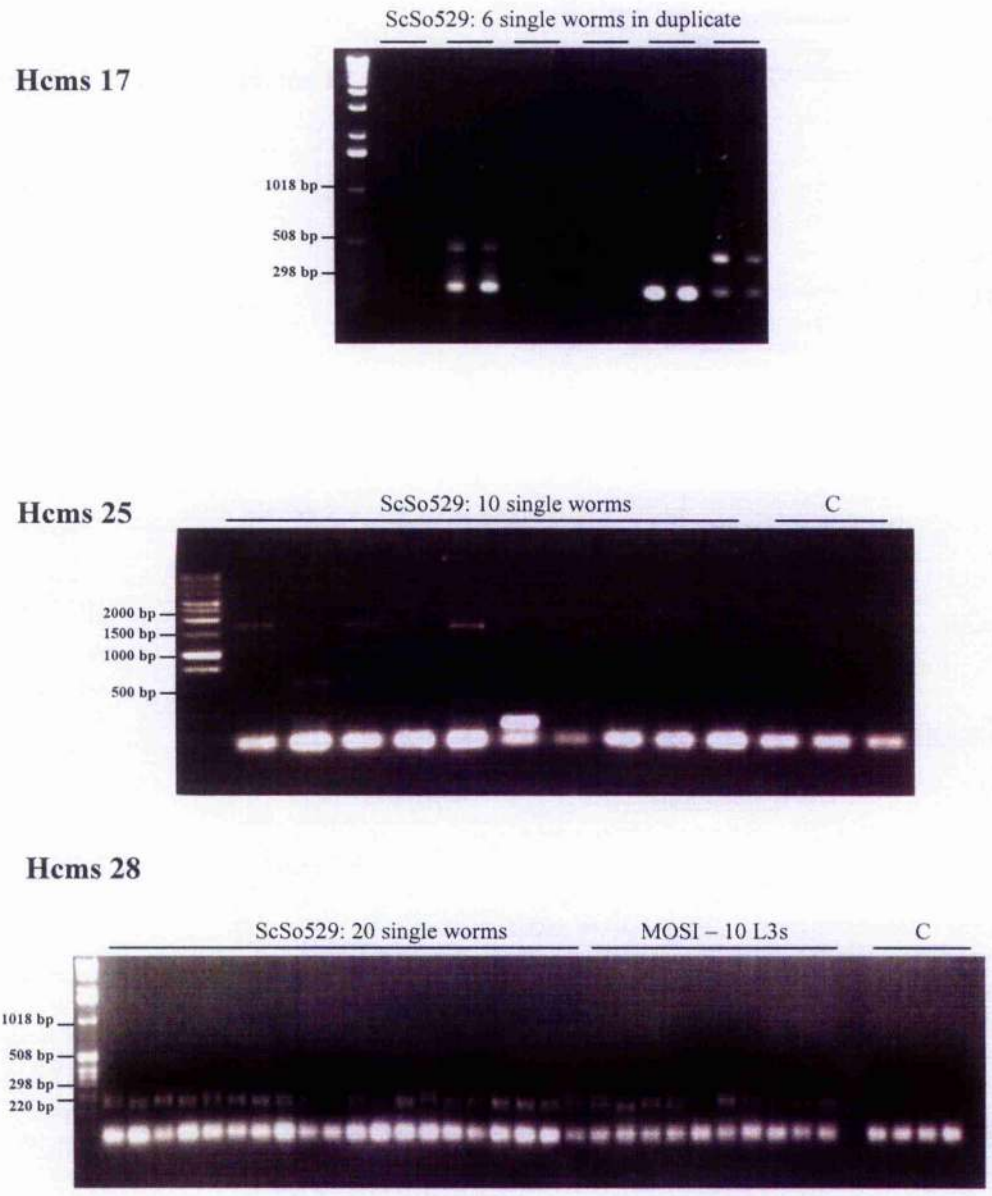


Table 4.5: Summary of results from single worm PCR panel – *H. contortus* microsatellites

Summary of results from single worm PCR panel for *T. circumcincta* orthologues of *H. contortus* microsatellites. Several criteria were used to designate markers as unsuitable for population genetic analysis. The main categories were: a) non-amplification of PCR product and b) inappropriately sized PCR products* compared to sequence data, these included amplification of products at least 200 bp greater than the expected size and amplification or more than two amplicons, which would be inconsistent with amplification from single worms. ScSo529, Tci 1 (MOSI) and FrMe indicate the *T. circumcincta* populations from which single worms were used. See table 2.1-2.3 for more information on populations.

Microsatellite	Association with HcRep	Single worm amplification			
		No. of worms tested	Products of expected size	Inappropriately sized products*	Non-amplification
Hcms 19	-	5 FrMe	-	5	-
		5 ScSo529	-	1	4
		5 MOSI	-	5	-
Hcms 22	-	5 FrMe	1	3	1
		5 ScSo529	2	1	2
		5 MOSI	3	2	-
Hcms 23	-	5FrMe	-	-	5
		5 ScS0529	-	-	5
		5 MOSI	-	2	3
Hcms 27	-	5FrMe	-	3	2
		5 ScSo529	1	-	4
		5 MOSI	-	3	2
Hcms 38	-	5FrMe	-	1	4
		5 ScSo529	-	1	4
		5 MOSI	-	5	-

Table 4.6: Summary table of microsatellites isolated from EST search

Summary of microsatellites isolated from the *T. circumcincta* EST database including the EST database cluster number, the full microsatellite repeat sequence and TRF data given for each microsatellite. TRF = Tandem Repeat Finder program. TRF found repeat arrays and calculated the number of repeats found (Copy Number) and the percentage match to a perfect array of the repeat type (Percent Match). * MTG 71 contains a compound microsatellite made up of a stretch of GTG upstream to a stretch of ACC, separated by 7 bp.

Microsatellite	Cluster Number	Full microsatellite repeat found by Tandem Repeat Finder	TRF Repeat size & type	TRF Copy Number	TRF Percent Match
MTG 60	TDC00331	$(TAAA)_7TA$	TAAA	7.5	100%
MTG 61	TDC00506	$(AGG)_2$AGG AAA AGA AGG AAG $(AGA)_2$ $(AGG)_2$ $(AGA)_2$ $(AGG)_2$ AGA ATA $(AGG)_3$ $(AGA)_2$ $(AGG)_2$ $(AGA)_2$ AGG AAG $(AGA)_2$ $(AGG)_3$ AAA AGA $(AGG)_2$ $(AGA)_2$ AGG AAG $(AGA)_2$ AGG AAG AGG $(AGA)_2$	AGG	49.3	73%
MTG 62	TDC00786	$(CCA)_2CCC(CCA)_2(CCT)_2(CCA)_8$	CAA	17	91%
MTG 63	TDC01068	$(ACA)_3$ $(GCA)_2$ GCC $(ACA)_2$ GCA $(ACA)_5$ GCA $(ACA)_3$	ACA	18	84%
MTG 64	TDC01338	$(GA)_{31}$	GA	31	100%
MTG 65	TDC01687	CCA CCG CCA $(CCT)_2$ CCA CCG $(CCA)_5$ CCG $(CCA)_3$	CCA	16	82%
MTG 66	TDC02355	$(AAAT)_5$ ATAT $(AAAT)_3$	AAAT	9.5	94%
MTG 67	TDC02355	$(CT)_{14}$	CT	14	100%
MTG 68	TDC02250	$(ACA)_2$ $(ACC)_2$ TCG $(ACA)_4$ ACT ACA ACC ACA ACT ACG (ACA $ACC)_2$	ACA	18.7	73%
MTG 69	TDC01786	CCA GCG $(CCG)_2$ CCA $(CCA)_6$ CC	CCA	11.7	87%
MTG 70	TDC00872	AGCC AGTC AGCC CACC AGCC ACCA $(AGCC)_4$	AGCC	10	72%
MTG 71*	TDC00550	$(GTG)_2$ GGT TTG $(GTG)_2$ GCT TTG $(GTG)_2$ GCG TTG $(GTG)_2$ GCT TCG $(GTG)_2$ GT	GTG	18.7	62%
MTG 71*	TDC00550	ACC TCC $(GCC)_3$ $(TCC)_2$ $(ACC)_3$ $(TCC)_3$ $(ACC)_4$ GCC $(ACC)_4$ A	ACC	22.3	87%
MTG 73	TDC00441	$(TGC)_2$ TGT TGC $(TGT)_2$ $(TGA)_3$ TG $(TGA)_2$ TAA $(TGA)_2$ AA ATA $(TGA)_2$	TGA	19.3	74%
MTG 74	TDC00289	$(TGT)_2$ TGC TGA $(TGT)_2$ TGA TGT TGA $(TGT)_4$	TGT	13	80%

Table 4.7: Summary of results from single worm PCR panel – EST search

Summary of results from single worm PCR panel for microsatellites isolated from *T. circumcincta* EST database. MTG 60 had insufficient sequence flanking the microsatellite for primer design. MTG 61 and MTG 63 were not tested on the single worm panel as products amplified from genomic *T. circumcincta* DNA on gradient PCR were > 800 bp, therefore unsuitable for further analysis. Several criteria were used to designate markers as unsuitable for population genetic analysis. The main categories were: a) non-amplification of PCR product and b) inappropriately sized PCR products* compared to sequence data, these included amplification of products at least 200 bp greater than the expected size and amplification or more than two amplicons, which would be inconsistent with amplification from single worms. ScSo529, Tci1 (MOSI) and FrMe indicate the *T. circumcincta* populations from which single worms were used. See table 2.1-2.3 for more information on populations.

Microsatellite	No. of primer sets tested	Single worm amplification			
		No. of worms tested	Products of expected size	Inappropriately sized products*	Non-amplification
MTG 62	4	5 FrMe	1	-	4
		5 ScSo529	4	-	1
		5 MOSI	3	-	2
MTG 64	4	5 FrMe	1	-	4
		5 ScSo529	1	-	4
		5 MOSI	3	-	2
MTG 65	1	5FrMe	5	-	-
		5 ScS0529	5	-	-
		5 MOSI	4	-	1
MTG 66	1	5FrMe	-	-	5
		5 ScS0529	3	-	2
		5 MOSI	1	-	4
MTG 67	1	5FrMe	5	-	-
		5 ScSo529	5	-	-
		5 MOSI	5	-	-
MTG 68	4	5FrMe	5	-	-
		5 ScSo529	5	-	-
		5 MOSI	5	-	-
MTG 69	1	5FrMe	5	-	-
		5 ScSo529	5	-	-
		5 MOSI	5	-	-
MTG 70	1	5FrMe	-	2	3
		5 ScSo529	1	-	4
		5 MOSI	1	-	4
MTG 71	4	5FrMe	4	-	-
		5 ScSo529	3	-	-
		5 MOSI	3	-	-
MTG 73	4	5FrMe	5	-	-
		5 ScSo529	5	-	-
		5 MOSI	5	-	-
MTG 74	4	4 FrMe	4	-	-
		4 ScSo529	4	-	-
		5 MOSI	4	-	1

Figure 4.15: PCR products amplified from single worms using the eight microsatellites selected for further analysis

PCR products amplified from single adult *T. circumcincta* males or L3s from the ScSo529, Tci1 (MOSI) and FrMe populations using the microsatellites MTG 15, Hcms 28, MTG 65, MTG 67, MTG 68, MTG 69, MTG 73 and MTG 74, visualised on a 2 % agarose gel. The microsatellites MTG 65, MTG 67, MTG 68, MTG 69, MTG 73 and MTG 74 show PCR products amplified from 5 single adult *T. circumcincta* males from each of the three populations. C = controls using dH_2O in replacement for template. For population information see tables 2.1-2.3. The predicted size of the amplified products for the original sequences was; MTG 15 = 256 bp, Hcms 28 = 185 bp, MTG 65 = 213 bp, MTG 67 = 194 bp, MTG 68 = 133 bp, MTG 69 = 140 bp, MTG 73 = 157 bp, and MTG 74 = 162 bp.

Figure 4.15: PCR products amplified from single worms using the eight microsatellites selected for further analysis

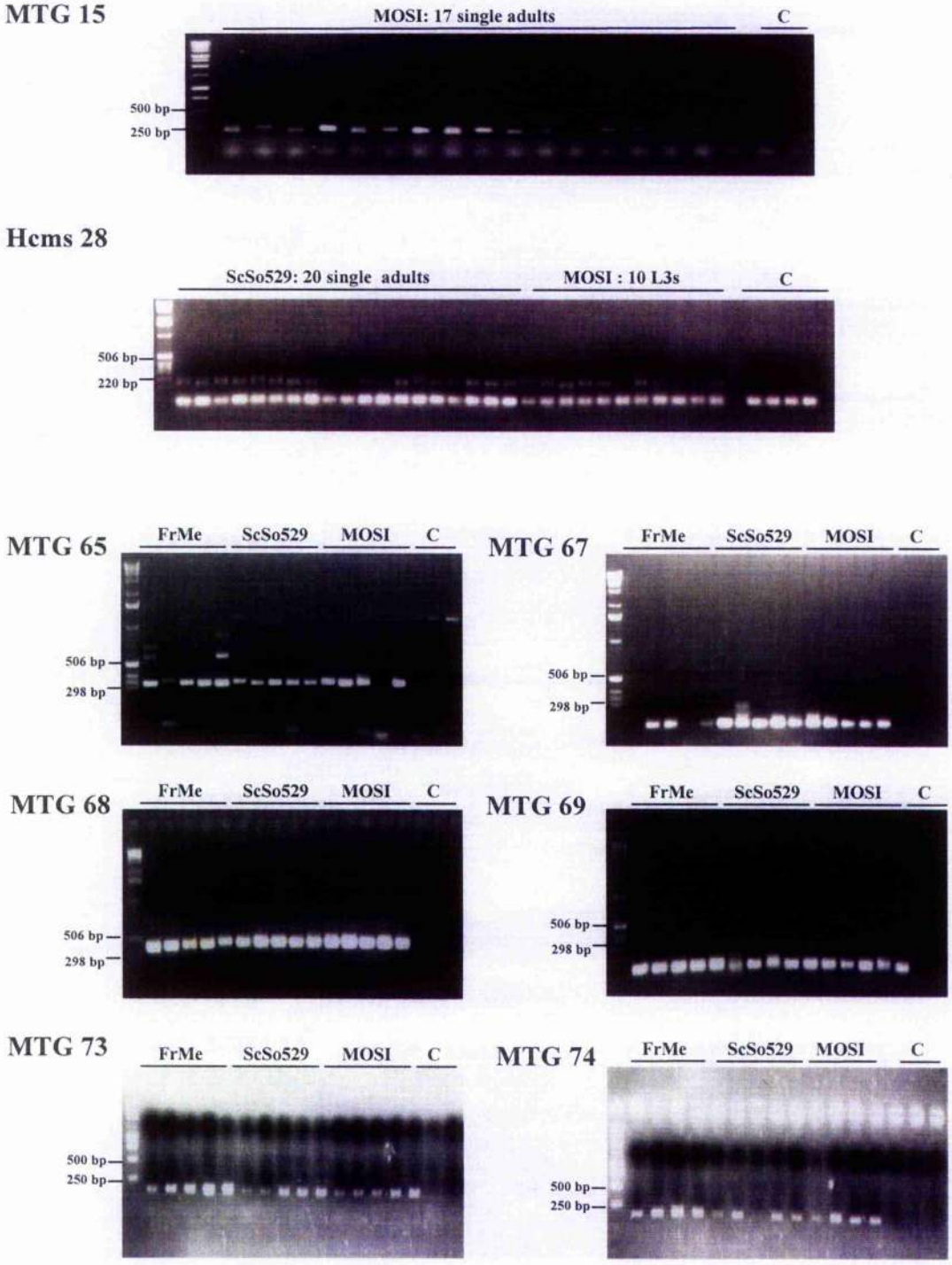


Table 4.8: Polymorphism of eight microsatellites selected as potential population genetic markers

The table shows a summary of the eight microsatellites assessed for polymorphism. Polymorphism of each microsatellite is based on genotyping three populations; Tci1 (MOSI) (25 males), ScSo529 (30 males) and FrMe (33 males). Forward primers flanking these eight microsatellites were synthesised with a FAM (Blue) or HEX (Green) 5' fluorescent dye label end (MWG). * expected size for *H. contortus* (Otsen *et al.* 2000). ** Binning ranges for individual alleles were ambiguous and so the number of alleles for MTG 68 is approximate. # five alleles were identified, however one allele (122) had a mean frequency of 0.96 based on the three populations genotyped.

Microsatellite	Type of repeat	Flourescent Dye	Annealing Temperature	Size expected (bp)	Size range of alleles (bp)	No. of alleles	Method of isolation
MTG 15	GT / CA	FAM	58°C	256	233-275	19	gDNA screening
Hcms 28	GT / CA	FAM	55°C	185*	155-189	12	*H. contortus*
MTG 65	CCA / GGT	HEX	50°C	213	322-357	10	EST
MTG 67	CT / GA	HEX	58°C	194	172-192	10	EST
MTG 68	ACA / TGT	FAM	50°C	133	420-453	10**	EST
MTG 69	CCA / GGT	FAM	58°C	140	122-131	5#	EST
MTG 73	TCA / AGT	HEX	45°C	157	148-157	4	EST
MTG 74	ACA / TGT	FAM	45°C	162	156-186	6	EST

Figure 4.16: Example Genescan chromatograms for the eight microsatellite markers selected as potential population genetic markers

Example Genescan chromatograms corresponding to products amplified from single adult male *T. circumcincta* using the microsatellites: MTG 15, Hcms 28, MTG 67, MTG 68, MTG 69, MTG 73, MTG 74 and MTG 65. The first seven boxes show examples of a homozygote (Upper) and heterozygote (Lower) for each microsatellite. MTG 69 was monomorphic, therefore no heterozygotes could be demonstrated. MTG 65 shows an individual amplifying three products (Upper; green arrow), a heterozygote (Middle) and homozygote (Lower). The non-template addition of an adenosine base by Taq polymerase at the 3' end of the PCR product can result in a doublet peak for each allele, referred to as a 'plus-A effect' (Breen *et al.* 1999). A 'plus-A effect' peak is demonstrated in Hcms 28 and MTG 73.

Figure 4.16: Example Genescan chromatograms for the eight microsatellite markers selected as potential population genetic markers

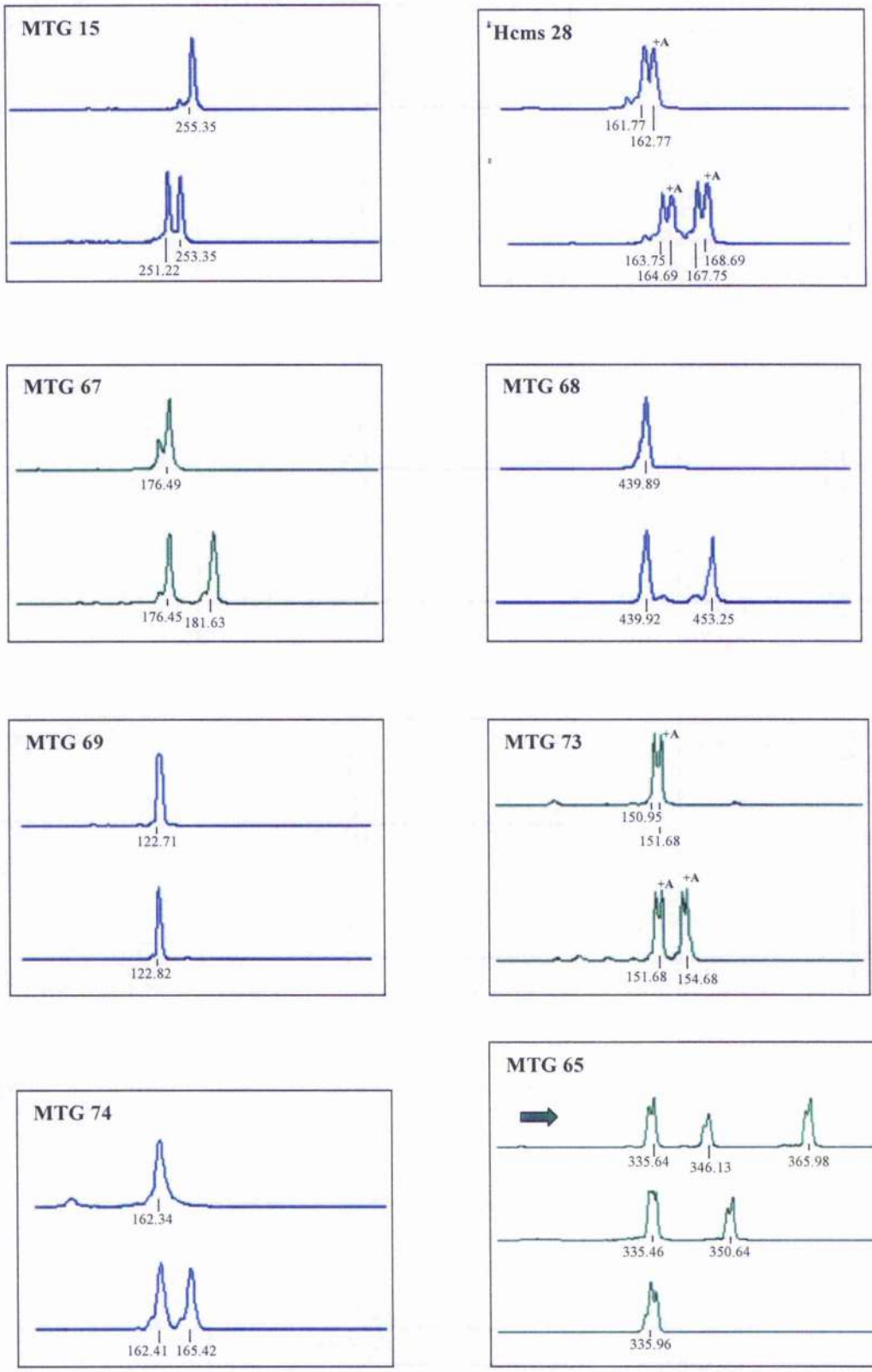


Figure 4.17a: Allele sequencing for Hcms 28

A schematic representation of individual alleles amplified using Hcms 28 from the individual adult *T. circumcincta* males; 1E4 and 1A7 (Population FrMe) and from Tci1 (MOSI) genomic DNA (gDNA). A = forward primer and B = reverse primer. Genescan size corresponds to allele bin in which the chromatogram reading was binned. The Sequence size refers to the exact numbers of base pairs calculated from sequence data. The lower figure shows the corresponding alignments of these sequences. Orange font – poor sequence read, Light blue shading - microsatellite sequence alignment, Yellow shading - base pair mismatch between sequences.

Figure 4.17a

Hcms 28

Schematic representation	Genescan Size	Sequence size
1E4 A → (GTCT) $(GT)_5$ - 85bp - $(GT)_6$ ← B	163	166
1A7 A → $(GT)_6$ - 85bp - $(GT)_8$ ← B	165	168
gDNA A → $(GT)_8$ - 85bp - $(GT)_6$ ← B	NK	168

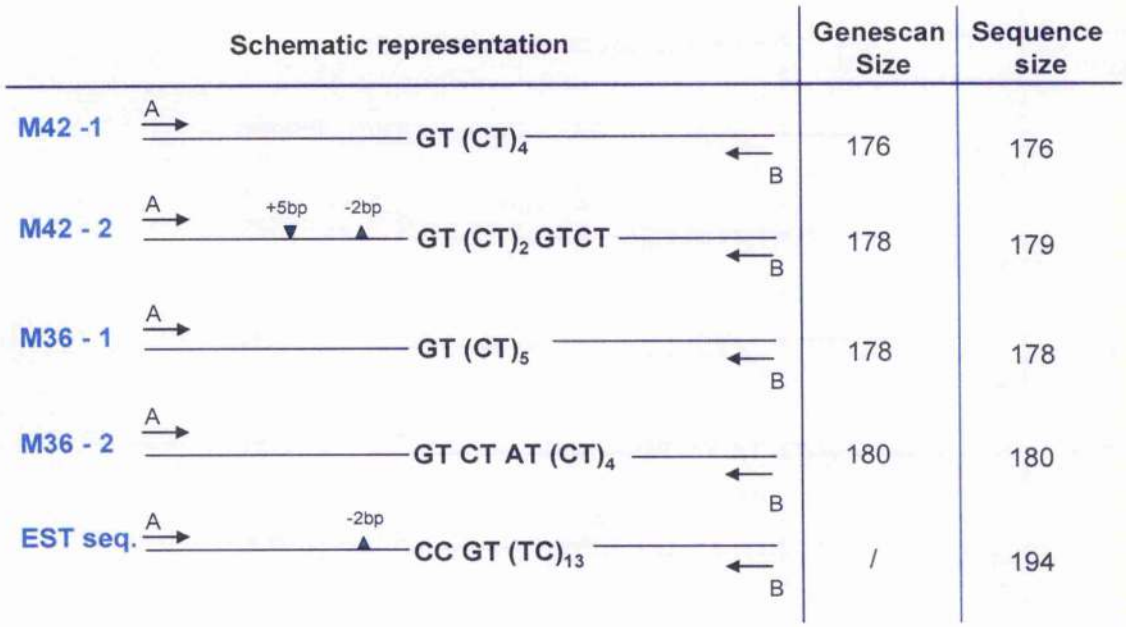
```
                 Hcms 28b primer
1E4    CCGACTAATCACTTCTTGTTTGTCATTTTCTGTCTGTGTGTGTGT--(44)
1A7    CCGACTAATCACTTCTTGTTTGTCGTTTTCTGTGTGTGTGTGT----(43)
gDNA   CCGACTAATCACTTCTTGTTTGTCGTTTTCTGTGTGTGTGTGTGT(47)

1E4    GCTCTGTTGTGGAAGGCGGTCTCTGCTATTGGCGGTGGCGTATCCGT(47)
1A7    GCTCTGTTGTGGAAGGCGGTCTCTGCTATTGGCGGTGGCGTATCCGT(47)
gDNA   GCTCTGTTGTGGAAGGCGGTCTCTGCTATTGGCGGTGGCGTATCCGT(47)

1E4    CCGTGTGGTGCAGTGCTGCGTCCTCTTTGATTGCTTGCCTGTGTGT(46)
1A7    CCGTGTGGTGCAGTGCTGCGTCCTCTTTGATTGCTTGCCTGTGTGT(46)
GDNA   CCGTGTGGTGCAGTGCTGCGTCCTCTTTGATTGCTTGCCTGTGTGT(46)

1E4    GTGTGT----TTGCTCTCTCTCATCTCCACAC(28)      (166)
1A7    GTGTGTGTGTTTGCTCTCTCTCATC       (25+7)    (168)
gDNA   GTGTGT----TTGCTCTCTCTCATCTCCACAC(28)      (168)
                      Hcms 28a primer
```

Figure 4.17b: Allele sequencing for MTG 67

A schematic representation of individual alleles amplified using MTG 67 from the individual adult *T. circumcincta* males; M42 and M36 (Population ScSo529), in addition to the EST sequence from the database. M42-1, M42-2 and M36-1 and M36-2 correspond to the two alleles amplified from the same individual worm, M42 and M36, respectively. A = forward primer and B = reverse primer. Indels: ▲ = sequence deleted and ▼ = sequence inserted Genescan size corresponds to allele bin in which the chromatogram reading was binned. The Sequence size refers to the exact numbers of base pairs calculated from sequence data. The lower figure shows the corresponding alignments of these sequences. Orange font – poor sequence read, Light blue shading - microsatellite sequence alignment, Yellow shading - base pair mismatch between sequences.

Figure 4.17b

MTG 67



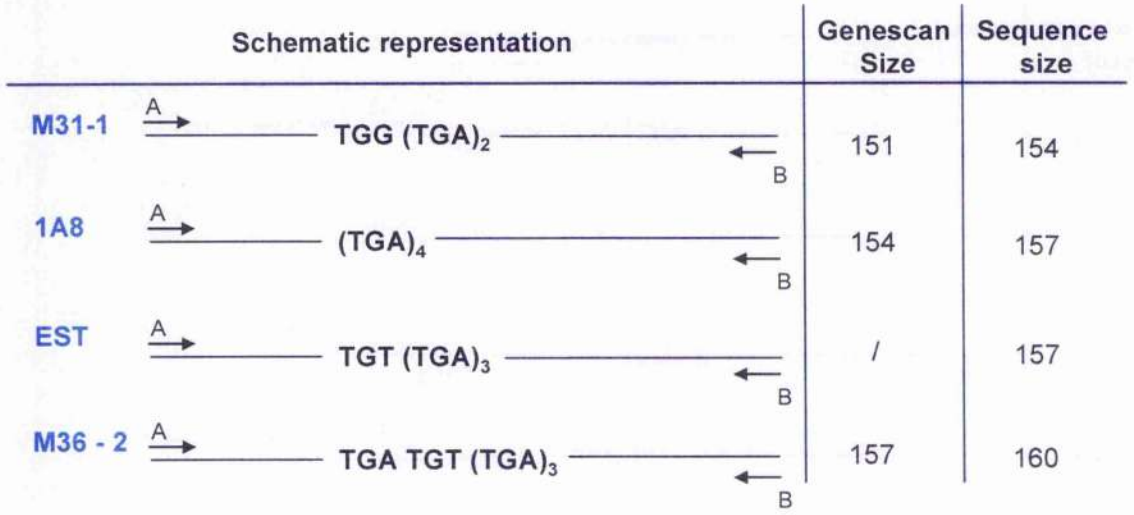

```
          Pr67a primer
M42-1 CAAGTCGTTTAGGCACGTCTGGCTACATTATTCTGCTCGTCTTAGC(46)
M42-2 CAAGTCGTTTAGGCACGTCTGGCCACATTATTCTGCTCGTTTTAGC(46)
M36-1 CAAGTCGTTTAGGCACGTCTGGCCACATTATTCTGCTCATCTTAGC(46)
M36-2 CAAGTCGTTTAAGCACGTCTGGCTACATTATTCTGCTCGTCTTAGC(46)
EST   CAAGTCGTTTAGGCACGTCTGGCCACATTATTCTGCTCGTCTCAGC(46)

M42-1 TAT-----CTAAGCTGGTGATCGCTTTCCCG--------------(26)
M42-2 TATGCTATCTAAGCTGGTGAT--CTTCTCCC--------------(29)
M36-1 TAT-----CTAAGCTGGTGATCGCTTCCCCG--------------(26)
M36-2 TAT-----CTAAGCTGGTGATCGCTTCCCCG--------------(26)
EST   TAT-----CTAAGCTGGTGAT--CTTCCCCCCTCTCTCTCTCTCT(39)

M42-1 -TC----TCTCTCTCGTTGATGACTCGCTCTCCAACTCTGAAACTT(41)
M42-2 -TC----TCTCTCTCGCTGATGACTCGCTCTTCAACTCTGCAACTT(41)
M36-1 -TC--TCTCTCTCTCGTTGATGACTCGCTCTTCAACTCTGAAACTT(43)
M36-2 -TCTATCTCTCTCTCGTTGATGACTCGCTCTTCAACTCTGAAACTT(45)
EST   CTCTCTCTCTCTCTCGTTGAAGACTCGCTCTTCAACTCTGAAACTT(46)

M42-1 TTCATCGGTGTCATGCTCTCCTTGTCGACGGGTTTATTTACCGATC(46)
M42-2 TTCATCGGTGTCATGCTCTCCTTGTCGACAGGTTTATTTACCGATC(46)
M36-1 TTCATCGGTGTCATGCTCTCCTTGTCGGCGGGTTTATTTACCGATC(46)
M36-2 TTCATCGGTGTCATGCTCTCCTTGTCGACGGGTTTATTTACCGATC(46)
EST   TTCATCGGTGTCATGCTCTCCTTGTCGGCGGGTTTATTTACCGATC(46)

M42-1 AATTGGGTTCCGCCCTG(17)    (176)
M42-2 AATTGGGTTCCGCCCTG(17)    (179)
M36-1 AATTGGGTTCCGCCCTG(17)    (178)
M36-2 AATTGGGTTCCGCCCTG(17)    (180)
EST   AATTGGGTTCCGCCCTG(17)    (194)
        Pr67b primer
```

Figure 4.17c: Allele sequencing for MTG 73

A schematic representation of individual alleles amplified using MTG 73 from the individual adult *T. circumcincta* males; M31, M36 (Population ScSo529), and 1A8 (Population FrMe), in addition to the EST sequence from the database. M31-1 and M31-2 correspond to the two alleles amplified from the same individual worm, M31. A = forward primer and B = reverse primer. Genescan size corresponds to allele bin in which the chromatogram reading was binned. The Sequence size refers to the exact numbers of base pairs calculated from sequence data. The lower figure shows the corresponding alignments of these sequences. Light blue shading - microsatellite sequence alignment, Yellow shading - base pair mismatch between sequences.

Figure 4.17c

MTG 73




```
          Pr73d primer
M31-1 GTAGTAGTGATTAACTTCCGTCATCATCCTTTTAGATAACCGCCATGGT(49)
1A8   GTAGTAGTGATTAACTTCCGTCATCATCCTTTTAAATAATCGCCATGGT(49)
EST   GTAGTAGTGATTAACTTCCGTCATCATCCTTTTAAATAAACGCCATGGT(49)
M31-2 GTAGTAGTGATTAACTTCCGTCATCATCCTTTTAAATAAACGCCATGGT(49)

M31-1 CACCAAAATGCTGCTGTTGCTGTTG------GTGATGATGTGATGATAA(43)
1A8   CACCAAAATGCTGCTGTTGCTGTTGA---TGATGATGATGTGATGATAA(46)
EST   CACCAAAATGCTGCTGTTGCTGTTG---TTGATGATGATGTGATGATAA(46)
M31-2 CACCAAAATGCTGCTGTTGCTGTTGATGTTGATGATGATGTGATGATAA(49)

M31-1 TGATGAAAATATGATGAGAGAACTAAGAACTCGAGAGAGAACGGCTTCG(49)
1A8   TGATGAAAATATGATGAGAGAACTAAGAACTCGAGAGAGAACGGCTTCG(49)
EST   TGATGAAAATATGATGAGAGAACTAAGAACTCGAGAGAGAACGGCTTCG(49)
M31-2 TGATGAAAATATGATGAGAGAACTAAGAACTCGAGAGAGAACGGCTTCG(49)

M31-1 AATTTATACAAGG(13)        (154)
1A8   AATTTATACAAGG(13)        (157)
EST   AATTTATACAAGG(13)        (157)
M31-2 AATTTATACAAGG(13)        (160)
       Pr73a primer
```

Figure 4.17d: Allele sequencing for MTG 74

A schematic representation of individual alleles amplified using MTG 74 from the individual adult *T. circumcincta* males; M26 (Population ScSo529) and the EST sequence from the database. A = forward primer and B = reverse primer. Genescan size corresponds to allele bin in which the chromatogram reading was binned. The Sequence size refers to the exact numbers of base pairs calculated from sequence data. The lower figure shows the corresponding alignments of these sequences. Light blue shading - microsatellite sequence alignment, Yellow shading - base pair mismatch between sequences.

Figure 4.17d

MTG 74

Schematic representation	Genescan Size	Sequence size
M26-1 A → TGT CGT $(TGT)_2$ ← B	159	162
EST A → $(TGT)_4$ ← B	/	162
M26-2 A → $(TGT)_6$ ← B	165	168

```
          Pr74b primer
M26-1 TCAATAACGAACTAAATATGGACAAAGAAATTTGGTTTAGTGTTGTTGC(49)
EST   TCAATAACGAACTAAATATGGACAAAGAAATTTGGTTTAGTGTTGTTGC(49)
M26-2 TCAATATCGAACTAAATATGGACAAAGAAATTTGGTTTAGTGTTGTTGC(49)

M26-1 TAATGTTGTTGATGTTGATGTCGTTGTT------GTTATTGAAGCACTC(43)
EST   TGATGTTGTTGATGTTGATGTTGTTGTT------GTCATTGAAGCACTC(43)
M26-2 TGATGTTGTTGATGTTGATGTTGTTGTTGTTGTTATTGAAGCACTC(49)

M26-1 TGATGTCTCCTCCCTCCTCGATTTCATGCCCACACATGCCTAGTACTC(48)
EST   TGATGTCTCCTCCCTCCTCGATTTCATGCCCACACATGCCTAGTACTC(48)
M26-2 TGATGTCTCCTCCCTCCTCGATTTCATGCCCACACATGCCTAGTACTC(48)

M26-1 TTCGCTGTACCACGAGTCCATC(22)     (162)
EST   TTCGCTGTACCACGAGTCCATC(22)     (162)
M26-2 TTCGCTGTACCACGAGTCCATC(22)     (168)
          Pr74c primer
```

Figure 4.17e: Schematic diagram for MTG 15 allele sequencing

A schematic representation of individual alleles amplified using MTG 15 from the individual adult *T. circumcincta* males; M25, M21, M23 (Population ScSo529) and K23, K25, K22 and K5 (Population Tci1 (MOSI)), in addition to the original microsatellite clone sequence. A = forward primer and B = reverse primer. Genescan size corresponds to allele bin in which the chromatogram reading was binned. Indels: ▲ = sequence deleted and ▼ = sequence inserted. The Sequence size refers to the exact numbers of base pairs calculated from sequence data assuming 64 bp from the end of the sequence obtained, to the end of the pr15b primer. Figure 3.17e shows the corresponding alignments of these sequences.

Figure 4.17e

MTG 15

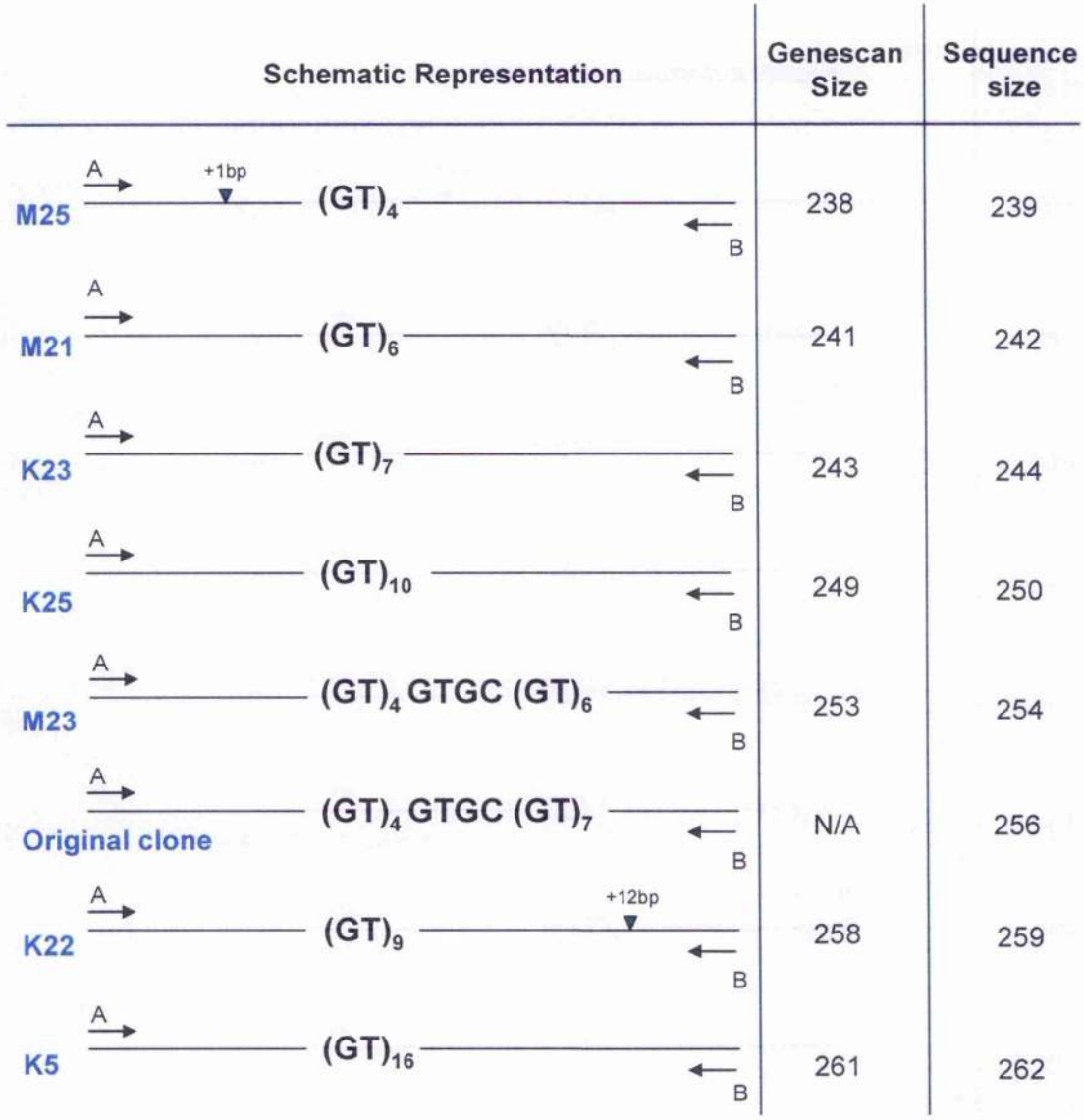


Figure 4.17f: Allele sequencing for MTG 15

The corresponding sequence alignments for Figure 3.17d for MTG 15. Orange font – poor sequence read, Light blue shading - microsatellite sequence alignment, Yellow shading - base pair mismatch between sequences.

Figure 4.17f

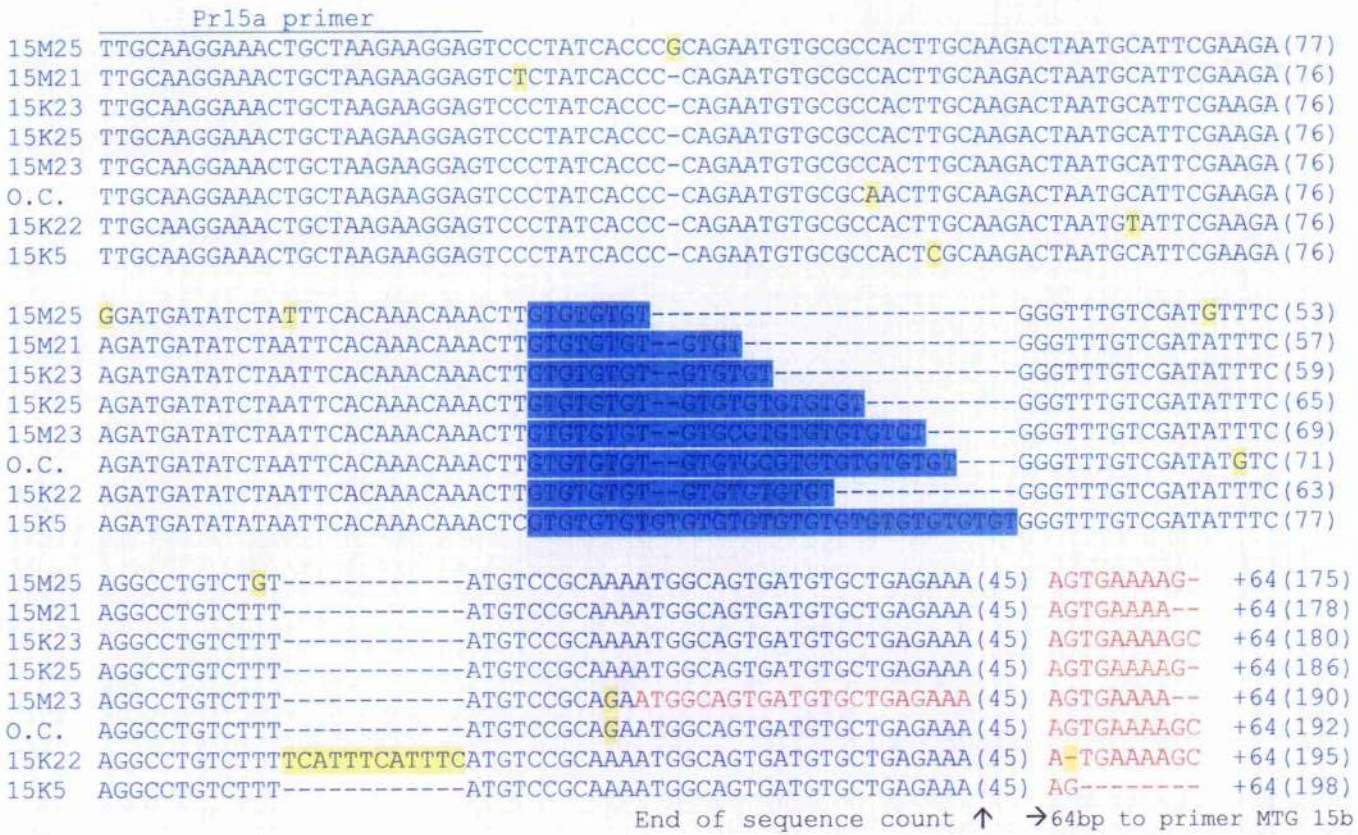

Pr15a primer
15M25 TTGCAAGGAAACTGCTAAGAAGGAGTCCCTATCACCCGCAGAATGTGCGCCACTTGCAAGACTAATGCATTCGAAGA(77)
15M21 TTGCAAGGAAACTGCTAAGAAGGAGTCTCTATCACCC-CAGAATGTGCGCCACTTGCAAGACTAATGCATTCGAAGA(76)
15K23 TTGCAAGGAAACTGCTAAGAAGGAGTCCCTATCACCC-CAGAATGTGCGCCACTTGCAAGACTAATGCATTCGAAGA(76)
15K25 TTGCAAGGAAACTGCTAAGAAGGAGTCCCTATCACCC-CAGAATGTGCGCCACTTGCAAGACTAATGCATTCGAAGA(76)
15M23 TTGCAAGGAAACTGCTAAGAAGGAGTCCCTATCACCC-CAGAATGTGCGCCACTTGCAAGACTAATGCATTCGAAGA(76)
O.C. TTGCAAGGAAACTGCTAAGAAGGAGTCCCTATCACCC-CAGAATGTGCGCAACTTGCAAGACTAATGCATTCGAAGA(76)
15K22 TTGCAAGGAAACTGCTAAGAAGGAGTCCCTATCACCC-CAGAATGTGCGCCACTTGCAAGACTAATGTATTCGAAGA(76)
15K5 TTGCAAGGAAACTGCTAAGAAGGAGTCCCTATCACCC-CAGAATGTGCGCCACTCGCAAGACTAATGCATTCGAAGA(76)
15M25 GGATGATATCTATTTCACAAACAAACTTGTGTGTGT----------GGGTTTGTCGATGTTTC(53)
15M21 AGATGATATCTAATTCACAAACAAACTTGTGTGTGT--GTGT----------GGGTTTGTCGATATTTC(57)
15K23 AGATGATATCTAATTCACAAACAAACTTGTGTGTGT--GTGTGT----------GGGTTTGTCGATATTTC(59)
15K25 AGATGATATCTAATTCACAAACAAACTTGTGTGTGT--GTGTGTGTGTGT----------GGGTTTGTCGATATTTC(65)
15M23 AGATGATATCTAATTCACAAACAAACTTGTGTGTGT--GTGCGTGTGTGTGTGT------GGGTTTGTCGATATTTC(69)
O.C. AGATGATATCTAATTCACAAACAAACTTGTGTGTGT--GTGTGCGTGTGTGTGTGT----GGGTTTGTCGATATGTC(71)
15K22 AGATGATATCTAATTCACAAACAAACTTGTGTGTGT--GTGTGTGTGT----------GGGTTTGTCGATATTTC(63)
15K5 AGATGATATATAATTCACAAACAAACTCGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGGGTTTGTCGATATTTC(77)
15M25 AGGCCTGTCTGT----------ATGTCCGCAAAATGGCAGTGATGTGCTGAGAAA(45) AGTGAAAAG- +64(175)
15M21 AGGCCTGTCTTT----------ATGTCCGCAAAATGGCAGTGATGTGCTGAGAAA(45) AGTGAAAA-- +64(178)
15K23 AGGCCTGTCTTT----------ATGTCCGCAAAATGGCAGTGATGTGCTGAGAAA(45) AGTGAAAAGC +64(180)
15K25 AGGCCTGTCTTT----------ATGTCCGCAAAATGGCAGTGATGTGCTGAGAAA(45) AGTGAAAAG- +64(186)
15M23 AGGCCTGTCTTT----------ATGTCCGCAGAATGGCAGTGATGTGCTGAGAAA(45) AGTGAAAA-- +64(190)
O.C. AGGCCTGTCTTT----------ATGTCCGCAGAATGGCAGTGATGTGCTGAGAAA(45) AGTGAAAAGC +64(192)
15K22 AGGCCTGTCTTTTCATTTCATTTCATGTCCGCAAAATGGCAGTGATGTGCTGAGAAA(45) A-TGAAAAGC +64(195)
15K5 AGGCCTGTCTTT----------ATGTCCGCAAAATGGCAGTGATGTGCTGAGAAA(45) AG-------- +64(198)
End of sequence count ↑ →64bp to primer MTG 15b

Figure 4.17g: Allele sequencing for MTG 68

A schematic representation of individual alleles amplified using MTG 68 from the individual adult *T. circumcincta* males; M30 (Population ScSo529) and the EST sequence from the database. A = forward primer and B = reverse primer. Genescan size corresponds to allele bin in which the chromatogram reading was binned. ▼ = sequence insertions. The Sequence size refers to the exact numbers of base pairs calculated from sequence. Light blue shading - microsatellite sequence alignment, Yellow shading - base pair mismatch between sequences data. Black arrows indicate the start and end of intron sequence.

Figure 4.17g

MTG 68

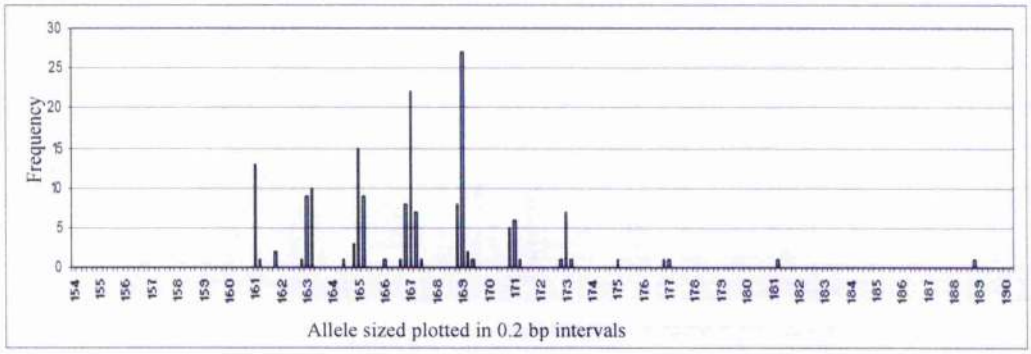
	Schematic representation	Genescan Size	Sequence size
EST	A → $(ACA)_2(ACC)_2TCG\ (ACA)_4\ ACT\ ACA\ ACC\ ACA\ ACT\ ACG\ (ACA\ ACC)_2$ ← B	/	153
M30-3	A → +238 bp ▼ $(ACA)_2(ACC)_2TCG\ (ACA)_3\ (ACT)_2\ ACA\ ACT\ ACA\ ACC\ ACG\ (ACA\ ACC)_2$ +52 bp ▼ ← B	441	443

```
        Pr68c primer      Start of Intron ↓
EST     ATCACCAGGCGGCTGCTACGTCAAGCACAG----------------------------------------------------------------------(30)
M30-3   ATCACCAGGCGGCTGCTACGTCAAGCACAGGCAAGTCGTCAATGTTTTGCAGAAGAGGGGTGCTGGACGGGTGTCGAATCAACTGCGACTGATTT(95)

EST     ----------------------------------------------------------------------------------------------(30)
M30-3   ATTAGCTCCTGGCCTGCTTACCTGCAATATCTTTTATTACCCTTCCAACTACCTGTGTACGGGTAATTGTGGAATGTCTGTGGTTGCTTTTTTGT(190)

                                                                                  ↓ End of Intron
EST     ------------------------------------------------------------------------GAGAAATGTCGTACAAC(47)
M30-3   TCGGGTATGGATGGAGATGGAGAGAAAACTGTGAGCAGTCACTTGAAACAGCCCTCTAGGATATTCTGGTTCTTTCAGGAGAAATGTCGTACAAC(285)

                                                                  Start of Intron ↓
EST     AACCACCTCGACAACAACAACAACTACAACCACAACTACGACAACCACAACCACGAATCCAATTACTG--------------------------(115)
M30-3   AACCACCTCGACAACAACAACTACTACAACTACAACCACGACAACCACAACCACGAATCCAATTACCGGTACTGAAGCGCATTTTTTTGTCCTTC(380)

                                  ↓ End of Intron
EST     -------------------------GATGTGAAATGGATCTGGTGCTCATACTCGACTTTTCG(153)
M30-3   CTAGGAAATACCTATTATTTTCCAGGATGTGAAATGGATCTGGTGCTCATACTCGACTTTTCG(443)
                                                  Pr68d primer
```

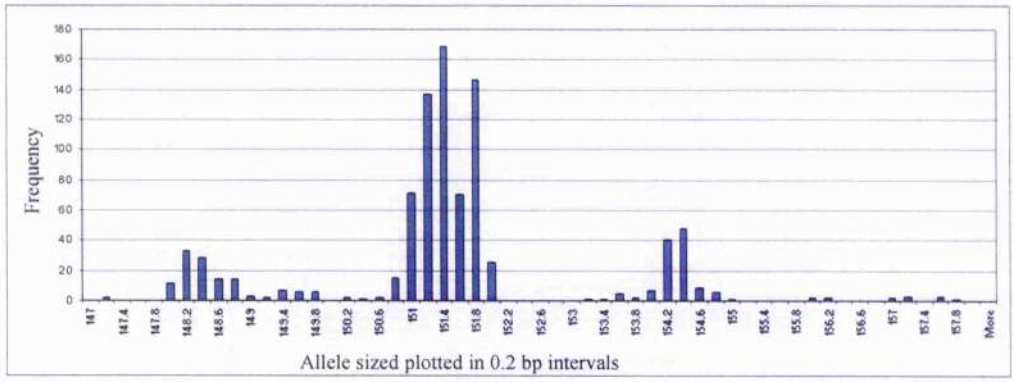
Figure 4.18: Allele binning for the microsatellites Hcms 28, MTG 73 and MTG 74
The figure shows histograms for the microsatellites Hcms 28, MTG 73 and MTG 74, demonstrating allele binning. Each individual allele sized, based on the decimal point reading from Genotyper, was plotted at 0.2 bp or 0.5 bp intervals in order to ascertain to appropriate allele size in which to bin data. All 896 alleles (from 448 individuals genotyped) plotted. The plots demonstrate clear allele bins for each microsatellite.

Figure 4.18: Allele binning for the microsatellites Hcms 28, MTG 73 and MTG 74

Hcms 28



MTG 73



MTG 74

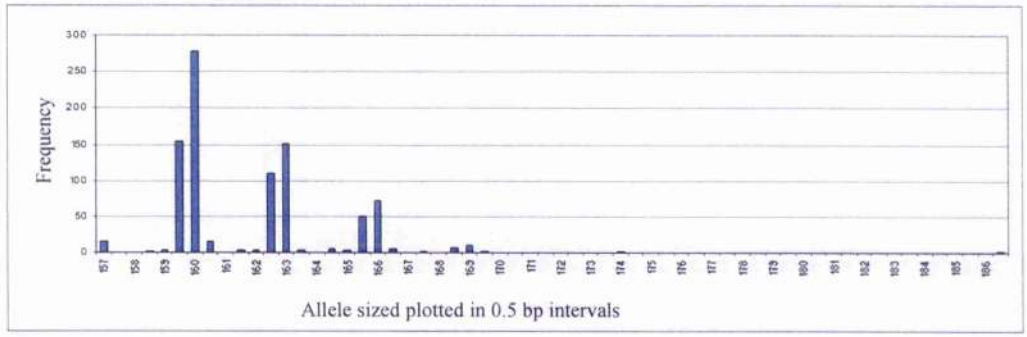
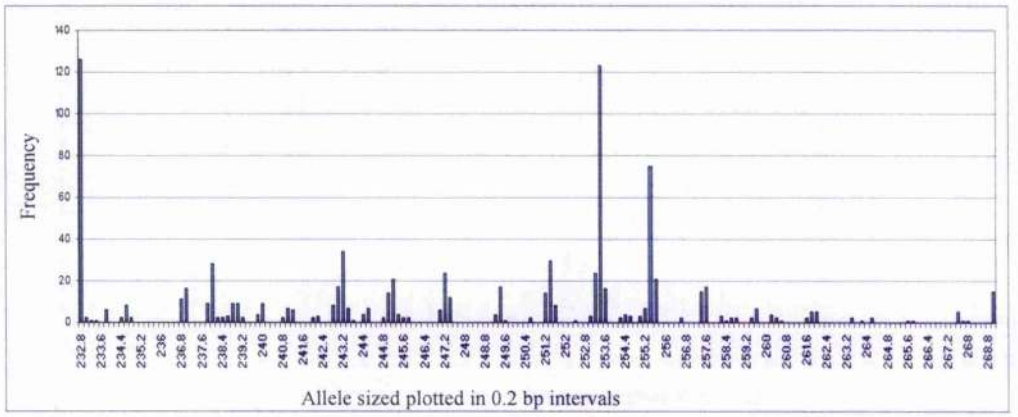


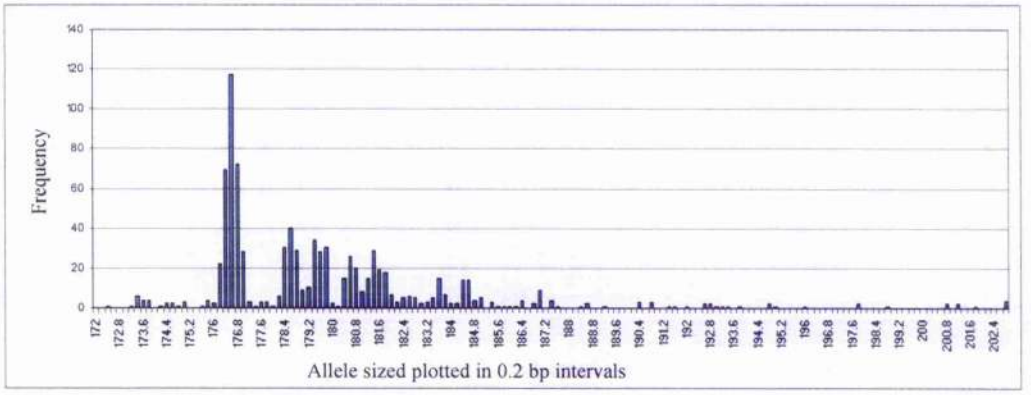
Figure 4.19: Allele binning for the microsatellites MTG 15, MTG 67 and MTG 68
The figure shows histograms for the microsatellites MTG 15, MTG 67 and MTG 68, demonstrating allele binning. Each individual allele sized, based on the decimal point reading from genotyper, was plotted at 0.2 bp, in order to ascertain to appropriate allele size, in which to bin data. All 896 alleles (from 448 individuals genotyped) plotted. These microsatellite markers demonstrate alleles which did not fall into obvious bins.

Figure 4.19: Allele binning for the microsatellites MTG 15, MTG 67 and MTG 68

MTG 15



MTG 67



MTG 68

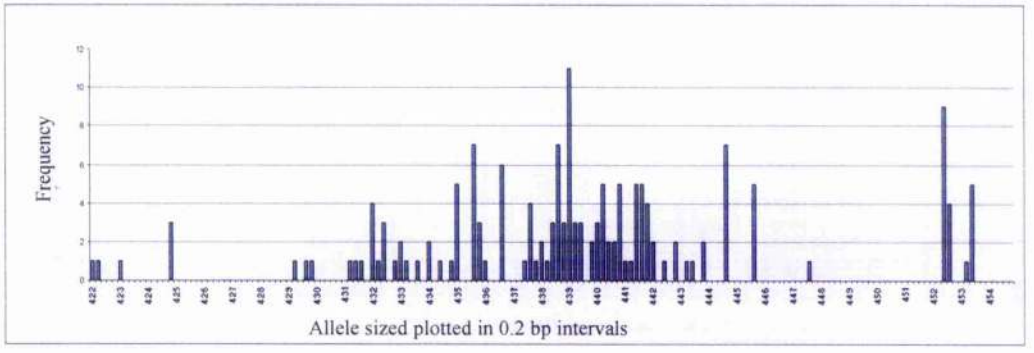


Figure 4.20: Example of numerical sorting for allele binning

This shows an example of numerical sorting performed for MTG 15 in order to find natural breaks in allele sizes and define allele bin sizes. All 896 alleles (from the 448 individuals genotyped) were numerically sorted in ascending order, first by the allele size it was binned as, secondly by the decimal point reading given by Genotyper. Each natural break is indicated by a purple arrow.

Figure 4.20: Example of numerical sorting for allele binning

Worm ID	Binned allele	Genescan Allele
ScSo-529-A-M24	236	236.85
ScSo-529-A-M24	236	236.85
ScSo-529-A-M28	236	236.95
ScSo-529-A-M28	236	236.95
FrGa-5852-A-9	237	237.92
Mosi-1-A-K31	237	237.95
Mosi-1-A-K31	237	237.95
ScSo-529-A-M25	237	238.1
ScSo-529-A-M25	237	238.1
FrGa-5852-A-3	239	239
FrGa-5852-A-3	239	239
FrGa-5852-A-4	239	239.7
FrGa-5852-A-4	239	239.7
Mosi-1-A-K33	241	241
Mosi-1-A-K33	241	241
ScSo-529-A-M21	241	241.01
ScSo-529-A-M21	241	241.01
Mosi-1-A-K40	241	241.16
FrGa-5852-A-8	243	243.02
Mosi-1-A-K35	243	243.15
Mosi-1-A-K36	243	243.16
Mosi-1-A-K36	243	243.16
FrGa-5852-A-10	245	245.19
FrGa-5852-A-7	247	247.14
FrGa-5852-A-2	247	247.15
FrGa-5852-A-6	247	247.18
ScSo-529-A-M27	247	247.24
ScSo-529-A-M30	247	247.27
ScSo-529-A-M27	249	249.21
FrGa-5852-A-6	249	249.21
Mosi-1-A-K32	249	249.32
Mosi-1-A-K34	249	249.36
Mosi-1-A-K39	249	249.36
ScSo-529-A-M23	253	253.16
ScSo-529-A-M23	253	253.16
ScSo-529-A-M29	253	253.17
ScSo-529-A-M29	253	253.17
FrGa-5852-A-1	253	253.25
FrGa-5852-A-1	253	253.25
FrGa-5852-A-5	253	253.26
FrGa-5852-A-10	253	253.33
Mosi-1-A-K38	253	253.37
Mosi-1-A-K38	253	253.37
ScSo-529-A-M26	253	253.4
Mosi-1-A-K35	253	253.47
Mosi-1-A-K34	255	255
FrGa-5852-A-7	255	255.2
FrGa-5852-A-8	255	255.24
ScSo-529-A-M30	255	255.39
ScSo-529-A-M26	255	255.4
ScSo-529-A-M22	255	255.41
Mosi-1-A-K32	255	255.43
FrGa-5852-A-2	257	257.43
ScSo-529-A-M22	257	257.48
Mosi-1-A-K40	259	258.49
Mosi-1-A-K37	259	258.62
Mosi-1-A-K37	259	258.62
FrGa-5852-A-5	259	259.42
FrGa-5852-A-9	259	259.5
Mosi-1-A-K39	265	265.62

Table 4.9: The range, mean and standard deviation of allele sizes

The range, mean and standard deviation of allele sizes calculated for each allelic bin of each marker using Excel 2003. Minimum and maximum values for each bin are also shown, in addition to 'count' (the number of individual alleles on which the data are based). All data incorporates duplicate data generated from homozygote individuals.

Table 4.9: The range, mean and standard deviation of allele sizes

Dinucleotides

MTG 15	227.00	233.00	236.00	237.00	239.00	241.00	243.00	245.00	247.00	249.00	251.00	253.00	255.00	257.00	259.00	261.00	263.00	265.00	267.00	269.00	271.00	273.00	275.00	279.00	286.00	290.00
Mean	227.64	234.03	236.82	237.91	239.19	240.99	243.11	245.09	247.14	249.28	251.24	253.29	255.24	257.36	258.95	261.17	263.68	265.57	267.56	269.60	271.69	273.70	275.70	279.13	285.96	290.08
Standard Deviation		0.61	0.10	0.15	0.53	0.14	0.46	0.20	0.12	0.08	0.18	0.12	0.28	0.22	0.55	0.77	0.46	0.08	0.17	0.14	0.14				0.06	0.11
Minimum		232.81	236.61	237.67	238.55	240.77	241.95	244.79	246.84	249.13	250.49	252.40	254.09	256.55	258.17	260.00	263.22	265.51	267.41	269.51	271.61				285.92	290.00
Maximum		234.71	236.95	238.38	240.00	241.20	244.17	245.63	247.36	249.48	251.60	253.47	255.52	257.59	259.50	261.86	264.13	265.62	267.85	269.81	271.86				286.00	290.15
Count		22.00	27.00	41.00	36.00	15.00	83.00	45.00	42.00	22.00	54.00	167.00	115.00	34.00	17.00	19.00	5.00	2.00	7.00	4.00	3.00				2.00	2.00

Hcms 28	155.00	159.00	161.00	163.00	165.00	167.00	169.00	171.00	173.00	175.00	177.00	179.00	181.00	183.00	185.00	189.00
Mean	155.02	159.07	160.87	162.89	164.84	166.80	168.80	170.77	172.76	174.74	176.73	178.70	180.82	182.98	184.61	188.62
Standard Deviation		0.27	0.23	0.19	0.19	0.14	0.19	0.14	0.13	0.19	0.12	0.08	0.25	0.45	0.10	
Minimum		158.75	160.48	162.57	164.22	166.43	168.43	170.48	172.50	174.43	176.49	178.62	180.57	182.41	184.47	
Maximum		159.92	161.70	163.90	165.85	167.11	169.84	171.13	173.02	175.07	177.00	178.82	181.06	183.44	184.69	
Count		33.00	66.00	98.00	88.00	181.00	231.00	89.00	43.00	25.00	20.00	5.00	4.00	5.00	4.00	

MTG67	172.00	174.00	176.00	178.00	180.00	182.00	184.00	186.00	188.00	190.00	192.00	194.00	196.00	198.00	200.00	202.00	204.00	206.00	210.00
Mean	173.38	174.98	176.54	178.99	181.05	183.11	184.65	186.84	188.60	190.85	192.94	195.06	197.62	198.61	201.02	202.62	205.04	206.84	211.60
Standard Deviation	0.32	0.59	0.27	0.63	0.49	0.54	0.40	0.40	0.36	0.59	0.40	0.62	0.00		0.46				
Minimum	172.35	174.13	175.97	178.04	180.08	182.08	184.18	186.11	188.24	190.23	192.56	194.61	197.62		200.62				
Maximum	173.71	175.76	177.83	179.89	181.89	183.97	185.91	187.52	189.10	191.83	193.70	195.96	197.62		201.73				
Count	16.00	14.00	321.00	219.00	158.00	53.00	44.00	21.00	4.00	9.00	8.00	4.00	2.00		5.00				

Tri nucleotides

MTG 73	148.00	151.00	154.00	156.00
Mean	148.47	151.33	154.19	156.86
Standard Deviation	0.53	0.30	0.29	0.67
Minimum	147.03	150.08	153.05	155.88
Maximum	149.80	151.90	154.98	157.62
Count	126.00	638.00	119.00	13.00

MTG 74	153.00	156.00	159.00	162.00	165.00	168.00	173.00	186.00
Mean	152.93	156.57	159.59	162.54	165.49	168.56	173.62	186.33
Standard Deviation		0.20	0.23	0.24	0.34	0.35		
Minimum		156.29	158.50	161.29	164.29	167.49		
Maximum		156.99	160.35	163.35	166.19	169.04		
Count		15.00	453.00	269.00	135.00	20.00		

Table 4.10: Analysis of ambiguous genotypes and repeatability of allele binning

Column A shows the number of individuals for which genotyping was repeated (out of a total of 448 individuals genotyped) based on the criteria explained in section 4.2.7. Column B shows the error rate of the potentially ambiguous genotypes (from column A). This was calculated using the formula (a/2b) x 100 where a = the number of alleles which 'changed' (evidence of allelic dropout) between genotypes and b = the number of genotypes that were repeated. Column C shows the mean variation of the allele sizing (bold print) between genotypes repeated on the same individual. Column D shows the number of alleles that changed bins as a result of differences in allele sizing between repeated genotypes.

Microsatellite	A	B	C	D
	Number of individuals for which genotyping was repeated	**Percentage of repeated genotypes with a discrepancy compared to original genotype**	**The mean variation in allele sizing between repeat genotypes of same individual**	**Number of individual alleles where binning changed**
MTG 15	93 (20.7 %)	1.1 %	**0.14 bp** (± 0 - 0.79)	0 (n = 186)
Hcms 28	63 (14 %)	0 %	**0.19 bp** (± 0.01 - 0.63)	0 (n = 126)
MTG 67	110 (24.5 %)	2.7 %	**0.2 bp** (± 0 - 0.76)	0 (n = 220)
MTG 73	29 (6.4 %)	1.7 %	**0.29 bp** (± 0 - 1.13)	1 (n = 58)
MTG 74	88 (19.6 %)	7.4 % (5 % due to 162 allele)	**0.19 bp** (± 0 - 0.8)	0 (n = 176)

4.4 Discussion

4.4.1 Isolation and characterisation reviewed

Although the isolation of microsatellites from *T. circumcincta* was relatively simple, identifying loci that were potentially useful population genetic markers was less straightforward. This is evident when the numbers of microsatellites isolated is compared to the numbers finally selected for use as population genetic markers. From the three techniques used, isolation of microsatellites from the EST database was the most efficient approach in terms of producing usable markers. It is interesting to consider the relative success of the three different approaches used to isolate the markers.

4.4.1.1 Genomic library screening

From a total of 45 putative positive clones isolated from the genomic library screening only one was selected for further population genetic analysis. A high attrition rate is not an uncommon feature of other studies in which microsatellites have been isolated from parasitic nematodes by screening genomic libraries. For example in the plant parasitic nematode, *Heterodera schactii*, 79 putative positive clones were isolated from a genomic library by hybridisation but only five of these were used in subsequent population genetic investigations (Plantard and Porte 2003). Similarly in a second plant parasitic nematode, 48 positive clones were isolated from a genomic *Globodera pallida* library, from which only 14 were subsequently used in investigations (Thiery and Mugniergy 2000). A slightly higher success rate was found in a human parasitic nematode; where six microsatellites were used for further analysis from the 17 CA / GT microsatellites isolated from *Trichuris trichiura* using an enriched library screening (Barker and Bundy 2000). In contrast, eight microsatellites were isolated from *Strongyloides ratti*, none found to be suitable for population genetics analysis (Fisher and Viney 1996). In a review by Zane *et al.* (2002) of 170 primer notes using traditional methods for microsatellite isolation from a range of target species (i.e. non-enriched libraries), it was considered that up to 50 % of positive clones could be discarded during the isolation and characterisation phases. The most likely reasons being, lack of suitable flanking sequence for primer design, absence of expected repeat, or unreliable amplification (Zane *et al.* 2002). However, in some studies, microsatellites were not considered if they did not meet certain threshold criteria, including perfection and length of repeat (Schlotterer 1998), which can make it difficult to compare true attrition rates and percentages of usable markers isolated.

One of the main reasons for microsatellites being discarded from further use in this study was the association with the TecRep repeat which was found to be associated with 61 % of the microsatellites isolated from the genome library. Although some of these microsatellites might have been usable for population genetic analysis, they were not pursued due to potential complications with their use as markers, which would make their development difficult and labour intensive. As discussed earlier, the complex nature of the sequence polymorphisms posed potential difficulties in interpretation of genotypes and in some cases the size range of individual alleles would have been unsuitable for analysis using capillary electrophoresis with Genescan genetic analysis. Another source of microsatellite loss was the five putative positive clones that were not fully sequenced due to the large size of their inserts. This problem could have been improved by using a size selected genomic library containing inserts no larger than 1 - 1.5 kb. However this is likely to have increased the number of microsatellites discarded due to lack of flanking sequence. In this study, there was only one microsatellite of the 45 isolated from genomic screening, which was discarded due to lack of adequate flanking sequence for primer design; a benefit of not using a size selected genomic library.

The other major reason for isolated microsatellites being considered unsuitable as population genetic markers was the failure of primer sets to consistently amplify clear products of the appropriate size from single worm lysates. This accounted for seven microsatellites that were discarded from those isolated from the genomic library. Similar results have been found isolating microsatellites from other nematodes. Microsatellites isolated from *H. contortus* showed evidence of non-amplification from single worms for all 27 polymorphic loci, specific populations were problematic with six of the microsatellites (Otsen *et al.* 2000). Non-amplification of products from 4 / 10 and 5 / 9 microsatellites for which primers were designed, led to markers being discarded from the plant nematodes, *H. schachtii* (Plantard and Porte 2003) and *G. pallida* (Thiery and Mugniergy 2000), respectively. From the six *S. ratti* microsatellites for which primers were designed, five were discarded due to non-amplification or multiple products amplified from gDNA, and the final microsatellite was discarded due to poor or non-amplification from single worms (Fisher and Viney 1996).

In summary, hybridisation screening of a short insert library turned out to be a very inefficient way of isolating usable microsatellite markers from *T. circumcincta.* If this approach were to be used to isolate additional markers in the future it would have to be

modified to enable efficient isolation of a much larger number of markers. One approach would be to use enriched libraries to increase the yield of positive clones per colonies screened (Zane *et al.* 2002). However, a large amount of labour would still be required to characterise isolated markers to determine their suitability for population genetic analysis. Consequently, it appears that other approaches will be necessary to isolate larger numbers of usable markers for this parasite species.

4.4.1.2 Nature of microsatellites isolated from genomic library screen

The nature of microsatellite sequence repeats is an important issue for their use in population genetics. There are a wide variety of analytical techniques developed for the analysis of microsatellite variation, all of which make assumptions regarding the mutation patterns of microsatellites. Microsatellite mutation patterns are frequently assumed to follow a stepwise mutation model (SMM), in which length changes between alleles are based on the gain or loss of one repeat unit (Anderson *et al.* 2000;Kimura and Ohta 1978). Whereas, the infinite allele model (IAM) considers mutations which generate new alleles previously not present in the population and the *K*-allele model (KAM) model, is based on there being K allelic states possible and any allele has a constant probability of mutating towards any K-1 allelic state (Kimura and Crow 1964). However, the dynamics of microsatellite mutations has been suggested to differ for each loci and species investigated (Ellegren 2004;Estoup *et al.* 2002;Schlotterer 2000). In particular, the repeat number, repeat length, sequence of repeat motif, flanking sequence and interruptions in repeat sequence may all influence the mutational processes involved in microsatellite evolution (Estoup *et al.* 2002;Schlotterer 2000). The majority of microsatellites isolated from the *T. circumcincta* small insert library were imperfect dinucleotide repeats. Since library clones that hybridised most strongly with a $(CA)_{25}$ probe were sequenced, it seems likely that the majority of CA / GT microsatellites in the *T. circumcincta* genome are imperfect in nature. This is supported by the fact that the majority of CA / GT microsatellites previously isolated from the related nematode *H. contortus* also consisted of imperfect repeats (Hockstra *et al.* 1997;Otsen *et al.* 2000). Microsatellites isolated by screening genomic libraries of other nematode species include both imperfect repeats found in *Trichuris trichiura* (Barker and Bundy 2000) and a mixture of perfect and imperfect repeats found for *Strongyloides ratti* (Fisher and Viney 1996), *Heterodera schachtii* (Plantard and Porte 2003) and *Globodera pallida* (Thiery and Mugniergy 2000). In the relatively few other cases of microsatellites isolated from nematodes, mainly found during other sequence analysis investigations [*Metastronglyus*

(Conole *et al.* 2001); *Trichenella pseudospiralis* (Zarlenga *et al.* 1996); *Brugia malayi* (Underwood *et al.* 2000); *Meloidogyne artiella* (De Luca *et al.* 2002)] both imperfect, perfect and compound repeats have been isolated. Yet, it is difficult to make accurate comparisons of the microsatellite types present in these different species based on these results for a number of reasons, including differences in the methods of isolation and also in the criteria applied to choose microsatellites used in final investigations and therefore published. In addition, mutation rates of microsatellites (polymorphism) may differ between taxa and species (Ellegren 2004;Estoup *et al.* 2002;Schlotterer 2000) and so the nature of repeat types may well differ between species and not simply reflect the method of isolation.

The definition of a minimum number of repeats that constitutes a dinucleotide microsatellite is somewhat arbitrary, with a threshold repeat length of 8 - 12 nucleotides with a single nucleotide mismatch used as the defining boundaries in previous comparative studies (Katti *et al.* 2001). The average stretch of uninterrupted perfect dinucleotide repeat units within the imperfect CA / GT repeats isolated from the *T. circumcincta* genomic library was 5.7 (ranging from 2 to 31 repeat units). Hence, some of the microsatellites do not meet the minimum criteria commonly used in terms of perfect repeat lengths. Instead they consist of stretches of perfect repeats interrupted by imperfect repeat sequences spanning 21 - 80 bp. Although each site of perfect repeats potentially allows slippage during DNA replication, studies have shown that indels and point mutations in microsatellite arrays may have a stabilising influence, making them less polymorphic than those with perfect repeats (Goldstein and Clark 1995). For example, imperfect dinucleotide repeats in mammalian genomes tend to be less polymorphic than perfect repeats (Brinkmann *et al.* 1998;Primmer and Ellegren 1998). However, the results from microsatellites isolated from *T. circumcincta* are consistent with previous work on *H. contortus* suggesting that the majority of imperfect GT dinucleotide repeats in these trichostrongylid nematodes appear to be sufficiently polymorphic to be useful genetic markers (Hoekstra *et al.* 1997;Otsen *et al.* 2000). For example, the imperfect microsatellite, MTG 15, is highly polymorphic with 26 alleles found so far.

4.4.1.3 Homologous microsatellites from *H. contortus*

Primer pairs designed to sequence flanking nineteen previously characterised *H. contortus* microsatellites were tested on *T. circumcincta* genomic DNA in an attempt to isolate orthologus microsatellites. Eight of these, i.e. 42 %, amplified convincing PCR products from *T. circumcincta*. However only one of these, Hcms 28, was deemed suitable for

population genetic analysis, as the other seven failed to consistently amplify products from single worms. The success rate of using primers designed against *H. contortus* sequence to isolate orthologus microsatellites from *T. circumcincta* is somewhat less than that of similar approaches described in vertebrates. For example, 127 out of 173 (73.4 %) and 129 out of 174 (74.1 %) primer pairs corresponding to sequence flanking bovine microsatellites could amplify products from sheep and deer respectively, of which 42.5 % and 55.8 % were polymorphic (Slate *et al.* 1998). Similarly, twenty-eight (58.3 %) of the 48 primers pairs, designed for chicken sequence, amplified products from Japanese quail, of which eleven (22.9 %) were polymorphic (Pang *et al.* 1999). The success of the approach in vertebrates probably reflects the relatively close phylogenetic relationship of the species concerned. It is estimated that deer and cattle last had a common ancestor less than 20 - 25 million years ago (Modi *et al.* 1996) and the last common ancestor of chicken and quail existed between 28 - 37 million years ago (Dimcheff *et al.* 2002). In contrast, although there has been relatively little work on the evolutionary history of the Nematode phylum, *H. contortus* and *T. circumcincta* are likely to be more phylogenetically distant to each other than the vertebrate examples described above. Vanfleteren *et al.* (1994) estimated that strongylid and rhabditid genera, within Clade 5 (Figure 1.1), diverged approximately 400 million years ago. Clearly the species within the strongylid branch, such as those within the *Haemonchus* and *Teladorsagia* genera, could have diverged enormously from each other over this time. In addition, recent work has shown that *C. elegans* mitochondrial and nuclear DNA mutates faster than that of other taxa (Denver *et al.* 2000;Denver *et al.* 2004), and this could also contribute to the lack of usable homologous microsatellites. The main reason homologous *H. contortus* microsatellites from *T. circumcincta* were deemed unsuitable was due to the inability to amplify products of the expected size from single *T. circumcincta* worms. Hence this is likely to be a consequence of poor conservation of sequence flanking microsatellites as a result of the relatively large evolutionary distance between the species and may also be due to increased mutation rate relative to other groups of organisms. This project did not exhaustively examine the use of homologous microsatellites between these two species and further work is required to test these conclusions. Unpublished work by Dr Paul Johnson (University of Glasgow) found that the microsatellites, MTG 67 and MTG 74, isolated from *T. circumcincta* could amplify products from *H. contortus* bulk DNA but there is no data regarding polymorphism or single worm analysis (Dr Paul Johnson, pers. comm.).

4.4.1.4 Isolation of microsatellites from EST sequences

The majority of microsatellites isolated from the EST database using Tandem Repeat Finder (TRF) were trinucleotide repeats of variable perfection. In addition, two perfect dinucleotide repeats were found. The predominance of trinucleotide repeats is not surprising as open reading frames will be maintained despite variation in the number of repeat units. This was noted as a possible explanation for the alleles of a dinucleotide repeat found within an open reading frame, varying in multiples of 3 bp in *Brugia malayia* (Underwood *et al.* 2000). When studying the abundance of microsatellites in a number of other taxa, trinucleotide repeats were seen more often than other repeat types in exon regions (Toth *et al.* 2000). Interestingly, in contrast to those isolated from the genomic library, the two dinucleotide repeats which were isolated from the EST were perfect repeats, $(CT)_{14}$ and $(GA)_{31}$.

Inappropriate size amplification from microsatellites was the main reason for discarding markers isolated from the EST database. Other studies isolating microsatellites from EST sequences also experienced inappropriate size amplification (Yu *et al.* 2004), similar to results found here, with the most likely reason being insertion of introns causing extreme product sizes. Two microsatellites were discarded (MTG 61 and MTG 63) due to the extreme size of amplicons being inappropriate for Genescan analysis. One trinucleotide microsatellite was discarded because it was monomorphic (MTG 69). In the large scale isolation of microsatellites from porcine EST sequences, Rohrer *et al.* (2002) found that there was a higher percentage of monomorphic PCR products from microsatellites containing repeat motifs of three to six bases compared to dinucleotide repeats. Four microsatellites in this study (MTG 62, MTG 64, MTG 66 and MTG 70) were discarded due to non-amplification from single worms (Table 4.7). Complete failure to amplify from gDNA and single worms could have been the result of primer sequence interrupted by introns or large introns disrupting PCR extension, as suggested by Yu *et al.* (2004). This is unlikely to be the situation in this study as all microsatellites amplified products from gDNA, with non-amplification only occurring when markers were tested on single worms. Extreme levels of polymorphism found within this species could result in polymorphisms occurring in primer sequences resulting in the non-amplification of products. Despite using various annealing temperatures and designing additional primer pairs, this remained a major reason for markers isolated from the EST database to be discarded, similar to results from the other isolation methods. However, from all the isolation approaches used, the EST search proved to be the most efficient with 3 / 14 markers being usable for population

genetic analysis compared to 1 / 46 markers isolated from the genomic screening and 1 / 19 *H. contortus* microsatellites usable for *T. circumcincta*.

4.4.2 Polymorphism of the microsatellites

MTG 73 and MTG 74 are considerably less polymorphic than MTG 15, MTG 67 and Hcms 28, which could be due to the fact that they are derived from EST sequence or they are trinucleotide repeats (Table 4.8). There are a number of studies which have shown that dinucleotide repeats are more polymorphic than microsatellites with repeat motifs ranging from three to six base pairs (Anderson *et al.* 2000;Rohrer *et al.* 2002;Schug *et al.* 1998). In addition, MTG 67 also isolated from the EST database, is more polymorphic, suggesting the lack of alleles for MTG 73 and MTG 74 is not due to method of isolation. However, the location of each marker isolated from the EST database may also influence the polymorphism, being less polymorphic if isolated from coding regions. Although it was not possible to clearly determine the position of these microsatellites. Interestingly, MTG 67 is a perfect dinucleotide repeat which might suggest it would be more polymorphic than the imperfect dinucleotide repeats MTG 15 and Hcms 28 (Brinkmann *et al.* 1998;Goldstein and Clark 1995). However, MTG 67 is actually less polymorphic (10 alleles) than both MTG 15 (19 alleles) and Hcms 28 (12 alleles) (Table 4.8).

4.4.3 Microsatellite abundance estimated from genomic screening

In eukaryotes, microsatellite abundance varies across taxonomic groups from 223 per megabase (1 per 4.5 kb) in mammals, close to the average value for vertebrates, to 92 microsatellites per megabase (Mb) (1 per 10 kb) in fungi (Toth *et al.* 2000;Zane *et al.* 2002). *C. elegans* has been shown to have relatively few microsatellite sequences per million base pairs compared with other taxonomic groups, estimated to be 88 microsatellites per mega base of sequence (Toth *et al.* 2000;Zane *et al.* 2002). Therefore, the total number of microsatellite repeats can be estimated to be approximately 8,800 in the entire 100 Mb *C. elegans* genome. This estimate is based on microsatellites defined as stretches of tandem repeats (2 - 6 nucleotides) longer than 12 bp (Toth *et al.* 2000;Zane *et al.* 2002). If a less stringent definition is used, allowing for a mismatch every 10 bp, a slightly higher estimate of 100 microsatellites per Mb is made for the *C. elegans* genome (Katti *et al.* 2001). Both Katti *et al.* (2001) and Toth *et al.* (2000) found that GA / CT microsatellites were the most frequent type of dinucleotide repeat in *C. elegans* followed by CA / GT repeats, for which estimates were in the region of 10 per Mb and 7.5 per Mb, respectively (Katti *et al.* 2001).

The density of CA / GT microsatellites in the *T. circumcincta* genome can be estimated, albeit very approximately from the results of the library screening. Screening approximately 25 Mb of genomic sequence (9 - 12,000 colonies with the average insert size of 2.35 kb) yielded 45 positive clones suggesting a density of 1.8 CA / GT microsatellites per Mb. The *T. circumcincta* genome size has been estimated at 58.6 Mb (Leroy *et al.* 2003). Therefore, this equates to approximately 105 CA / GT microsatellites being present in the entire *T. circumcincta* genome. However, it is unlikely that all positive clones were identified in the screen and so this represents a minimum value. A similar calculation for the isolation of microsatellites from *H. contortus* suggested a density of GT microsatellites of 1.8 CA / GT microsatellites per Mb (Hoekstra *et al.* 1997). Hence, although the estimates based on library screening are clearly very approximate, and likely to be an underestimate, they suggest the density of CA / GT microsatellites is similar in the two parasite species. It will be interesting to compare microsatellite abundance and distribution between *C. elegans* and *H. contortus* more accurately in the future, once the entire genome has been sequenced for *H. contortus*.

4.4.4 The TecRep repeat sequence associated with CA / GT microsatellites

Approximately 61 % of the *T. circumcincta* microsatellites isolated were associated with a repetitive element of 146 bp, which has been named TecRep. These results are similar to the work carried out by Hoekstra *et al.* (1997) and Osten *et al.* (2000), for *H. contortus*, where 34 (48 %) of the 71 microsatellites isolated were found to be associated with the tandem repetitive sequence, HcRep. Both TecRep and HcRep are similarly found directly downstream to a GT microsatellite. Work carried out by Callaghan and Beh (1994), found a similar tandem repetitive sequence of 145 bp (TcRep), in another trichostrongylid nematode, *Trichostronglus colubriformis* and this was, at least in one case, similarly positioned downstream from a GT repeat. The proportion of GT microsatellites in *T. colubriformis* that are associated with TcRep is not known since this element was not identified as a result of isolating GT microsatellite sequences, as was the case for TecRep and HcRep. The first clone in which the *T. colubriformis* TcRep was identified also contained a GT microsatellite immediately upstream of the TcRep sequence. However, although additional independent TcRep containing clones were isolated, the nature of the upstream sequence was not reported. Further work showed that primers specifically designed to this repeat would amplify genomic DNA from a number of nematodes species including; *Cooperia oncophora, Haemonchus contortus, Nematodirus spathiger, Oesphagostomum radiatum* and

Teladorsagia circumcincta, in addition to five trichostrongylus species; *T. vitrinus, T. retortaeformis, T. axei, T. colubriformis* and *T. tenius* (Gasser *et al.* 1995). This provides strong evidence that this repeat has related elements present in many other trichostrongylid nematodes. Furthermore, recent work isolating microsatellites from the trichostrongylid nematodes of birds, *T. tenuis,* suggests that repetitive elements associated with microsatellites are also present (Dr Paul Johnson, pers. comm.). However, blast searching of NCBI public databases found no evidence of this repetitive element in other nematode species outside the trichostrongylid group. Also extensive blast searching of the *C. elegans* genome failed to find convincing homologies in the *C. elegans* genome. Also, Dot plot (Vector NTI) and Fasta (GCG, Wisconsin) analysis of twenty randomly chosen GT microsatellites from the *C. elegans* genome revealed no evidence of a downstream repetitive element either related or unrelated to TecRep (Dr J. Gilleard, pers. comm.). Hence it appears that, although this repetitive element is present in most trichostrongylid species examined to date, it is not present in nematodes outside this particular clade.

Microsatellite association with other repetitive elements has been documented previously in vertebrates and plants (Gallagher *et al.* 1999;Kostia *et al.* 1997;Temnykh *et al.* 2001). In vertebrates, these are mainly SINEs (short interspersed nucleotide elements). SINEs are DNA sequences originating from RNA molecules that have developed the ability to undergo retrotransposition within the genome (Gallagher *et al.* 1999). Microsatellites are commonly associated with the 3' end of the SINE, although they can also be associated with the 5' end or internal regions (Ramsay *et al.* 1999). Many SINEs are also associated with dinucleotide repeats, especially CA / GT repeats, referred to as the C-A family of SINEs. Yet, the TcRep and HcRep did not show any features linked with transposition (Hockstra *et al.* 1997), suggesting that they are not SINE elements. Wilder and Hollocher (2001) found another type of retrotransposon associated with microsatellite repeats in *Drosophila.* These 'mini-me' elements did not show the typical features of SINEs, such as coding for transposition enzymes, nor did these new mobile elements show any structural similarity to other known mobile elements (Wilder and Hollocher 2001). Mini–me elements have a number of shared features, including perfect inverted repeats at both end termini, a highly conserved 33 bp core sequence with two microsatellite repeat motifs found either side of the core sequence. They are highly abundant in the genome of a number of *Drosophila* species, with over 3,000 copies, and are suggested to have a role in the widespread genesis of microsatellite sequences. Unlike mini-me elements, the repetitive elements found in trichostrongylids (TecRep, TcRep, HcRep) are typically found in tandem arrays with the microsatellites

found upstream, and these repeat elements do not share any of the features highlighted by Wilder and Hollocher (2001).

4.4.5 Allele sequencing and Size Homoplasy

Electromorph size homoplasy (ESH) is when two alleles are identical in state (IIS) determined by electrophorectic sizing using analyses such as Genescan, but are different at the DNA sequence level, meaning that they are not identical by descent (IBS) (Adams *et al.* 2004). Sequence differences can be either within the microsatellite array or flanking regions. Where alleles are IIS but not IBD then there is a potential to underestimate population subdivision (Adams *et al.* 2004;Viard *et al.* 1998). Conversely, two alleles can have identical sequences within the repeat and so may be IBD but are scored as different by electrophoresis due to variation in the flanking sequence (Grimaldi and Crouau-Roy 1997). This situation can lead to overestimates of population subdivision (Adams *et al.* 2004).

Sequencing of individual alleles was carried out in this project to investigate the nature of allele size polymorphisms. Firstly, the allele sequencing confirmed that products amplified by a particular primer pair contained a microsatellite from a single locus in all cases. Electromorph size homoplasy was evident in all microsatellite loci except MTG 15. For example, MTG 73 and MTG 74 had SNPs between alleles of the same size within the microsatellite sequence whereas MTG 67 showed evidence of allelic variation due to indels and deletions in sequence flanking the microsatellite sequence. In addition, there was evidence of sequence differences between alleles of the same sequence size due both to variation in sequence flanking the microsatellite and variation of the microsatellite repeat (MTG67 : 178). Electromorph size homoplasy due to variation within the microsatellite array, either as point mutations or repeat numbers, is documented in both invertebrates (Viard *et al.* 1998) and trees (Adams *et al.* 2004). Viard *et al.* (1998) found that the number of variant sequences in each allele class, as defined by length, was dependent on the number of alleles sequenced. Therefore, it is likely that if more alleles had been sequenced for each microsatellite here then more sequence variants may have become evident. SSCP is a simple approach which could be used to assess variation between alleles of the same size.

It is important to be aware of the possible presence of electromorph size homoplasy of markers within and between populations when interpreting the results of population genetic studies. Evidence of within population electromorph size homoplasy would suggest that overall gene diversity is likely to be underestimated. Whereas, electromorph size homplasy that is structured between populations could lead to underestimates of population

differentiation. Viard *et al.* (1998) found that when the number of electromorphs shared between populations is low, then the detection of electromorph size homoplasy is unlikely to have a major effect on the population structure. However, if the number of electromorphs shared between populations is large and size homoplasy is detected between populations, then the overall population structure would be underestimated and is some cases altered (Viard *et al.* 1998). This latter situation could potentially apply to trichostrongylid nematode populations since previous mitochondrial DNA marker studies have suggested relatively low levels of population differentiation (Blouin *et al.* 1995). One way to minimise the potential problems caused by electromorph size homoplasy is to use the maximum number of independent markers possible. Adams *et al.* (2004) suggested that single locus investigations of the effect of electromorph size homoplasy may exaggerate the potential impact of homoplasy. Estoup *et al.* (2002) also concluded that electromorph size homoplasy was unlikely to cause significant problems for population genetic analysis, when a large number of microsatellite markers are used.

4.4.6 Apparent triploid genotypes with the MTG 65 marker

When the MTG 65 locus was amplified with the originally designed primer pair, three alleles were apparently detected in up to 16 % of individuals of a population. These results are not unique and have been noted in other studies, including the plant parasitic nematode, *H. schachtii,* (Plantard and Porte 2003) and the tick, *Ixodes ricinus* (de Meeus *et al.* 2004). However, in these previous studies there was very little discussion regarding the reasons why this might occur. There are several possible explanations. The first explanation is that MTG 65 amplified additional products from contaminating DNA. This is unlikely because no more than two alleles were ever amplified from those individuals apparently triploid at the MTG 65 locus when genotyped with the other five microsatellites. The second explanation is that amplicons were derived from two separate loci in the genome due to misannealing of the primer pair. This is perhaps more likely since a second set of primers that were tested did not amplify more than two alleles from any individual worm. However, this second primer set was not usable due to high numbers of non-amplifying alleles. Individual allele sequencing for this locus could have been performed in order to ascertain whether or not amplification was from more than one locus. One microsatellite which amplified multiple products from single *H. schachtii* nematodes was suggested to be due to amplification from more than one locus but this was not confirmed (Plantard and Porte 2003). The third possibility could be that the locus has been duplicated in the genome, as

was suggested by de Meeus *et al.* (2004). An investigation of the inheritance of this microsatellite marker in single female / progeny broods could be performed to resolve these issues.

4.4.7 Allele Scoring

There is very little literature discussing the problems associated with scoring alleles. This is either because the results presented in this chapter are particularly unusual or that the problem is common but rarely discussed. The latter view seems most likely since it is difficult to imagine that binning ranges are easily obtained in most other species given that there is an abundance of sequence evidence showing that the basis of the polymorphism between microsatellite alleles is often complex (see section 4.3.5). It is clearly evident that allele scoring using Genescan with internal size standards is much more accurate than using polyacrylamide gels (Bennett 2000). Nevertheless, Idury and Cardon (1997) have noted that the allele sizes found for individuals in a large data set will not necessarily fall into discreet groups when using ABI Genescan technology. This could be caused by a number of factors including 'plus-A effect', gel to gel variability, and non-specificity of primers. In some cases, PCR conditions can be optimised to increase the accuracy of allele binning, such as increased final extension time to reduce 'plus-A effect'.

Hcms 28, MTG 73 and MTG 74 were all relatively easy to score. Although Hcms 28 and MTG 73 both had 'plus-A effect', this was a predictable locus-specific pattern. Sequencing from Hcms 28, MTG 73 and MTG 74 showed that allelic size variation was due to variation in number of repeat units only (Figure 4.17a, 4.17c and 4.17d). In contrast, MTG 15 and MTG 67 contained size variation between alleles due to both variation in the number microsatellite repeat units, but also due to indels in sequence flanking the microsatellite. Hence, the amplicon size was not only representative of a set number of repeat units (Figure 4.17b, 4.17e and 4.17f). This may suggest that loci are easy to score if variation between alleles is mainly due to repeat number variation only. Furthermore, the amplicon size for MTG 15 was relatively large (227 – 290 bp) compared to the other markers which may have contributed to problems in binning due to the nature of larger products (Idury and Cardon 1997).

For the reasons outlined above, the scoring of alleles required some care and so automated allele scoring was considered to be inappropriate, especially for the markers MTG 15 and MTG 67. Consequently, all allele binning and scoring for each of the five microsatellites was performed manually. This has resulted in a high degree of confidence in the genotyping

results presented in chapter 5. This has been confirmed by the use of all five microsatellites in two other laboratories in which allele scoring was found to be reliable based on binning ranges and electrophoresis conditions determined from this work (Lindsey Stenhouse, Moredun Institute, Edinburgh and Dr Paul Johnson, Division of Evolutionary and Environmental Biology, University of Glasgow, pers comm.).

4.4.8 Genotyping errors

In practice, genotyping errors are defined as differences observed between two or more molecular genotypes obtained independently from the same sample (Bonin *et al.* 2004). Many population genetic studies do not report error rate calculations and error rate is often defined differently between studies, from the proportion of PCRs yielding one incorrect allele to the proportion of alleles which are incorrect (Hoffman and Amos 2005). It is important to acknowledge genotyping errors and be aware of their potential impact on population genetic analysis, in particular an artificial excess of homozygotes (Taberlet *et al.* 1996), deviations from Hardy-Weinberg equilibrium (de Meeus *et al.* 2004;Miller *et al.* 2002), false evidence of inbreeding and incorrect parentage assignments (Hoffman and Amos 2005). Genotyping errors can occur at any stage from DNA template to typing errors. Studies using extremely low quantities or quality of DNA template are prone to heterozygotes appearing to carry one allele, due to amplification failure, referred to as 'allelic dropout' (Morin *et al.* 2001;Taberlet *et al.* 1996). In this study, successful amplification using at least five markers on each single worm DNA sample was evidence of good quality and quantity of DNA template. Non-DNA based errors included non-amplification of alleles due to primer binding site mutations referred to as 'null alleles' (Dakin and Avise 2004;Pemberton *et al.* 1995), mis-scoring of alleles due to stutter patterns typical of microsatellites, short allele dominance (de Meeus *et al.* 2004;Wattier *et al.* 1998), and human errors such as data entry. Bonin *et al.* (2004) recommended a minimum of 5 - 10 % of blind samples are repeated in order to calculate an accurate error rate. This seems a sensible recommendation but was not performed in this study. In hindsight, this would have been very useful in order to calculate an accurate error rate. Nevertheless, stringent criteria were used to determine whether repeat genotyping of single worm samples was necessary and these were outlined earlier in this chapter (section 4.2.7). The severity of these criteria meant that high numbers of single worm genotypes were repeated per locus (6.4 – 24.4 %). Also, human error and typing errors were minimised by manually checking all data at least three times prior to exporting data for population genetic analysis, but this was not recorded

for error rate calculation. Manual checking and allele scoring analysis ensured correct binning of individual alleles. The computer program Micro-Checker ver 2.2.1 allowed all data to be analysed for evidence of typographical, stutter and short allele dominance errors. Evidence of 'null alleles' was found as a potential source of error but will be discussed in the next chapter, as it was further investigated.

The only error rate which could be calculated was the proportion of alleles which "changed" for those genotypes which were repeated. It is important to emphasise that "changed" means a loss or appearance of an allele and not a size change per se. This calculation presents a "worst case scenario" since the genotypes that were repeated were those considered to be of inadequate standard i.e. it is an error rate in which only the ambiguous genotypes are considered. Overall even this error rate of the "ambiguous genotypes" was low, ranging from 0 % for Hcms 28 to 2.7 % for MTG 67, with MTG 74 having a higher error rate of 7.4 %. In real terms the number of alleles which changed between repeat genotypes, 0 for Hcms 28 to 13 alleles for MTG 74, is very small and unlikely to affect population genetic analysis significantly. These error rate are more likely to be significant in a study considering paternity analysis (Bonin *et al.* 2004;Hoffman and Amos 2005).

The higher error rate found for MTG 74 was mainly due to the poor amplification of the allele 162. Analysis of the marker MTG 74 found that the allele 162 was poorly amplified in 71 (33 %) out of 216 single worms heterozygous with this allele. Of the 71 single worms, 66 of these were heterozygous with allele 159, four were heterozygous with the allele 165 and one was heterozygous with the allele 168. Since in the majority of cases in which allele 162 failed to amplify it was present with a smaller allele, this could be evidence of preferential amplification of the smaller allele, i.e. "short allele dominance".

In conclusion, five microsatellite markers were isolated from *T. circumcincta* and thoroughly characterised to demonstrate their utility as robust population genetic markers; MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74. Although it would be desirable to isolate more microsatellite markers to obtain a larger panel it was considered that five markers were sufficient to initiate population genetic analysis of *T. circumcincta*. These studies are presented in the next chapter.

Chapter 5: Population Genetic Analysis of *T. circumcincta*

5.1 Introduction

5.1.1 *T. circumcincta*: single species or species complex?

Teladorsagia circumcincta is found in a wide geographical distribution and in a range of domesticated ruminant species (Hoberg *et al.* 2000). Based on morphological criteria, *T. circumcincta* has traditionally been considered a single species. However, recent molecular studies suggest that the population structure is more complex with both morphologically discernable and cryptic species being identified (Gasnier *et al.* 1997;Hoberg *et al.* 1999;Leignel *et al.* 2002). Hoberg *et al.* (1999) discovered that nematodes of muskoxen (*Ovibos moschatus*) from the central Canadian Artic, previously regarded as *T. circumcincta*, were a separate species which they named *Teladorsagia boreoarcticus*, based on a number of morphological measurements and mtDNA ND4 sequences. Morphological measurements were carried out on a total 151 male and 100 female *T. boreoarcticus* individuals isolated from muskoxen and compared to morphological measurements made for 58 *T. circumcincta* and 20 *T. trifurcata* males and 34 *T. circumcincta* females isolated from domesticated sheep from worldwide locations. Based on these measurements, male and female *T. boreoarcticus* individuals were considerably larger than those of *T. circumcincta*, with statistically significant differences found between the mean values for morphometric measurements of the oesophageal valve, spicules, gubernaculum and bursa. Molecular analysis was conducted by sequencing the mtDNA ND4 gene from 11 *T. boreoarcticus* males from muskoxen and three *T. circumcincta* from domesticated sheep from Canada, identified using measurement of the male spicules. These sequences were aligned with a further 40 mtDNA ND4 sequences isolated from domestic sheep from the United States by Blouin *et al.* (1995). *T. boreoarcticus* is distinguishable from *T. circumcincta* by virtue of a 13 % sequence divergence of the mtDNA gene ND4, including 31 fixed differences. These results are of a similar magnitude to those found between *H. contortus* and *H. placei* which differ by a 16 % sequence divergence with 34 fixed differences (Blouin *et al.* 1997). Hence there is good evidence that *Teladorsagia boreoarcticus* and *Teladorsagia circumcincta* are separate species.

There is also evidence that some *T. circumcincta* populations found in goats differ genetically from those found in sheep (Gasnier and Cabaret 1996;Leignel *et al.* 2002).

Gasnier and Cabaret (1996) examined a number of isoenzyme loci and found the malate dehydrogenase (MDH-2) locus provided evidence for *T. circumcincta* population sub-structuring. Malate dehydrogenase has three alleles; A, B, and C. In *T. circumcincta* populations from goats all three alleles were found, yet the frequency of alleles A and B was low and there was a distinct deficiency of the heterozygote genotypes A / C and B / C. However in *T. circumcincta* populations from sheep only two alleles were found; A and B, but never C. They interpreted these results as evidence of two sub-populations of *T. circumcincta* existing in sheep and goats: one which was capable of infecting both sheep and goats which did not contain the C allele (which they termed a 'sheep / goat line' or *T. circumcincta* 'standard') and another population which was capable of infecting only goats which did contain the C allele (termed the 'goat line' or 'cryptic species' or *T. circumcincta* 'goat'). Leignel *et al.* (2002) subsequently provided further data in support of this hypothesis using several independent molecular markers; the mtDNA ND4 gene, the β-tubulin isotype 1 gene and the second transcribed region of the rRNA cistron (ITS-2). Male worms were collected from 5 sheep farms and 3 goat farms in France and genotyped using these markers. Results from the MDH-2 locus supported previous work, with A, B and C alleles identified (in addition to a very low frequency D allele). The C allele was found in worm populations from the three goat farms (allele frequencies ranging from 0.16 to 0.57) and there was a distinct lack of the heterozygote genotypes A / C and B / C. Sequencing of the β-tubulin isotype 1 gene of 190 individuals revealed that those typed with the MDH-2 A and B alleles had distinct β-tubulin sequences compared to those individuals with the MHD-2 C allele; these two allelic forms of the β-tubulin locus were designated Type I and Type II, respectively. Sequencing of the ITS-2 region from 32 individuals found two distinct types of ITS-2 sequence. In the 21 worms with the MDH-2 A and B alleles, the length of the ITS-2 sequence was 246 bp, where as that for the the 11 worms with the MDH-2 C allele was 252 bp. Finally, sequencing of the mtDNA ND4 gene further supported the hypothesis. Three main mtDNA ND4 sequence types were found in the *T. circumcincta* populations, individuals with MDH-2 A and B alleles being identified by Type I and Type II sequences and individuals with MDH-2 C alleles identified by Type III sequences, respectively. A summary of the results presented by Leignel *et al.* (2002) distinguishing *T. circumcincta* 'standard' and *T. circumcincta* 'goat' based on the four markers described above is presented in table 5.1. These results provide persuasive evidence that the current definition of *T. circumcincta* as a species masks a more complex population genetic structure including the presence of cryptic species.

5.1.2 Relationship between *Teladorsagia circumcincta*, *Teladorsagia davtiani* and *Teladorsagia trifurcata*

Teladorsagia davtiani, *Teladorsagia trifurcata*, and *Teladorsagia circumcincta* have been traditionally considered as three separate species based on the morphology of the male spicules (Lichtenfels *et al.* 1988;Lichtenfels and Hoberg 1993;Urquhart *et al.* 1996) (Figure 5.1). The females of these "species" cannot be distinguished morphologically. However, the validity of this distinction has been questioned. For example, *T. trifurcata* and *T. davtiani* were proposed to be morphological variants of *T. circumcincta* based on a number of observations. Firstly, the three male morphological variants always occurred together, with one (*T. circumcincta*) constituting a major proportion of the population (Lichtenfels *et al.* 1988). Secondly, allozyme electrophoresis studies failed to provide genetic evidence of different species (Andrews and Beveridge 1990;Gasnier *et al.* 1993). Andrews and Beveridge (1990) examined 250 *T. circumcincta*, 49 *T. davtiani* and 10 *T. trifurcata* single worms at thirty-four enzyme loci. A total of twenty-two loci were invariant with twelve loci being polymorphic. However, no fixed differences were detected among the three morphological variants. Gasnier *et al.* (1993) used five polymorphic enzyme loci to show low genetic distances between *T. circumcincta* and *T. trifurcata* (0.001), than compared to that found between *Ostertagia spp.* and *Teladorsagia spp.* (0.607) using Nei's unbiased genetic distance (Nei 1978). Furthermore, Gasnier *et al.* (1993) showed that *T. circumcincta* and *T. trifurcata* could interbreed, by producing males of both morphological variants from a cross between a female *T. circumcincta*, obtained from a monomorphic lab strain, and male *T. trifurcata*. A more recent study using ITS-2 sequence analysis did find intra-individual and interspecific variation in *T. circumcincta*, *T. trifurcate* and *T. davtiani* but failed to find any fixed sequence differences between these three putative species (Stevenson *et al.* 1996). In contrast, fixed sequence differences were found in the same study between *Teladorsagia* and *Ostertagi*, with the ITS-2 sequence for *O. ostertagi* being 238 bp compared to 246 bp for *Teladorsagia*. However, it should be noted that the results were based on sequences obtained from a small number of individual worms; four *T. circumcincta*, two *T. davtiani* and one *T. trifurcata* and one *O. ostertagi*. It is also worth noting that *T. circumcincta*, *T. davtiani* and *T. trifurcata* all have indistinguishable karyotypes when metaphase spreads are microscopically examined (Mutafova 1999).

Hence there is as yet no convincing genetic or morphological evidence to support the traditional designation of *T. circumcincta*, *T. trifurcata* and *T. davtiani* as separate species but more research is required to investigate this in more detail.

5.1.3 Geographical sub-structure of *T. circumcincta* populations

There have only been three major studies looking at the population sub-structure of the trichostrongylid nematodes, including *T. circumcincta*. Blouin *et al.* (1995) examined the population structure of five trichostrongylid species across North America; *O. ostertagi* and *H. placei* in cattle, *T. circumcincta* and *H. contortus* in sheep and *Mazamastrongylus odocoilei* in white-tailed deer. The analysis was conducted using a single locus; the mtDNA ND4 gene. A 463 bp fragment of the ND4 coding region was sequenced from nine to eleven individual worms from each of four or five populations per species per location in the United States (Figure 5.2). Blouin *et al.* (1995) found that the trichostrongylid nematode of white-tailed deer, *M. odocoilei*, had a very high within population diversity and significant geographical sub-structuring between populations. The pattern for *M. odocoilei* corresponded to geographical structuring based on mtDNA of the host species. In contrast, the trichostrongylid nematodes of sheep and cattle had very high within population variation but no subdivision / structuring between geographically separated populations. There are two broad alternative explanations for these results. Firstly, it may be that parasitic nematodes of North American livestock were derived from the same parasite population several hundred of years ago and genetic drift has occurred too slowly to allow these populations to have differentiated over subsequent time. Alternatively, it could be that high levels of contemporary gene flow, due to livestock movement, allows geographically separated parasite populations to behave as one large population, unlike the wild deer parasite populations since these hosts are much less mobile than domestic animals. Blouin *et al.* (1995) preferred the latter hypothesis primarily because of the very high levels of within population diversity and the lack of sub-structuring of the livestock parasites.

A more recent study was conducted investigating the genetic diversity of *T. circumcincta* populations within the U.K. from three sheep populations using mtDNA. Similar to the results of Blouin *et al.* (1995), they found all three populations had high levels of within population diversity and very little genetic differentiation between the geographically separate populations (Braisher *et al.* 2004). However, the population sampling was different to that of Blouin *et al.* (1995) in one respect. One population of worms was collected from Soay sheep that have been isolated on the island of Hirta off the West Coast of Scotland since 1932. Consequently, contemporary gene flow between populations could not be invoked to explain these results. Braisher *et al.* (2004) concluded instead that genetic drift

has occurred too slowly to allow fixation of new alleles or loss of haplotypes due to the extremely high effective population size of this parasite.

A third study on population sub-structure of *T. circumcincta* was conducted in France (Leignel and Humbert 2001). In common with the two studies previously described, they found a high level of within population diversity. However, in contrast to both Braisher *et al.* (2004) and Blouin *et al.* (1995), they found evidence of geographical sub-structuring between some of the parasite populations on goat farms in France. Although no population subdivision was seen between individual farms within the same region, there was sub-structuring on a larger scale between geographical regions of central and southern France and Morocco. This study is particularly interesting because the history of the livestock on these farms is well documented. Farms within a region were originally derived from the same stock approximately 10 - 20 years ago but are now largely closed (i.e. no movement of animals onto the farm). However, the isolation between each geographical region is at least 50 years (i.e. between North, South and Morocco). Consequently, the results might suggest that the genetic drift in these populations is sufficient to allow population sub-structure to be apparent in populations separated for more than 50 years, but not within populations separated by only 10 - 20 years. However, the authors also point out that the environmental conditions are very different between these regions, which could also have an influence on the population dynamics. Nevertheless, these results are different to those of Braisher *et al.* (2004), in which there was no evidence of genetic drift in the parasite population isolated in Soay sheep on Hirta for over 70 years.

The studies outlined above have all used single-locus mitochondrial markers to investigate the structure of *T. circumcincta* populations. This project aims to use a multilocus approach, using the five microsatellite markers isolated and described in Chapter 4, to investigate the population genetic structure of *T. circumcincta* and compare these results to those of previous studies. Analysis using the microsatellites isolated was restricted to *T. circumcincta* populations that were readily available. Consequently the samples consisted of parasite populations from a number of different countries, including both laboratory and field isolates from goats and sheep. A map outlining the geographical location of each population and their relation to each other is shown in figure 5.3. As there have been no previous studies of this species using microsatellite markers, an investigation of the basic population genetic structure is required prior to answering more complex questions. Therefore, the results presented in this chapter focus on answering the following questions.

Does any population sub-structuring exist? Are there differences between infra-populations obtained from hosts grazing the same area? Are there geographical differences between populations? Is there evidence of cryptic speciation? Is there any evidence of genetic differences between *T. circumcincta, T. trifurcata* and *T. davtiani*? Answers to these questions will provide a framework for future studies regarding more complex questions.

5.2 Results

5.2.1 Genotyping of *T. circumcincta* Populations

A total of fourteen *T. circumcincta* populations consisting of 22 to 48 individual adult males or females were genotyped using the following five microsatellite markers; MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74. All populations are listed in table 2.1–2.3. It should be noted here that the Motri populations, MotriPT, MotriPB, MotriPI, and MotriPL, are not discussed in detail in this chapter for the reasons explained in Chapter 2, section 2.2.1.

All individual adult male *T. circumcincta* were identified according to the Manual of Veterinary Parasitology Laboratory Techniques (MAFF 1986) using male spicule morphology. In addition, the species-specific PCR amplification of the rRNA Intergenic Transcribed Spacer (ITS-2) previously described by Wimmer *et al.* (2004) was conducted on all individual males and females from all populations to confirm the morphological identification of *Teladorsagia sp.*. It should be noted that this PCR does not distinguish between *Teladorsagia davtiani*, *Teladorsagia trifurcata*, and *Teladorsagia circumcincta* (Wimmer *et al.* 2004). Therefore the populations isolated from Soay sheep (ScKiTT, ScKiTD, and ScKiTC) were identified as being one of the three *Teladorsagia* morphological variants by Dr B. Craig at Edinburgh University, based on male tail morphology.

5.2.2 Measuring Hardy-Weinberg Equilibrium

Each population and microsatellite loci was tested to see if they deviated from Hardy-Weinberg Equilibrium (HWE) in order to see if the markers were behaving appropriately, to determine if there was any evidence of genetic linkage between any of the markers and to see if there was any evidence of sub-structuring within populations. Estimates of expected heterozygosity, H_e (Nei's unbiased, 1987), and observed heterozygosity, H_o (observed direct count), and the corresponding p-value was calculated for each population across each loci

using Excel Microsatellite Toolkit (Park 2001) and GDA ver 1.1 (Table 5.2). Overall, most of the loci for most of the populations are in HWE. No one population deviates across all loci, which would potentially indicate within population sub-structuring (admixture). In addition, four of the five microsatellites, Hcms 28, MTG 67, MTG 73, and MTG 74 do not show a pattern of deviation across all the populations. The p-values calculated for each population over all loci deviate from HWE due to individual microsatellite deviations.

There is significant departure from Hardy-Weinberg equilibrium at locus MTG 15 in all populations (except NzWs and FrMe) which is potentially due to the presence of non-amplifying alleles (null alleles) at this locus. The definition of a null allele for a microsatellite is any allele which consistently fails to be PCR amplified to detectable levels (Dakin and Avise 2004). Individuals where one of the two alleles has failed to amplify (i.e. have a single null allele) are 'false' homozygotes and such genotypes would consequently lead to an over estimate of homozygotes and departure from expected heterozygosity based on allele frequencies. This seems likely for the MTG 15 locus since for a number of individuals in each population a product could not be amplified, in spite of successful amplification at other loci, suggesting these individuals are null homozygotes. The Micro-Checker ver 2.2.1 program was used to investigate whether or not the heterozygote deficiencies as determined by GDA 1.1 were likely to be due to null alleles. This program determines whether heterozygote deficiencies occur equally for all combinations of alleles of a locus, as would be expected if heterozygote deficiency was due to the presence of null alleles. This principle is discussed in more detail in section 2.6.5. Micro-Checker ver 2.2.1 results were consistent with the presence of null alleles for all populations genotyped by locus MTG 15 which is in agreement with p-values calculated in GDA ver 1.1. This was also the case for 18 of the 22 population / loci combinations, shown in bold in table 5.2, which showed significant departure from HWE in GDA ver 1.1. The few population / loci combinations which did not show agreement between the two programs had GDA ver 1.1 p-values very close to 0.05 and were hence marginal results. This high level of correspondence between the prediction of null alleles by Micro-Checker ver 2.2.1 and the departures from HWE detected by GDA ver 1.1 supports a hypothesis that the deviations from HWE are due to the presence of null alleles.

Although marker MTG 15 clearly has a large number of null alleles in most populations, it is a very polymorphic and potentially informative marker. Consequently, all further analysis was conducted both including and excluding MTG 15 in calculations.

One further point to consider is that due to the nature of the analysis done by most population genetic analysis programs, including GDA ver 1.1 and Micro-Checker ver 2.2.1, it is not possible to enter 'blank' genotypes. Consequently individual worms that failed to amplify with a marker (i.e. potential null homozygotes) are not accounted for. For example, the HWE estimates calculated for the FrMe population for MTG 15, were calculated based on only seven individuals as the remaining 26 individuals were genotyped as null homozygotes at this loci. As a result, deviation from HWE is most likely to be underestimated by these programs in populations where individuals homozygous for null alleles were present. An indication of the extent of this can be seen from the number of worms successfully genotyped for each population / loci combination in table 5.2.

5.2.3 Investigation of Microsatellite Inheritance

As outlined above, it was considered that the presence of heterozygote deficiencies in some of the population / loci combinations was likely to be due to the presence of null alleles. One way to determine whether null alleles are present or not, is to study the inheritance of markers between female parents and their progeny. Such inheritance studies can also be used to determine whether sexual reproduction is obligate in these parasites, that there is Mendelian inheritance of alleles for each microsatellite, to check the correct allele assignment for each marker and to study the paternal origin of alleles.

A total of seven adult female *T. circumcincta* and between 6 and 10 of their offspring were genotyped using the five microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74. Adult female *T. circumcincta* were removed directly from the host abomasum at slaughter and singley placed in individual chambers of a 24-well plate and incubated at 37°C for 1 - 2 hours followed by incubation at 20°C overnight to allow egg laying. Adult females were then removed from the wells and their heads removed for DNA lysate preparation and genotyping. Individual DNA lysates were then prepared for larvated embryos or hatched larvae, avoiding any unfertilised offspring, and genotyping performed. The seven adult females consisted of two females from the Moredun triple resistant strain Tci5 (Motri) and 5 females from the Moredun 'fecund' strain (Tci2). Although *T. circumcincta* females produce large numbers of eggs *in vivo*, only small numbers of viable eggs could be harvested using our *in vitro* method. Genotypes for females and their offspring are presented in table 5.3a - c.

Equal representation of maternal alleles in the young from a heterozygous parent is consistent with Mendelian inheritance (McCoy and Tirard 2002). Within the limitations of the small sample sizes it was generally the case that where the female was heterozygous at a particular locus the two alleles were represented in roughly equal numbers of the progeny. Hence the results are broadly consistent with Mendelian inheritance with no evidence of selfing. If obligate sexual reproduction and Mendelian inheritance is occurring then every individual progeny should carry one maternal allele. However in a few cases there are examples in which a maternal allele is apparently absent from one or more members of a brood, which can be termed a Maternal-Offspring pair mismatch (e.g. females: Fe19 and Fe26 for MTG 15 and Fe26 for MTG 73). An example is shown in more detail in figure 5.4a, using the female Fe26 and all corresponding progeny. The most simple explanation of this result is that the maternal genotype is a 'false homozygote' i.e. heterozygous for the visible allele and a null allele. If this is the case the genotype of the progeny is then explicable because they all carry either the 243 or null maternal allele. The results are all consistent with this interpretation because Maternal-Offspring pair mismatches are only seen when the maternal genotype and the individual mismatched progeny are apparently homozygous. If we then take Maternal-Offspring pair mismatches as evidence of null alleles then the overall results of the inheritance analysis demonstrates their presence for the markers MTG 15 and MTG 73 in the females Fe19 and Fe26 and their respective offspring (Table 5.3a - c).

The identity of paternal alleles present in progeny can be deduced by a process of elimination based on knowledge of the maternal genotype using the approach explained in figure 5.4b. This process was used to deduce the minimum and maximum number of paternal alleles for each brood from a single female for each marker (Table 5.3a - c). For each brood the locus with the highest number of potential paternal alleles per female was then used to estimate the minimum number of males that must have mated with the female parent in question in order to produce the progeny genotyping results. The results show that all females mated with at least two males; adult female F21 must have mated with at least 3 males and the adult females F22, Fe19, Fe26 Fe37, Fe1 and Fe8, with at least 2 males respectively. However it should be noted that this is the minimum number and the results are also consistent with larger numbers of males mating with single females. In summary, the inheritance studies confirmed correct assignment of allele sizes for each microsatellite, were consistent with Mendelian inheritance of these alleles and demonstrated multiple

mating of female worms. In addition, this experiment provided direct evidence for the presence of null alleles for microsatellites MTG 15 and MTG 73.

5.2.4 Estimating Null Allele Frequencies

As a number of population / loci combinations not only deviated from HWE as calculated GDA ver 1.1, but also showed evidence of null alleles as predicted by Micro-Checker ver 2.2.1, null allele frequencies were estimated using GENEPOP ver 3.3 (Raymond and Rousset 1995) (Table 5.4). Null allele frequencies can also be calculated from apparent heterozygote deficiencies using expected and observed heterozygosities. This was first described by Chakraborty *et al.* (1992) and referenced as equation 3 (Brookfield 1996) and then modified by Brookfield (1996) as equation 4. Both equations were applied in Microsoft Excel to the data for each population and microsatellite locus. All estimates of null allele frequencies for all populations with all microsatellites were similar to those found by GENEPOP ver 3.3 (results not shown).

5.2.5 Investigation of linkage disequilibrium between markers.

Hardy-Weinberg disequilibrium can sometimes be accounted for by non-random association of markers. In order to investigate this linkage disequilibrium estimates were calculated by using GDA ver 1.1 preserving genotypes for each. There were no major departures from linkage equilibrium for any particular combination of loci across all populations, suggesting all five loci are randomly associating and not genetically linked (data not shown).

5.2.6 Genetic Diversity within parasite populations

All the populations have a high degree of genetic diversity which agrees with previous studies for *T. circumcincta* and other trichostrongylid species. All populations were polymorphic at all loci, with the number of alleles per locus per population ranging from 3 to 18 (Table 5.5). Average expected heterozygosity was greater than 0.5 in all populations across all loci and across all populations both including and excluding MTG 15. Average number of alleles per locus, calculated over all five loci ranged from 5.4 to 9.4. The NzWs population has a low average number of alleles (5.4) compared to all the other populations which range from 6.8 to 9.4. However, expected heterozygosity for this population is similar to all the other populations. The FrMe population has an average number of alleles of 6, which is artificially low due to the number of individuals successfully genotyped using the MTG 15 locus. Overall, no one population stands out as being less diverse than the others from these parameters. Alleles which are only seen in one population are referred to as

unique alleles. The number of unique alleles per population seen was never greater than 2 for any one population / loci combination. All unique alleles were found to be at a frequency of 0.07 or lower, except for the 172 allele in the French Meunier (FrMe) population at MTG 67. This is discussed in further detail in section 5.2.8.1 and section 5.2.8.3.

5.2.7 Genetic Distance and Differentiation

5.2.7.1 Genetic differentiation between individual hosts from the same farm

Previous studies investigating the population genetic structure of nematode populations have often used the nematode populations from one host as a representative sample of the entire farm. In order to test the extent of genetic variation between nematode populations isolated from different hosts from the same farm, three *T. circumcincta* populations from three individual goats from the Sourhope farm were genotyped. The three goats had been co-grazed under identical management conditions for their entire lifespan of 20 months including having the same anthelmintic treatments (Table 2.2). A total of 21, 30 and 30 individual adult *T. circumcincta* males were genotyped from the three individual goats ScS0507, ScSo529 and ScSo210 respectively (Table 2.1 and 2.2). All three populations show similar levels of genetic diversity across all five loci based on the number of alleles per locus and the expected level of heterozygosity (Table 5.2 and Table 5.5). The distribution of allele frequencies for each of the three loci are broadly similar for each of the three populations (Figure 5.5). The Fst values, calculated by GDA ver 1.1, are very low between these three *T. circumcincta* populations (0.0024 - 0.0049) indicating very little genetic differentiation (Table 5.6a and 5.6b, highlighted in grey shading). Similar results have also been found for *H. contortus* populations of the same isolate passaged through different individual hosts, (Fst values ranging from 0.0048 to 0.0028) (Dr Erica Packard and Dr John Gilleard, pers. comm.). AMOVA analysis showed that 99.08 % of genetic variation found is within the three *T. circumcincta* populations (ScS0507, ScSo529 and ScSo210) rather than between the populations (0.92 %). Also overall Fst value estimated by Arelquin ver 2.0 was of similar magnitude to pairwise values found with GDA ver 1.1 (Fst = 0.00916, p-value = 0.219). In summary, all the data analysis shows evidence of little genetic differentiation between the three populations from different hosts from the same farm but high levels of genetic variation within each population. The results, supported by parallel work on *H. contortus* (Dr E. Packard and Dr J. Gilleard, pers. comm.), suggests that a nematode population from a single host provides a good representation of the nematode population from co-grazing animals. Consequently, the remainder of populations included

in this study consist of adult worms isolated from one host representing either a farm or laboratory isolate from a particular geographical location.

5.2.7.2 Genetic differentiation between populations

Pairwise Fst values were calculated between each of the fourteen populations genotyped in this project, based on all five microsatellites and then re-calculated excluding MTG 15 (Tables 5.6a and 5.6b). The majority of pairwise Fst values were very low, less than 0.04, indicating very little genetic differentiation between most of the populations. Low levels of genetic differentiation were found between all U.K. populations, despite different geographical locations, host species and origin of isolate (i.e. farm or lab). In addition, there were no significant levels of genetic differentiation between adult female and male *T. circumcincta* from the same hosts; Tci5 (MotriF - females) and Tci5 (MotriPT – males). The French Meunier (FrMe - first column, dark blue / blue in table 5.6a and 5.6b) population shows the highest level of genetic differentiation compared to all other populations with values ranging between 0.081 - 0.165, when pairwise Fst were calculated using all loci. Pairwise Fst values ranged from 0.104 – 0.215 when calculated excluding MTG 15. Moderate levels of genetic differentiation were also found between the New Zealand population (NzWs) and the other populations (not including FrMe); Fst values ranged from 0.323 – 0.672 and from 0.034 – 0.66 when the MTG 15 marker was included and excluded respectively. It is interesting to note that the levels of genetic differentiation among the three French populations, FrGa, FrSo and FrMn (Fst values from -0.0030 – 0.0014) were very low. Indeed, they are much lower than pairwise Fst values between these French populations and the UK populations (Fst values between 0.0005 – 0.0316).

Principle coordinate analysis (PCA) was performed to allow the Fst results, calculated from multiple variables using GDA ver 1.1, to be expressed in a multi-dimensional plot for all eighteen populations (Figure 5.6a and 5.6b). These plots clearly demonstrate that the French Meunier (FrMe) and New Zealand (NzWs) populations are the most genetically differentiated, whereas all the other populations cluster together. This is evident on PCA plots, based on Fst values calculated both including and excluding MTG 15. The data was then jacknifed by sequentially omitting one locus at a time and re-calculating Fst values and plotting using PCA (Figure 5.7). Both the French Meunier (FrMe) and the New Zealand (NzWs) populations were clearly separated from the other populations, which were clustered together in every case showing that the result was not due to any one marker. It is also apparent from the PCA plots that the three other nematode populations from French

goat farms, FrGa, FrSo, and FrMn, tended to cluster together rather than being mixed in with the U.K. parasite populations.

AMOVA analysis was conducted to examine the partitioning of genetic variation within and among populations (Tables 5.7a and 5.7b). Three main population groupings were analysed. For the initial analysis populations were grouped according to geographical country of origin; U.K., France and New Zealand. The second analysis excluded the FrMe population and the third analysis excluded both the FrMe and NzWs populations (Table 5.7a). Throughout all analyses conducted, the within population variation was very high, ranging from 95.44 % (all populations included) to 97.89 % (when FrMe and NzWs are omitted). Weak genetic structuring was suggested both among populations from different countries (3.35 % of total variation) and between populations from the same country (1.2 %). When the analysis was re-calculated with the FrMe population removed from the analysis; among country variation decreased to 2.56 % and the variation found among populations of the same country decreased to 0.87 %. This provides evidence that the FrMe population is genetically different to the other French populations as the genetic variation "among populations within countries" decreased markedly when the FrMe population was removed from this group. Similarly the genetic variation "among countries" decreased suggesting the FrMe population accounts for much of the genetic differentiation in this category. Further AMOVA analysis found that when the FrMe and NzWs populations were both removed from the analysis, variation between the remaining countries (France and the U.K.) fell further to 1.21 %. This result supports the previous evidence that the FrMe and NzWs populations account for the main source of population structuring seen in previous groupings. Results found when the AMOVA analysis was conducted again, omitting MTG 15 data, were very similar (Table 5.7b).

5.2.8 Further investigation of the FrMe *T. circumcincta* population

The results described above have shown that the FrMe population is highly genetically differentiated from all the other *T. circumcincta* populations in this study, including the other three French populations (FrGa, FrSo and FrMn). This genetic differentiation was clearly demonstrated both with the Pairwise Fst values (Tables 5.6a and 5.6b) and the PCA plots (Figures 5.6a - 5.7). In addition, AMOVA analysis found that the FrMe population accounted for a large proportion of the overall genetic structuring between the parasite populations (Table 5.7a and 5.7b). Consequently, this population (FrMe) was investigated in more detail to investigate the nature of this variation. The microsatellite data were examined

in more detail and in addition, the worms were genotyped using two additional, previously described markers; the isotype 1 β-tubulin locus and the rRNA ITS-2 marker (Leignel *et al.* 2002).

5.2.8.1 Further examination of FrMe microsatellite genotyping

The FrMe population differed very obviously from the other populations at two of the microsatellite loci; MTG 15 and MTG 67. A dramatic effect was seen when the FrMe population was genotyped using MTG 15. It was observed previously that MTG 15 deviates from Hardy Weinberg equilibrium for the majority of populations in this study and that the most likely cause is the presence of null alleles. When the FrMe population was genotyped using MTG 15, only seven of the 33 individuals could be amplified and the other 26 individuals were considered homozygous null (Figure 5.8). Due to the large number of individuals which could not be amplified, new primers were designed shifting both original primers either 5 bp upstream or 5 bp downstream from their original positions. Both of the new sets of primer pairs amplified products from the same seven individuals that had been previously amplified by the original primer pair but not from any of the 26 individuals for which no products could be originally amplified (results not shown). In addition, two more primers were designed to anneal to sequence well outside all the other primers on a subset of the FrMe individuals and again the PCR amplifications corresponded to the previous results. It should be noted that the primer sets for the other microsatellite loci robustly amplified products for the 26 individual templates that failed to amplify with these three independent sets of primers for the MTG 15 locus. These results suggest that the individuals that fail to amplify were true null homozygotes for the MTG 15 locus due to sequence polymorphism present in the flanking regions.

The microsatellite locus MTG 67 also showed a striking difference between the FrMe population and the other populations in this study. The FrMe population contained a unique allele, 172, at a relatively high frequency (0.26). This allele was not found in any worm from any of the other populations examined. Interestingly this allele was found in 11 of the 26 individuals considered homozygous null for MTG 15 but only 1 of the 7 individuals successfully amplified by MTG 15 (Table 5.8).

5.2.8.2 Isotype I β-tubulin and rRNA ITS-2 genotyping

Leignel *et al.* (2002) have proposed a hypothesis that *T. circumcincta*, as defined by traditional morphological criteria, is really a complex of several different species.

Specifically, that at least one 'cryptic species' may exist in goats in France which they termed *T. circumcincta* 'goat'. As discussed in the introduction to this chapter, the evidence for this is based on four independent markers which classify *T. circumcincta* individuals as either *T. circumcincta* 'standard' or as potentially *T. circumcincta* 'goat'. The FrMe worms come from a farm on which it has been suggested this 'cryptic species' of *T. circumcincta* exists and so it seemed possible that this might be the reason for the highly divergent nature of this population based on the microsatellite genotyping. In order to investigate this possibility, the worms genotyped from the four French goat farms (FrMe, FrGa, FrSo, and FrMn) with the microsatellite markers, were also genotyped with two of the markers suggested by Leignel *et al.* (2002) to discriminate the *T. circumcincta* 'goat' cryptic species from the *T. circumcincta* 'standard' species. The two markers used were the isotype 1 β-tubulin gene and the rDNA ITS-2 sequence.

i) The β-tubulin marker: Primers M2 and D7 amplify a fragment of the *T. circumcincta* isotype 1 β-tubulin gene encompassing 2 introns and sequencing of this fragment from individual worms previously revealed two distinct sequence types termed Type I and Type II (Leignel *et al.* 2002). In addition to a number of synonymous substitutions in the coding regions, the main difference between these two sequence types was the size of the first intron; 106 bp for Type I and 338 bp for Type II. The situation was a little more complex than this since the Type I and Type II sequences showed some "within type" polymorphism based on the presence or absence of small indels in intron 1. In summary, PCR amplification with primers M2 and D7 from single worms resulted in amplicons of 800 - 802 bp corresponding to Type I β-tubulin alleles or amplicons of 1032 - 1080 bp corresponding to Type II β-tubulin alleles. Leignel *et al.* (2002) reported that *T. circumcincta* 'standard' worms (as defined by the MDH isoenzyme genotype) all contained Type I β-tubulin alleles whereas *T. circumcincta* 'goat' all contained Type II β-tubulin alleles. Since there is a >200 bp size difference between the two β-tubulin allelic types then these can easily be distinguished on agarose gel electrophoresis allowing simple genotyping (Figure 5.9a).

ii) The rDNA ITS-2 marker: Primers NC1 and NC2, corresponding to 5.8S and 28S ribosomal DNA sequences of the free living nematode *C. elegans,* were first used on a parasitic nematode by Gasser *et al.* (1993) to amplify the rDNA ITS-2 region of *Trichostrongylus retortaeformis.* Leignel *et al.* (2002) found the ITS-2 sequence to be 246 bp from *T. circumcincta* 'standard' worms, as defined by the MDH isoenzyme genotype,

and to be 252 bp from *T. circumcincta* 'goat' worms. Since there is only a 6 bp difference between the two ITS-2 sequence types, these could not be reliably distinguished by agarose gel electrophoresis (unlike the β-tubulin locus). Consequently, in order to genotype worms in the French samples (FrMe, FrGa, FrSo and FrMn) the NC1 primer was 5' labelled with the fluorochrome FAM and PCR products were analysed on the ABI 3100 capillary sequencer. As the NC1 and NC2 primers actually correspond to sequence in the 5.8S and 28S ribosomal DNA coding regions, the amplified fragment is larger than the ITS-2 itself. The amplicon includes the primer sequences (40 bp) and 50 bp of the 28S subunit in addition to the ITS-2 region (Figure 5.9b). Consequently the fragment sizes actually amplified with the NC1 and NC2 primers are predicted to be 336 bp for the *T. circumcincta* 'standard' worms and 342 bp for the *T. circumcincta* 'goat' worms. These will be referred to as Type I and Type II respectively for clarity.

All worms in the French populations FrMe, FrGa, FrSo, FrMn and also in the NzWs, ScSo529, ScKiTT, ScKiTD and ScKiTC were genotyped with the β-tubulin and ITS-2 markers. All individuals in the FrGa, FrSo, FrMn, NzWs, ScSo529, ScKiTT, ScKiTD and ScKiTC populations were typed as Type I for the β-tubulin locus (800 - 802 bp product) and Type I for the ITS-2 locus (333 bp product), suggesting these populations of worms only contained the *T. circumcincta* 'standard' worms. The relative difference in size found between the Type I and Type II ITS-2 fragments using Genescan in this experiment was 8 bp rather than 6 bp as predicted from Leignel *et al.* (2002) sequence data. However it should be noted that size of an amplified fragment determined by an ABI 3100 may not reflect the actual sequence length of any particular product. Sequencing of amplicons could have been used to clarify this issue. For the FrMe population, 27 out of 33 worms were genotyped as Type II for both the β-tubulin and ITS-2 markers (341 bp product) and so appear to be the *T. circumcincta* 'goat' cryptic species based on these markers (Table 5.9). Only 3 worms were genotyped as having both Type I alleles for both markers, so appear to be *T. circumcincta* 'standard' worms (FrMe3, FrMe7 and FrMe23). One worm failed to amplify with the β-tubulin marker (FrMe17) and the remaining two worms were "intermediate" genotypes being Type 1 for the ITS-2 marker (333 bp) and Type 2 for the β-tubulin marker (FrMe2 and FrMe26) (Table 5.9).

5.2.8.3 Comparison of multilocus genotypes of the microsatellite, β-tubulin and ITS-2 markers for the FrMe population.

Principle Coordinate Analysis (PCA) was conducted using multilocus genotypes of the five microsatellite loci in order to plot the genetic distance between individuals in each of the French populations. PCA plots show the individual worms from the three French populations FrSo, FrGa, and FrMn mix together in one large cluster consistent with a lack of genetic sub-structuring between these populations (Figure 5.10a). This analysis shows that individual worms are as genetically similar to individuals from separate populations as are individuals within the same population. In contrast, the majority of worms from the FrMe population cluster together in a separate group (Figure 5.10a). This clustering is still evident when MTG 15 data are removed and PCA plot re-calculated (Figure 5.10b). Similarly when the data was jacknifed by sequentially omitting one locus at a time and re-calculating genetic distances based on the remaining four microsatellites, the resulting PCA plots demonstrated a similar pattern of clustering of the individuals from the FrMe population showing that no single microsatellite was responsible for the clustering (Figure 5.11).

In the PCA plot based on all five microsatellite loci (Figure 5.10a), six individual worms from the FrMe population do not cluster with the main group of FrMe worms but instead cluster within the main mix of individuals from the other four French populations. These individual worms are FrMe2, FrMe3, FrMe7, FrMe17, FrMe23, and FrMe26. It is striking that all six of these worms had a Type I ITS-2 genotype and 4 of the 6 had a Type I β-tubulin genotype (Table 5.9). Furthermore, these six worms all amplified with the MTG 15 microsatellite marker and none of them contained the unique 172 bp MTG 67 allele. The individual FrMe6 was also amplified with MTG 15, however is found to be Type II using both the ITS-2 and β-tubulin markers, in addition to the 172 bp MTG 67 allele. Hence there is remarkably strong agreement beyween the multilocus microsatellite genotypes and the ITS-2 / β-tubulin genotyping in assigning individual worms as *T. circumcincta* 'standard' or *T. circumcincta* 'goat' cryptic species.

5.2.9 Polymorphism of the rDNA ITS-2 marker

Previous studies investigating variation at the ITS-2 region of rDNA have used sequence alignments to compare and quantitate variation within and between nematode species. The main reason for genotyping worms using the ITS-2 marker in this study was to investigate the potential presence of the *T. circumcincta* 'goat' cryptic species in the populations we

had genotyped, in particular the FrMe population. The analysis of fluorescently labelled ITS-2 PCR products using a capillary sequencer and Genescan analysis enabled these amplicons to be accurately sized. As described earlier, the Type I and Type II sequences could be easily discerned, although an 8 bp rather than a 6 bp difference was observed. In the course of genotyping the samples using this technique, additional polymorphism of this marker was detected including a number of individuals from which multiple amplicons were observed (Figure 5.12 and Table 5.10). The simplest explanation for this is intra-individual polymorphism of the rDNA array in which there is more than one type of ITS-2 sequence within a single array (Figure 5.9b). All populations genotyped contained a 333 / 335 bp double product in roughly equal numbers as well as the more common single 333 bp product. Of particular note was a 337 bp product which was observed only in the French populations and almost exclusively in the FrGa population. Indeed 8 out of 40 individuals in the FrGa populations contained this 337 bp unit compared to none out of 164 individuals from the Scottish and New Zealand populations that were ITS-2 genotyped. Interestingly, this FrGa population has been suggested to contain a second cryptic species based on mtDNA ND4 sequence data (Leignel *et al.* 2002).

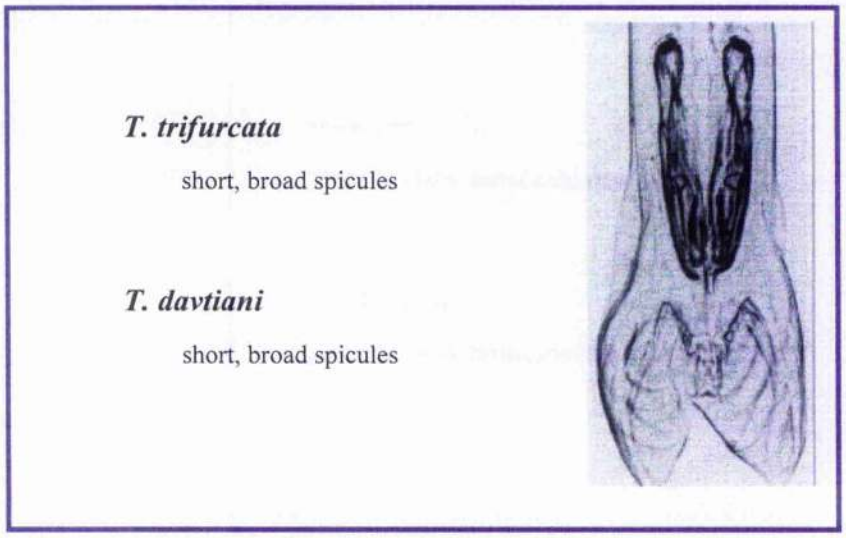
5.2.10 Comparison of *T. circumcincta, T. trifurcata* and *T. davtiani*

Teladorsagia davtiani, *Teladorsagia trifurcata*, and *Teladorsagia circumcincta* have been traditionally considered three separate species based on the morphology of the male tails (Figure 5.1) (Urquhart *et al.* 1996). However genetic differences between these three putative species have not been demonstrated. For example, Stevenson *et al.* (1996) could not find any fixed differences in ITS-2 sequences between 4 worms of *T. circumcincta*, one of *T. trifurcata* and 2 worms of *T. davtiani* sequenced. Consequently it is unclear whether these three morphologically distinct types represent true separate species. A total of 31 *T. circumcincta* (ScKiTC), 30 *T. trifurcata* (ScKiTT) and 31 *T. davtiani* (ScKiTD), as defined by male spicule morphology, were isolated from the same hosts, Soay sheep, on the Isle of Hirta (kindly performed by Dr B. Craig) (Table 2.1-2.3). These were genotyped with the 5 microsatellite markers. Similar levels of polymorphism and diversity were found for the *Teladorsagia davtiani*, *Teladorsagia trifurcata*, and *Teladorsagia circumcincta* populations. The average number of alleles per locus were similar; *T. davtiani* (8.2), *T. trifurcata* (7.6) and *T. circumcincta* (7.8) (Table 5.5). The allele frequencies for all five microsatellites are broadly similar between the three populations (Figure 5.13). A few potential differences were seen e.g.: MTG 15, allele 253 and MTG 67, allele 182, although

these could be artefacts due to the limitations of sample size. Fst values calculated between these three populations based on the 5 markers show very little genetic differentiation between the three types and are comparable to values seen between *T. circumcincta* populations isolated from different hosts on the same farm (Table 5.11). PCA was conducted using multilocus genotypes in order to plot and compare the genetic distance between individuals of each type of *Teladorsagia* (Figure 5.14) and showed individual worms of each type mixed in together with no obvious clustering of one type apparent. In order to compare these three populations with the other populations in this study, PCA plots using pairwise Fst values calculated by GDA ver 1.1 (Figure 5.15a and 5.15b) were re-calculated. These PCA plots are based on the same data as in figure 5.6a and 5.6b but with FrMe and NzWs populations excluded, in order to zoom into the main cluster of populations visible in previous PCA plots. Although *T. davtiani* appears to be slightly separated from the main cluster than are *T. circumcincta* and *T. trifurcata*, it is still within the same broad cluster, being no further separated than some of the other U.K. *T. circumcincta* populations.

Figure 5.1: Morphological identification of *T. circumcincta, T. davtiani* and *T. trifurcata*

The upper box shows the tail end of a male specimen of *T. trifurcata,* which demonstrates the short broad spicules found in both *T. trifurcata* and *T. davtiani.* The lower box shows the tail end of a male specimen of *T. circumcincta,* demonstrating long thin spicules.



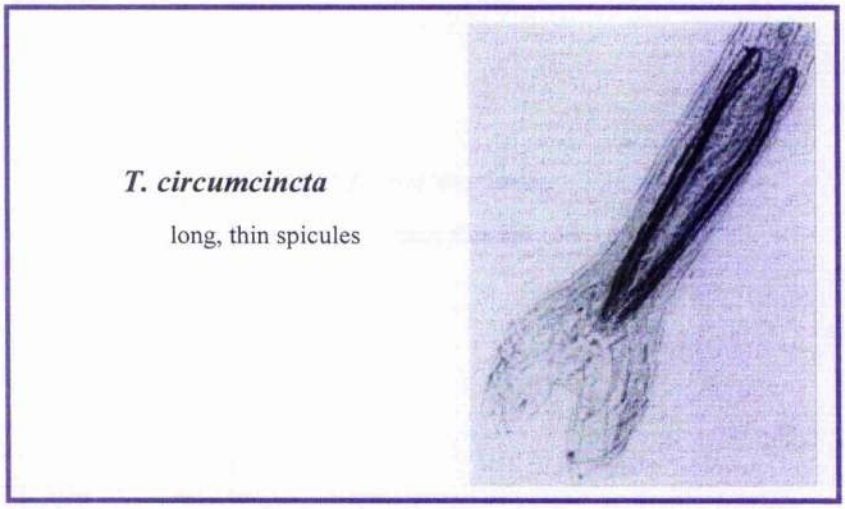


Figure 5.2: Map of North America

Map outlining the location of *M. odocoilei, H. contortus* and *T. circumcincta* populations sampled by Blouin *et al.* (1995).

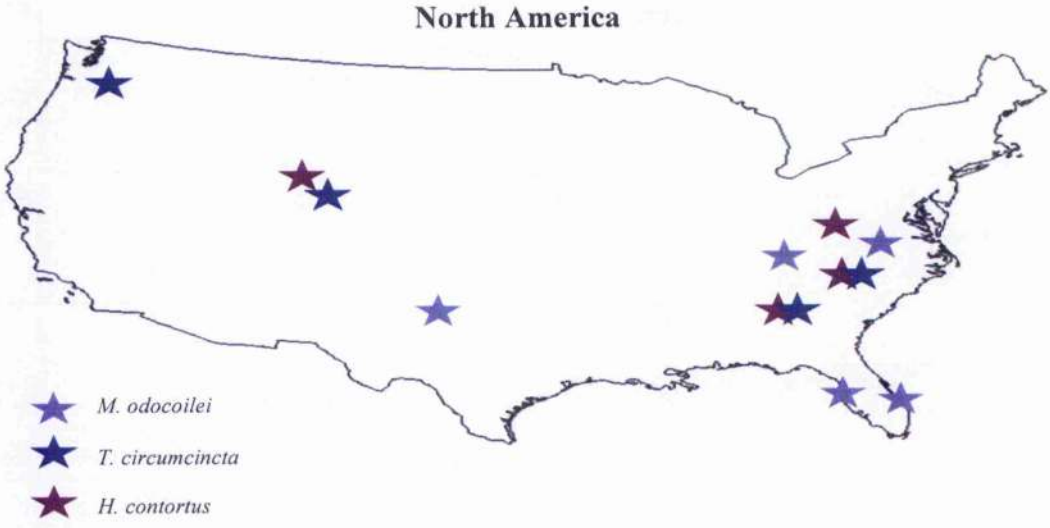


Table 5.1: Summary of results from Leignel *et al.* (2002)

Summary of results from Leignel *et al.*(2002) detailing the distinction between *T. circumcincta* 'standard' and *T. circumcincta* 'goat' based on the four independent markers; MDH-2, mtDNA ND4, β-tubulin isotype 1 and rDNA ITS-2.

	T. circumcincta 'standard'	*T. circumcincta* 'goat'
MDH-2	Alleles **A** and **B**	Allele **C**
β-tubulin isotype 1	Type I (al 1 and al 2)	Type II (al 1 and al 2)
ITS-2	246 bp (Type I)	252 bp (Type II)
mtDNA ND4	Type I & Type II	Type III

Figure 5.3: Map outlining populations studied in this project

The figure shows the 18 populations genotyped in this project, further details are found in Tables 2.1-2.3. The number in brackets is the number of individual adult males genotyped for each population. Note that the populations collected from: 1) Wild soay sheep are from the Island of Hirta and these included *T. circumcincta* (ScKiTC), *T. davtiani* (ScKiTD), and *T. trifurcata* (ScKiTT); 2) The Moredun Lab strains include one population isolated from strain Tci1 (MOSI) and five populations experimentally derived from the same Tci5 (Motri) strain (MotriF, MotriPT, MotriPB, MotriPI and MotriPL); 3) The Sourhope Farm includes three populations isolated from three goats of the same age, managed under identical management conditions (ScSo529, ScSo210 and ScSo509); 4) Carmarthen Farm represents a field population collected from one sheep; 5) The New Zealand population is a lab strain collected from one goat and 6) All populations collected from France are from goat farms in the field.

Figure 5.3: Map outlining populations studied in this project

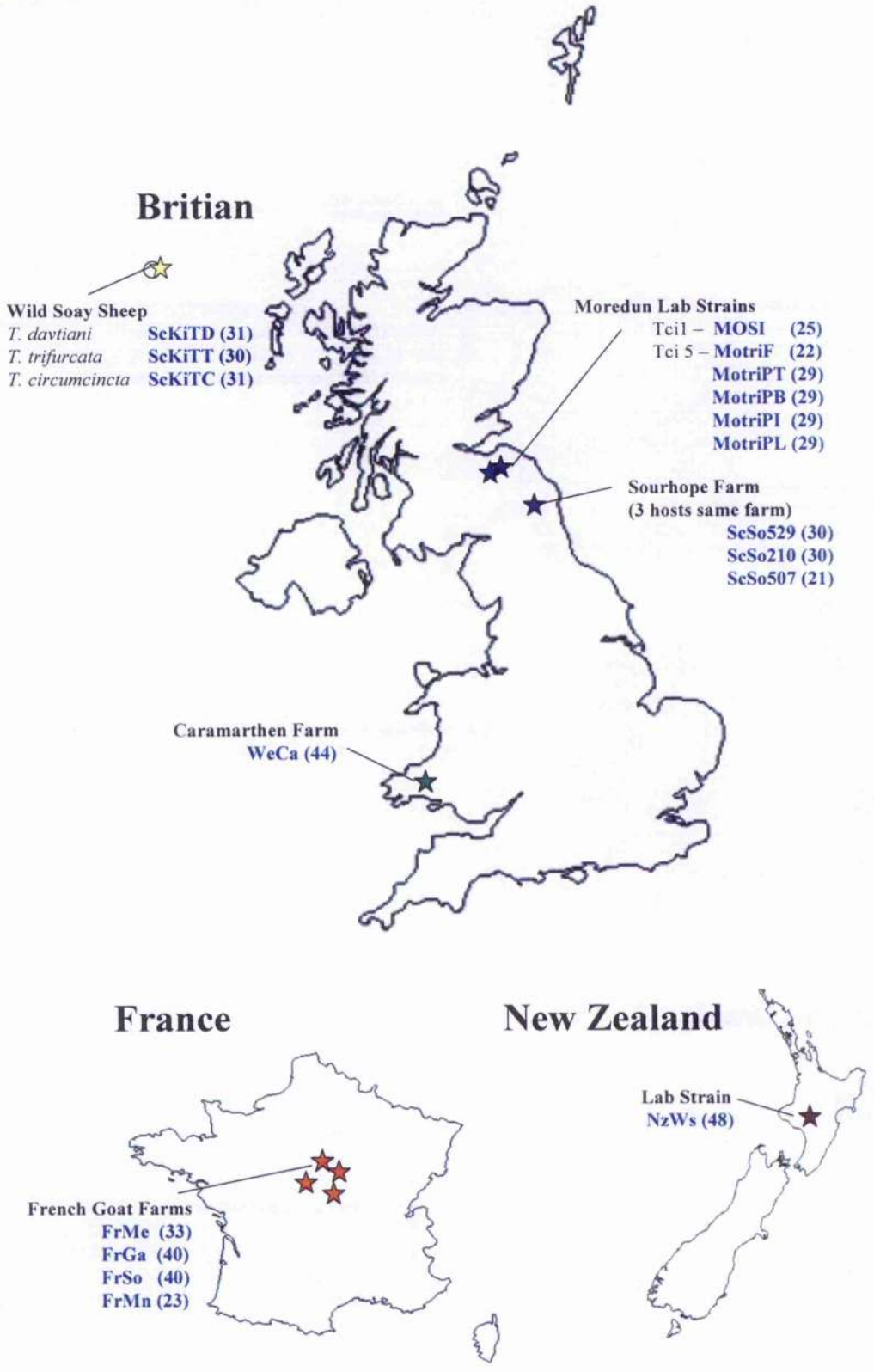


Table 5.2: Expected and observed heterozygosities across each population and each microsatellite marker

H_e = expected heterozygosity (Nei's unbiased, 1987) and H_o = observed heterozygosity calculated in Excel Microsatellite Toolkit (Park 2001). P-value analysis calculated in GDA ver 1.1 (Lewis 2001) using Fisher's Exact Test (10000 runs). A p-value of < 0.05 is evidence of deviation from Hardy-Weinburg equilibrium (HWE). P-values deviating from HWE are shown in bold type, and p-values close to 0.05 are shown in italics. Null homozygotes were not included in data analysis. N_T = total number of individuals typed for each population. N = Number of individual included in data analysis of each loci (i.e. the number of individuals successfully genotyped).

Populations	MTG 15					Hcms 28				MTG 67				MTG 73				MTG 74				All Loci			All Loci excluding MTG15		
	N_T	N	H_e	H_o	P - value	N	H_e	H_o	P - value	N	H_e	H_o	P - value	N	H_e	H_o	P - value	N	H_e	H_o	P - value	H_e	H_o	P - value	H_e	H_o	P - value
FrMe	**33**	7	0.868	0.571	*0.074*	33	0.822	0.758	0.753	30	0.702	0.433	**0.005**	33	0.668	0.545	**0.030**	33	0.571	0.455	*0.073*	0.726	0.552	0.053	0.691	0.548	**0.000**
FrGa	**40**	39	0.895	0.513	**0.000**	40	0.843	0.925	0.109	40	0.748	0.675	0.114	40	0.484	0.425	0.439	40	0.634	0.575	0.578	0.721	0.623	**0.000**	0.678	0.650	0.213
FrSo	**40**	36	0.914	0.444	**0.000**	40	0.873	0.700	**0.050**	40	0.772	0.725	0.921	40	0.412	0.275	**0.007**	40	0.698	0.600	0.257	0.734	0.549	**0.000**	0.689	0.575	**0.000**
FrMn	**23**	20	0.885	0.450	**0.000**	23	0.892	0.696	**0.001**	23	0.694	0.391	**0.004**	23	0.436	0.478	1.000	23	0.700	0.522	0.237	0.721	0.507	**0.000**	0.680	0.522	**0.000**
Mosi	**25**	25	0.908	0.600	**0.000**	25	0.807	0.800	0.874	25	0.686	0.800	0.617	25	0.438	0.320	0.143	25	0.500	0.520	0.127	0.668	0.608	**0.048**	0.608	0.610	0.571
MotriF	**22**	18	0.910	0.278	**0.000**	22	0.872	0.773	**0.033**	22	0.802	0.727	0.203	22	0.440	0.318	*0.063*	22	0.616	0.545	0.249	0.728	0.528	**0.000**	0.683	0.591	**0.013**
MotriPT	**29**	27	0.901	0.259	**0.000**	27	0.871	0.889	0.362	29	0.718	0.759	0.517	29	0.355	0.310	0.401	29	0.572	0.517	*0.063*	0.684	0.547	**0.000**	0.629	0.619	**0.603**
MotriPB	**29**	24	0.856	0.208	**0.000**	27	0.829	0.704	0.121	29	0.755	0.552	**0.012**	28	0.340	0.321	0.485	29	0.568	0.310	**0.002**	0.670	0.419	**0.000**	0.623	0.472	**0.000**
MotriPI	**29**	25	0.918	0.480	**0.000**	24	0.850	0.875	0.564	29	0.659	0.552	0.315	29	0.470	0.448	0.466	29	0.379	0.172	**0.001**	0.655	0.505	**0.006**	0.590	0.512	**0.050**
MotriPL	**29**	28	0.853	0.250	**0.000**	29	0.855	0.793	0.708	28	0.717	0.536	**0.021**	29	0.285	0.276	0.142	29	0.490	0.179	**0.000**	0.640	0.407	**0.000**	0.587	0.446	**0.000**
ScSo529	**30**	30	0.911	0.433	**0.000**	30	0.856	0.800	0.237	30	0.726	0.600	0.316	30	0.300	0.333	1.000	30	0.550	0.367	*0.065*	0.669	0.507	**0.000**	0.608	0.525	**0.010**
ScSo210	**30**	26	0.862	0.154	**0.000**	30	0.741	0.667	**0.041**	30	0.718	0.433	**0.003**	30	0.420	0.400	0.714	30	0.617	0.500	0.322	0.671	0.431	**0.000**	0.624	0.500	**0.000**
ScSo507	**21**	20	0.926	0.250	**0.000**	21	0.871	0.810	0.606	21	0.778	0.524	**0.010**	21	0.463	0.381	0.442	21	0.604	0.714	0.425	0.728	0.536	**0.000**	0.679	0.607	**0.047**
NzWS	**48**	48	0.765	0.646	0.167	48	0.768	0.792	0.860	47	0.800	0.617	**0.010**	48	0.520	0.646	0.218	48	0.563	0.396	*0.055*	0.683	0.619	**0.008**	0.663	0.613	**0.048**
WeCa	**44**	36	0.859	0.361	**0.000**	44	0.847	0.795	**0.050**	44	0.750	0.750	0.361	44	0.336	0.386	1.000	44	0.603	0.682	*0.070*	0.679	0.595	**0.000**	0.634	0.653	0.783
ScKiTD	**31**	28	0.913	0.321	**0.000**	31	0.829	0.677	**0.022**	31	0.766	0.677	0.642	31	0.260	0.097	**0.001**	31	0.645	0.387	**0.002**	0.683	0.432	**0.000**	0.625	0.460	**0.000**
ScKiTT	**30**	26	0.860	0.192	**0.000**	36	0.837	0.667	*0.069*	29	0.633	0.552	*0.093*	30	0.244	0.233	0.410	30	0.566	0.400	**0.018**	0.628	0.409	**0.000**	0.570	0.463	**0.002**
ScKiTC	**31**	27	0.864	0.407	**0.000**	30	0.869	0.800	0.626	30	0.671	0.567	0.247	31	0.328	0.323	1.000	31	0.535	0.323	**0.012**	0.653	0.484	**0.000**	0.601	0.503	**0.006**
Overall populations			**0.895**	**0.390**			**0.852**	**0.774**			**0.758**	**0.612**			**0.438**	**0.371**			**0.611**	**0.457**		**0.686**	**0.514**		**0.666**	**0.554**	

Table 5.3a - c: Inheritance of microsatellite alleles

The tables show the genotyping data from seven females (Female) (F22, F21, Fe19, Fe26, Fe37, Fe1 and Fe8) and their corresponding offspring (O) for MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74. Two females (Fe 1 and Fe 8) and a number of offspring failed to amplify with all microsatellites (shaded in yellow) or the quality of amplification was poor and therefore the genotype is provisional (shaded in light blue). Data from these individual samples are included in the table for information only. The number of paternal alleles was calculated as explained in Figure 5.4b, with minimum and maximum numbers shown here. M / O = evidence of maternal / offspring mismatch; Y = mismatch, N = no mismatch. Where problematic amplification made inheritance analysis inconclusive, data is shown in italics. N/A = data inconclusive. Underlined alleles correspond to alleles which are potential paternal alleles. Where the exact number of paternal alleles could not be deduced due to amplification problems, the number of paternal alleles was not determined.

Table 5.3a: Inheritance of microsatellite alleles

Worm ID	MTG 15		Hcms 28		MTG 67		MTG 73		MTG 74	
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
F21 (Female)	237	237	173	185	176	190	151	151	159	162
Offspring										
E11	237	237	169	185	176	176	151	151	159	162
E12	237	237	169	185	176	176	151	151	159	162
E13	237	237	161	173	176	178	151	151	162	162
E14	237	241	169	173	176	190	151	151	159	162
E15	237	237	169	185	176	176	151	151	159	165
E16	237	237	173	185	176	180	151	151	162	162
E17	237	237	171	173	178	190	151	151	159	162
E18	237	237	173	173	180	190	151	151	159	159
E19	237	253	167	173	176	190	151	151	162	162
No. of paternal alleles		3-4		5-7		3-5		1-2		2-4
M/O mismatch		N		N		N		N		N
F22 (Female)	234	245	161	171	176	178	151	151	159	162
Offspring										
E1	245	245	167	171	176	178	151	154	159	162
E2	245	253	161	167	176	176	151	151	159	162
E3	245	253	167	171	176	176	151	151	159	162
E4	234	239	169	171	178	178	151	151	162	168
E5	234	255	161	167	176	176	151	151	162	165
E6	245	245	161	165	176	180	151	151	159	159
E7	245	253	161	165	176	176	151	151	162	165
E8	0	0	0	0	176	176	0	0	0	0
No. of paternal alleles		4-5		3		3-5		2-3		3-5
M/O mismatch		N		N		N		N		N

Table 5.3b: Inheritance of microsatellite alleles

Worm ID	MTG 15		Hcms 28		MTG 67		MTG 73		MTG 74	
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
Fe19 (Female)	259	259	161	171	176	176	151	151	159	159
Offspring										
Fe20	249	249	161	167	176	176	151	151	159	159
Fe21	0	0	0	0	0	0	0	0	0	0
Fe22	253	253	169	171	176	184	151	151	159	159
Fe23	249	249	169	171	176	184	151	151	159	159
Fe24	241	241	167	171	176	178	151	151	159	159
Fe25	0	0	161	167	176	180	151	151	159	159
No. of paternal alleles		3-4		2		4		1-2		1-2
M/O mismatch		Y		N		N		N		N
Fe26 (Female)	243	243	169	175	176	178	148	148	159	159
Offspring										
Fe27	243	243	161	169	176	178	151	151	159	159
Fe28	243	251	175	175	176	176	151	151	159	159
Fe29	251	251	169	175	176	178	148	151	159	159
Fe30	243	243	161	175	176	180	151	151	159	159
Fe31	243	253	161	175	176	180	151	151	162	162
Fe32	243	243	169	175	176	176	151	151	159	159
Fe33	243	253	161	169	176	178	151	151	162	162
Fe34	0	0	161	169	176	178	151	151	162	162
Fe35	253	253	169	175	176	176	148	151	159	159
Fe36	251	251	161	175	176	178	148	151	159	159
No. of paternal alleles		3-4		2-4		2-4		1		2-3
M/O mismatch		Y		N		N		Y		N/A
Fe37 (Female)	237	253	171	173	176	178	148	151	159	159
Offspring										
Fe38	253	253	169	173	176	178	148	151	159	159
Fe39	237	237	161	173	176	180	151	151	159	159
Fe40	253	253	161	173	176	176	148	151	159	159
Fe41	237	237	161	171	178	180	151	151	159	159
Fe42	237	249	169	171	178	180	151	151	159	159
Fe43	237	249	161	171	178	178	151	151	159	159
Fe44	237	241	171	171	178	178	151	151	159	159
Fe45	241	253	171	173	176	186	148	151	159	159
No. of paternal alleles		3-5		3-4		4-5		1-3		1-2
M/O mismatch		N		N		N		N		N

Table 5.3c: Inheritance of microsatellite alleles

Worm ID	MTG 15		Hcms 28		MTG 67		MTG 73		MTG 74	
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2
Fe1 (Female)	0	0	169	171	0	0	0	0	159	159
Offspring										
Fe2	243	243	167	171	176	180	148	151	159	159
Fe3	241	241	169	175	176	176	148	151	159	162
Fe4	241	241	161	169	176	182	148	151	162	162
Fe5	243	249	161	171	176	180	151	151	162	162
Fe6	243	243	161	171	176	176	151	151	159	159
Fe7	0	0	0	0	0	0	0	0	0	0
No. of paternal alleles		N/A		3		N/A		N/A		2-3
M/O mismatch		N/A		N		N/A		N/A		N/A
Fe8 (Female)	0	0	0	0	0	0	151	151	0	0
Offspring										
Fe9	243	243	0	0	178	182	0	0	0	0
Fe10	243	259	0	0	180	180	148	151	0	0
Fe11	243	243	167	171	180	180	151	151	159	159
Fe12	243	243	161	167	178	178	151	151	159	159
Fe13	243	259	167	167	176	182	151	151	159	159
Fe14	243	259	167	167	180	182	151	151	159	159
Fe15	243	253	167	171	180	180	151	151	159	159
Fe16	243	243	161	167	180	182	151	151	159	159
Fe17	243	259	167	167	176	182	151	151	159	159
Fe18	243	243	167	181	180	180	151	151	159	159
No. of paternal alleles		N/A		N/A		N/A		2-3		N/A
M/O mismatch		N/A		N/A		N/A		N/A		N/A

Figure 5.4a: Example of Maternal-Offspring Mismatch

The maternal genotype (Fe26) is apparently homozygous for allele 243. However four progeny (Fe29, Fe34, Fe35 and Fe36) have not inherited the 243 maternal allele, referred to as maternal-offspring mismatch. This can be explained if the maternal genotype is heterozygous 243 / null. In this case, those progeny lacking the 243 maternal allele have instead inherited the maternal null allele. The progeny (Fe34) appears to be homozygous null, which could be explained by inheritance of a maternal 'null' allele and a paternal 'null' allele.

Figure 5.4b: Calculating the number of Paternal alleles per brood

Unambiguous designation of maternal and paternal alleles is seen in the progeny E11, E12, E13, E14, E15, E17 and E19. The following rules were applied to designate paternal alleles in potentially ambiguous cases: 1) Where the genotype of the offspring was heterozygous and identical to that of the mother, both alleles were considered as potential paternal alleles (e.g. E16) and 2) Where the genotype of the offspring was apparently homozygous for a maternal allele (e.g. E18), the paternal allele was taken as being either identical to the maternal allele or a null. Once the paternal alleles had been assigned for each individual progeny then the maximum and minimal number of paternal alleles was counted for each brood. In this case the minimum number of alleles would be five (161, 167, 169,171 and 173) and the maximum would be seven (161, 167, 169, 171, 173 (or 'null') and 185). As each male could contribute up to two alleles per brood, the minimum number of males in this brood must be 3.

Figure 5.4a: Example of Maternal-Offspring Mismatch

Worm ID	MTG 15	
	allele 1	allele 2
Fe26 (F)	243	/
Fe27 (O)	243	/
Fe28 (O)	243	251
Fe29 (O)	/	251
Fe30 (O)	243	/
Fe31 (O)	243	253
Fe32 (O)	243	/
Fe33 (O)	243	253
Fe34 (O)	/	/
Fe35 (O)	/	253
Fe36 (O)	/	251

Maternal – Offspring Mismatch

Four offspring apparently have no maternal allele
(Fe29, Fe34, Fe35 and Fe36)

Figure 5.4b: Calculating the number of Paternal alleles per brood

Worm ID	Hcms 28	
	allele 1	allele 2
F21 (F)	173	185
E11 (O)	169	185
E12 (O)	169	185
E13 (O)	161	173
E14 (O)	169	173
E15 (O)	169	185
E16 (O)	173	185
E17 (O)	171	173
E18 (O)	173	/
E19 (O)	167	173

Potential Paternal Alleles

169
161
173 or 185
171
173 (or null?)
167

Minimum number of paternal alleles = **5**

Maximum number of paternal alleles = 7

Therefore female mated with at least **3** individual males

Undetermined Maternal or Paternal allele

Maternal alleles

Paternal Alleles

/ = denotes an allele which could be identical to first allele or could be 'null' allele

Table 5.4: Calculations of null allele frequencies

N_f values shown in bold indicate deviation from HWE, based on calculations made in GDA ver 1.1 (Lewis 2001). Genepop ver 3.3 requires at least one null homozygote per population to estimate null frequency. Hence, one extra individual was added to each population and given a null homozygote genotype for all loci, and therefore null allele frequencies are over estimated. N_f: null allele frequency calculated by GENEPOP ver 3.3 (Raymond and Rousset 1995); N_e: number of null homozygotes estimated using null allele frequency calculated as follows: if p = allele frequency the p^2= frequency of homozygote for allele p, therefore (p^2)(no. of individuals in population) = expected number of homozygote nulls. N_o = no. of observed homozygote null individuals from data set not including the extra individual which was added to each population to allow Genepop ver 3.3 analysis.

Table 5.4: Calculations of null allele frequencies

Population	MTG 15			Hcms 28			MTG 67			MTG 73			MTG 74		
	N_f	N_e	N_o	N_f	N_e	N_o	N_f	N_e	N_o	N_f	N_e	N_o	N_f	N_e	N_o
FrMe	**0.837**	23	26	0.083	0.2	0	**0.301**	2.9	3	**0.114**	0.4	0	0.147	0.7	0
FrGa	**0.245**	2.4	1	0.036	0.05	0	0.090	0.3	0	0.114	0.5	0	0.092	0.3	0
FrSo	**0.344**	2.7	4	**0.126**	0.4	0	0.074	0.1	0	**0.168**	0.6	0	0.112	0.3	0
FrMn	**0.373**	5.6	3	**0.143**	0.8	0	**0.224**	2	0	0.112	0.3	0	0.169	1.1	0
Mosi	**0.195**	0.9	0	0.079	0.2	0	0.058	0.1	0	0.179	0.7	0	0.093	0.2	0
MotriF	**0.483**	5.1	4	**0.103**	0.2	0	0.123	0.3	0	0.187	0.8	0	0.148	0.5	0
MotriPT	**0.408**	4.8	2	0.129	0.48	2	0.065	0.1	0	0.137	0.5	0	0.124	0.4	0
MotriPB	**0.493**	7	5	0.196	1.1	2	**0.163**	0.8	0	0.193	1.1	1	**0.215**	1.3	0
MotriPI	**0.366**	3.8	4	0.233	1.6	5	0.138	0.6	0	0.107	0.3	0	**0.237**	1.6	0
MotriPL	**0.374**	4.1	1	0.083	0.2	0	**0.206**	1.2	1	0.119	0.4	0	**0.311**	2.8	1
ScSo529	**0.274**	2.3	0	0.085	0.2	0	0.120	0.4	0	0.103	0.3	0	0.187	1	0
ScSo210	**0.489**	7.1	4	**0.083**	0.2	0	**0.216**	1.4	0	0.114	0.4	0	0.142	0.6	0
ScSo507	**0.409**	3.5	1	0.101	0.2	0	**0.205**	1.3	0	0.164	0.6	0	0.082	0.1	0
NzWS	0.100	0.5	0	0.048	0.1	0	**0.159**	1.2	1	0.046	0.1	0	0.150	1.1	0
WeCa	**0.433**	8.2	8	**0.066**	0.2	0	0.056	0.1	0	0.069	0.2	0	0.063	0.2	0
ScKiTD	**0.401**	4.9	3	**0.113**	0.4	0	0.101	0.3	0	**0.232**	1.6	0	**0.207**	1.3	0
ScKiTT	**0.474**	6.7	4	0.136	0.6	0	0.163	0.8	1	0.126	0.5	0	**0.181**	0.9	0
ScKiTC	**0.379**	4.4	4	0.119	0.4	1	0.171	0.9	1	0.121	0.5	0	**0.199**	1.2	0

Table 5.5: Population diversity

H_e = expected heterozygosity (Nei's unbiased, 1987) calculated in Excel Microsatellite Toolkit (Park 2001). Average number of alleles per locus was calculated using all five microsatellites by GDA ver 1.1 (Lewis 2001). The total number of alleles found for each microsatellite are shown under each microsatellite title. Number of alleles per microsatellite per population are shown with the number of unique alleles per locus per population shown in brackets. * = based on only seven individuals. ** = Unique allele frequency is 0.26, all other unique alleles are at frequencies < 0.07.

Table 5.5: Population Diversity

Population	No. of samples	H_e All loci	H_e excluding MTG 15	Avg. No. of alleles / locus	No. of alleles (unique alleles) per population				
					MTG 15 26 total alleles	Hcms 28 18 total alleles	MTG 67 19 total alleles	MTG 73 6 total alleles	MTG 74 8 total alleles
FrMe	33	0.7263	0.6908	6	7 (1)*	9(1)	5 (1**)	4	5 (1)
FrGa	40	0.7210	0.6776	9.4	18	11	9 (1)	3	6 (1)
FrSo	40	0.7339	0.6888	9.4	14	11	12	4	6 (1)
FrMn	23	0.7213	0.6804	7.4	11	12	6	3	5
Mosi	25	0.6676	0.6076	6.8	13 (1)	8	6	3	4
MotriF	22	0.7280	0.6826	8.2	13 (1)	12	9	3	4
MotriPT	29	0.6837	0.6292	8	13	12	7 (1)	3	5
MotriPB	29	0.6696	0.6229	7.4	11	9	9	3	5
MotriPI	29	0.6552	0.5896	7.6	14	12 (1)	5	4	3
MotriPL	29	0.6399	0.5867	8.6	13	14 (2)	6	6 (2)	4
ScSo529	30	0.6688	0.6082	8.6	15	11 (1)	8	4	5
ScSo210	30	0.6714	0.6237	8	12	10	9 (2)	4	5
ScSo507	21	0.7284	0.6792	7.8	13	10	8	3	5
NzWS	48	0.6831	0.6626	5.4	8	7	6	3	3
WeCa	44	0.6790	0.6340	8.2	11	11	10 (1)	4	5
ScKiTD	31	0.6827	0.6251	8.2	16 (1)	11	7	3	4
ScKiTT	30	0.6280	0.5699	7.6	13	10	8	3	4
ScKiTC	31	0.6534	0.6007	7.8	14	11	7	3	4

Figure 5.5: Allele frequencies for ScSo529, ScSo210 and ScSo507 for each locus
Individual histograms for microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74, showing the allele frequencies for each host population, ScSo529, ScSo210, ScSo507, collected from three individual goats of the same age raised under identical management conditions.

Figure 5.5: Allele frequencies for ScSo529, ScSo210 and ScSo507 for each locus

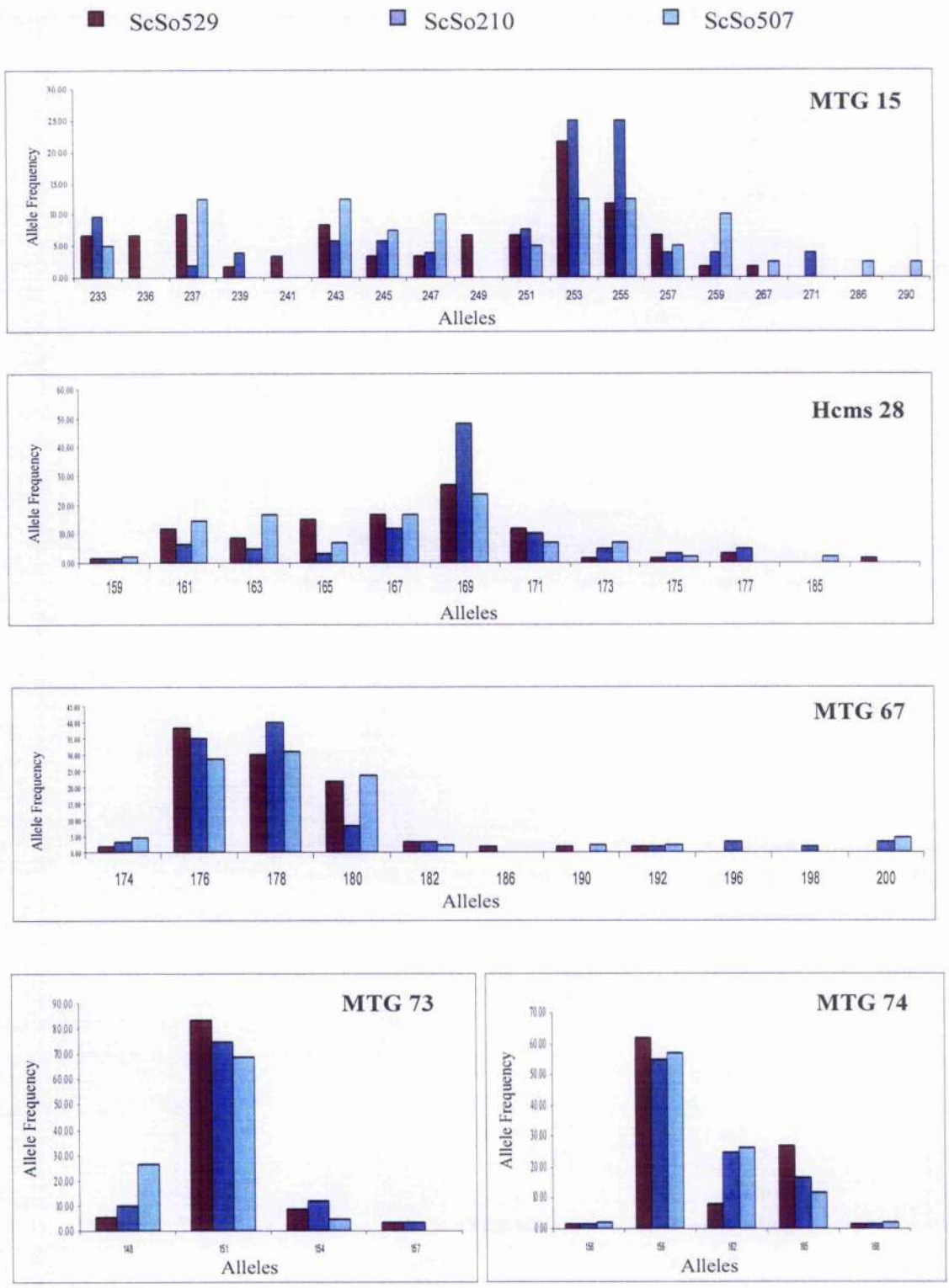


Table 5.6a: Pairwise Fst values calculated using all five loci

Pairwise Fst calculations between all 14 populations using the microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74. Calculations performed using GDA ver 1.1 (Lewis 2001). Dark blue shading – moderate / high levels of genetic differentiation (Fst > 0.1); pale blue shading – moderate levels of genetic differention (Fst range from 0.04 to 0.1); white shading – low levels of genetic differentiation (Fst < 0.04), based on Wright's guidelines (1978) as referenced by (Hartl and Clarke 1997). Purple shading highlights pairwise Fst values between the French populations: FrGa, FrSo and FrMn. Grey shading highlights pairwise Fst values between three individual goats on the same farm.

Table 5.6b: Pairwise Fst values calculated using all loci excluding MTG 15

Pairwise Fst calculations between all 14 populations using the microsatellites Hcms 28, MTG 67, MTG 73 and MTG 74. Calculations performed using GDA ver 1.1 (Lewis 2001). Shading as for table 5.6a.

Table 5.6a: Pairwise Fst values calculates using all five loci

	FrMe	FrGa	FrSo	FrMn	Mosi	MotriF	MotriPT	MotriPB	MotriPI	MotriPL	ScSo529	ScSo210	ScSo507	NzWS	WeCa	ScKiTD	ScKiTT
FrGa	0.0809																
FrSo	0.0967	0.0004															
FrMn	0.0850	0.0014	-0.0030														
Mosi	0.1477	0.0190	0.0245	0.0182													
MotriF	0.1164	0.0072	0.0025	0.0005	0.0115												
MotriPT	0.1226	0.0059	0.0083	0.0082	0.0134	-0.0064											
MotriPB	0.1439	0.0089	0.0149	0.0139	-0.0008	-0.0002	-0.0030										
MotriPI	0.1583	0.0228	0.0316	0.0308	0.0012	0.0149	0.0051	-0.0043									
MotriPL	0.1650	0.0203	0.0227	0.0179	0.0049	-0.0043	0.0005	-0.0065	0.0067								
ScSo529	0.1498	0.0190	0.0092	0.0222	0.0099	0.0055	0.0017	-0.0038	0.0013	0.0061							
ScSo210	0.1244	0.0145	0.0261	0.0202	0.0220	0.0061	-0.0020	-0.0019	0.0135	0.0125	0.0066						
ScSo507	0.1058	0.0011	-0.0013	0.0002	-0.0018	-0.0089	-0.0070	-0.0040	0.0023	0.0040	0.0024	0.0049					
NzWS	0.1221	0.0430	0.0524	0.0626	0.0652	0.0512	0.0449	0.0324	0.0547	0.0574	0.0586	0.0424	0.0459				
WeCa	0.1357	0.0070	0.0094	0.0038	0.0049	0.0027	-0.0031	-0.0049	0.0084	-0.0063	0.0049	0.0127	-0.0015	0.0511			
ScKiTD	0.1427	0.0210	0.0207	0.0149	0.0254	0.0204	0.0306	0.0242	0.0392	0.0250	0.0282	0.0340	0.0149	0.0672	0.0143		
ScKiTT	0.1619	0.0239	0.0307	0.0186	0.0066	0.0249	0.0148	0.0028	0.0048	0.0091	0.0056	0.0186	0.0237	0.0567	0.0056	0.0214	
ScKiTC	0.1573	0.0216	0.0201	0.0097	-0.0003	0.0124	0.0065	-0.0019	-0.0030	-0.0027	-0.0014	0.0137	0.0054	0.0594	-0.0015	0.0250	-0.0068

Table 5.6b: Pairwise Fst values calculates using all five loci excluding MTG 15

	FrMe	FrGa	FrSo	FrMn	Mosi	MotriF	MotriPT	MotriPB	MotriPI	MotriPL	ScSo529	ScSo210	ScSo507	NzWS	WeCa	ScKiTD	ScKiTT
FrGa	0.1041																
FrSo	0.1224	-0.0017															
FrMn	0.1125	-0.0054	-0.0063														
Mosi	0.1902	0.0196	0.0287	0.0221													
MotriF	0.1432	0.0058	0.0039	0.0012	0.0129												
MotriPT	0.1613	0.0082	0.0134	0.0145	0.0153	-0.0062											
MotriPB	0.1814	0.0116	0.0154	0.0152	0.0006	0.0021	-0.0022										
MotriPI	0.2067	0.0343	0.0465	0.0425	0.0029	0.0202	0.0127	-0.0051									
MotriPL	0.2042	0.0185	0.0224	0.0225	0.0042	-0.0007	-0.0015	-0.0038	0.0059								
ScSo529	0.1942	0.0253	0.0163	0.0287	0.0161	0.0088	0.0059	-0.0030	0.0088	0.0062							
ScSo210	0.1591	0.0180	0.0296	0.0259	0.0261	0.0115	0.0001	0.0048	0.0185	0.0209	0.0115						
ScSo507	0.1367	0.0003	-0.0008	0.0015	0.0033	-0.0082	-0.0074	-0.0032	0.0106	0.0063	0.0083	0.0083					
NzWS	0.1367	0.0387	0.0519	0.0593	0.0619	0.0471	0.0449	0.0341	0.0544	0.0567	0.0645	0.0476	0.0385				
WeCa	0.1736	0.0059	0.0065	0.0066	0.0045	-0.0021	-0.0020	-0.0042	0.0143	-0.0070	0.0051	0.0170	-0.0007	0.0466			
ScKiTD	0.1823	0.0208	0.0247	0.0228	0.0318	0.0284	0.0371	0.0246	0.0560	0.0314	0.0359	0.0388	0.0253	0.0590	0.0165		
ScKiTT	0.2152	0.0303	0.0398	0.0277	0.0066	0.0262	0.0243	0.0051	0.0114	0.0043	0.0116	0.0263	0.0313	0.0667	0.0077	0.0227	
ScKiTC	0.2016	0.0218	0.0226	0.0168	-0.0024	0.0113	0.0080	-0.0043	-0.0044	-0.0029	-0.0007	0.0191	0.0056	0.0582	-0.0005	0.0306	-0.0082

Figure 5.6a: Principle Coordinate Analysis using all five loci

Principle Coordinate Analysis using the microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74 based on Fst values calculated by GDA ver 1.1, with each population representing a separate data point. The percentage of variation explained by the first two coordinates is shown on the X and Y axis of the graph.

Figure 5.6b: Principle Coordinate Analysis using all loci excluding MTG 15

Principle Coordinate Analysis using the microsatellites Hcms 28, MTG 67, MTG 73 and MTG 74 based on Fst values calculated by GDA ver 1.1, with each population representing a separate data point. The percentage of variation explained by the first two coordinates is shown on the X and Y axis of the graph.

Figure 5.6a

All five microsatellite loci

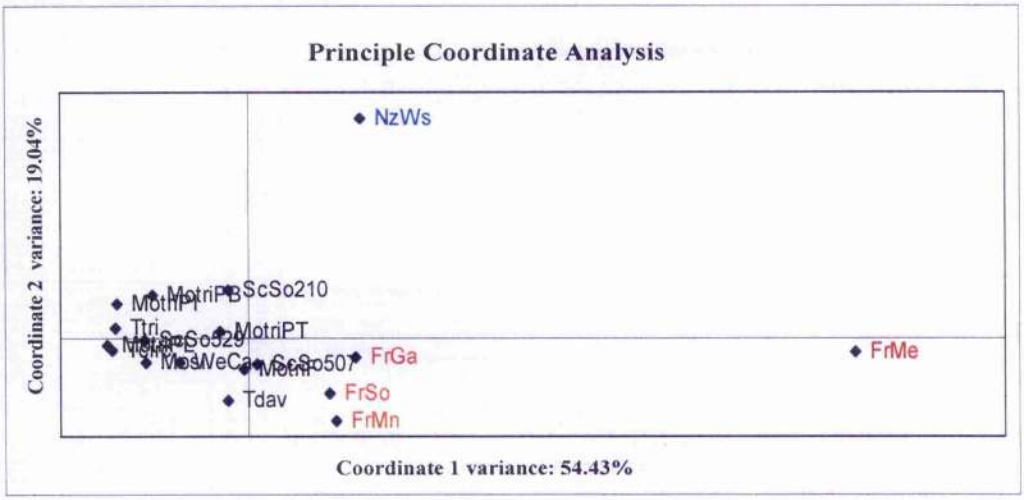


Figure 5.6b

All loci excluding MTG 15

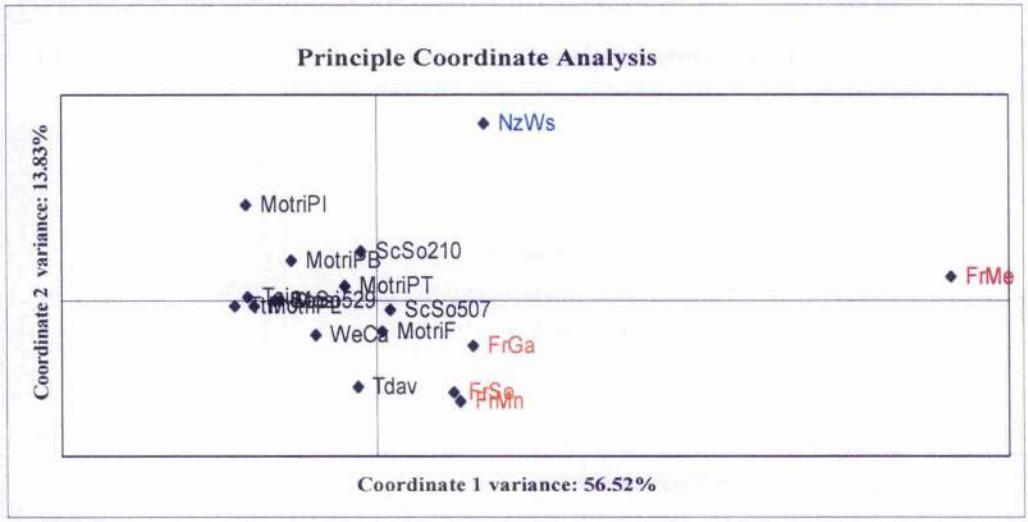
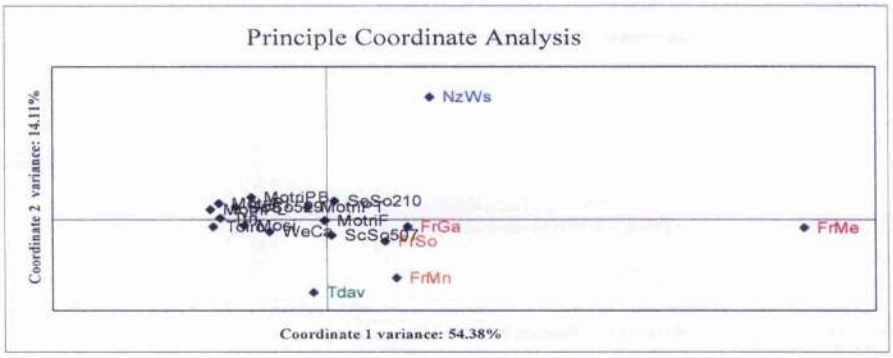


Figure 5.7: Jackknifing Principle Coordinate Analysis

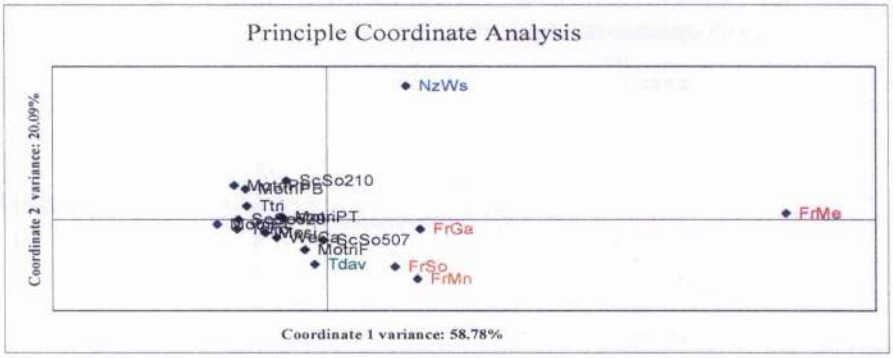
Principle Coordinate Analysis using the microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74, based on Fst values calculated by GDA ver 1.1, excluding one microsatellite at a time as indicated by the labelling. Each data point represents one population. Percentage of variation explained by the first two coordinates is shown on the X and Y axis of the graph.

Figure 5.7: Jackknifing Principle Coordinate Analysis

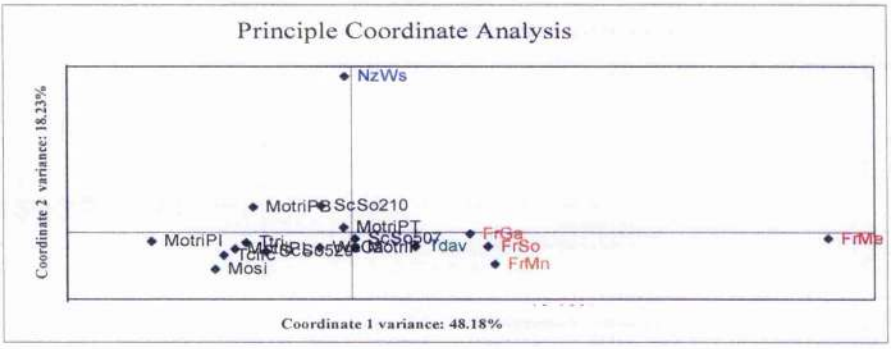
No Hcms 28



No MTG 67



No MTG 73



No MTG 74

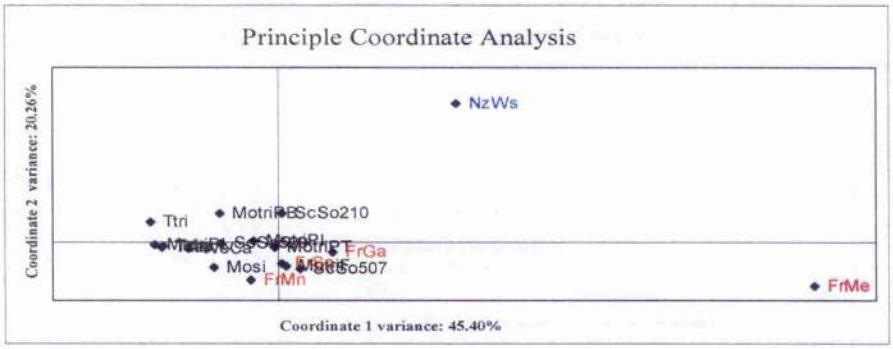


Table 5.7a: AMOVA analysis using all five loci

Analysis of Molecular Variance (AMOVA) calculated locus by locus using Arelquin ver 2.0 (Excoffier *et al.* 1992;Schneider *et al.* 2000;Weir 1996;Weir and Cockerman 1984). The following three population groupings were used for the countries: France (FrMe, FrGa, FrSo, and FrMn), U.K. (MOSl, MotriF, MotriPT, MotriPB, MotriPI, MotriPL, ScSo529, ScSo210, ScSo507, WeCa, ScKiTC, ScKiTT, and ScKiTD) and New Zealand (NzWs). The FrMe and NzWs populations were excluded from the calculations as stated in columns. ** = p-value < 0.001, * = p-value < 0.01

Table 5.7b: AMOVA analysis using all loci excluding MTG 15

Analysis of Molecular Variance (AMOVA) calculated locus by locus using Arelquin ver 2.0 (Excoffier *et al.* 1992;Schneider *et al.* 2000;Weir 1996;Weir and Cockerman 1984). Groupings are as outlined in Table 5.7a were used. ** = p-value < 0.001, * = p-value < 0.01

Table 5.7a: AMOVA analysis using all five loci

Source of Variation using all loci	All Populations		All Populations excluding FrMe		All Populations excluding FrMe & NzWs	
	Variance Components	Percentage of Variation	Variance Components	Percentage of Variation	Variance Components	Percentage of Variation
Within Populations	1.5948**	95.44 %	1.6108**	96.57 %	1.6027**	97.89 %
Among Populations within Countries	0.0203**	1.21 %	0.0145*	0.87 %	0.01468*	0.90 %
Among Countries	0.0559**	3.35 %	0.0427**	2.56 %	0.01985*	1.21 %

Table 5.7b: AMOVA analysis using all loci excluding MTG 15

Source of Variation using all loci except MTG 15	All Populations		All Populations excluding FrMe		All Populations excluding FrMe & NzWs	
	Variance Components	Percentage of Variation	Variance Components	Percentage of Variation	Variance Components	Percentage of Variation
Within Populations	1.2514**	94.08 %	1.6107**	96.57 %	1.6027**	97.89 %
Among Populations within Countries	0.02996**	2.25 %	0.01454*	0.87 %	0.01468*	0.90 %
Among Countries	0.04885	3.67 %	0.04269**	2.56 %	0.01985*	1.12 %

Figure 5.8: Amplification of microsatellite MTG 15 from the Tci1 (MOSI) and FrMe populations

A 2 % agarose gel showing amplification of the MTG 15 locus from 1) Upper panel: 17 individual worms from the Tci1 (MOSI) population. Amplicons are visible for all individuals. 2) Lower panel: 33 individuals from the FrMe population. Amplicons are visible from only seven individuals (6 dark purple arrows) All the PCR products were examined by Genescan analysis to confirm lack of amplification. The light purple arrow indicates an amplicon that was barely visible on the gel but clearly visible on Genescan analysis. C = controls using dH_2O in replacement for template.

Figure 5.8: Amplification of microsatellite MTG 15 from the Tci1 (MOSI) and FrMe populations

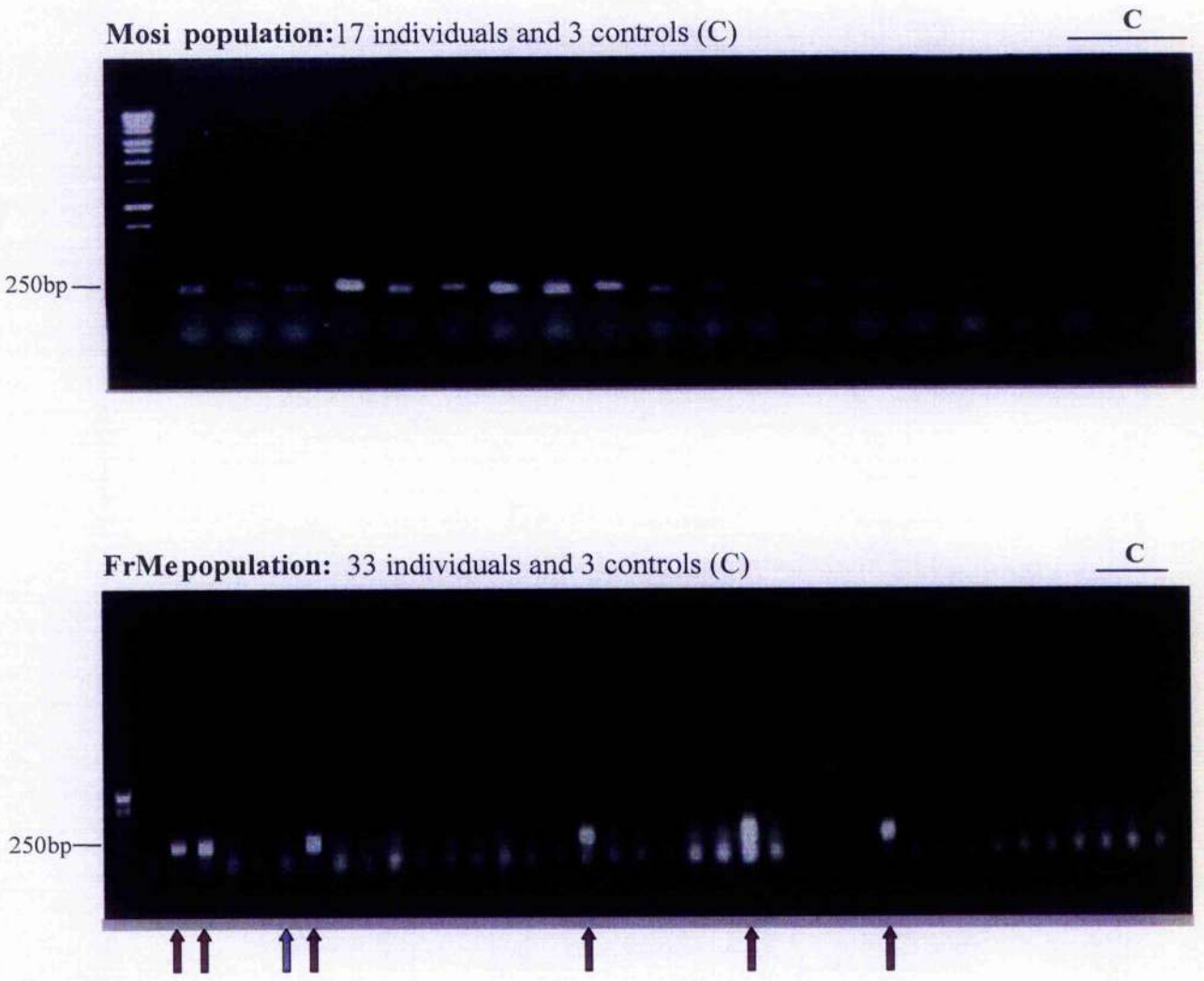


Table 5.8: FrMe genotyping results with MTG 15 and MTG 67

Summary of results for 33 individuals from the FrMe population genotyped with MTG 15 and MTG 67. Individuals highlighted in light blue were amplified successfully with MTG 15. The 172 allele of MTG 67 which was unique to the FrMe population is shown in purple font. Homozygous null refers to individuals which consistently failed to amplify with that marker.

FrMe Individuals	MTG 15	MTG 67
FrMe1	Homozygous Null	178 / 178
FrMe2	**253 / 253**	178 / 178
FrMe3	**243 / 243**	176 / 178
FrMe4	Homozygous Null	Homozygous Null
FrMe5	Homozygous Null	176 / 178
FrMe6	**258 / 258**	**172 / 172**
FrMe7	**241 / 263**	178 / 178
FrMe8	Homozygous Null	178 / 178
FrMe9	Homozygous Null	178 / 178
FrMe10	Homozygous Null	**172** / 176
FrMe11	Homozygous Null	**172** / 178
FrMe12	Homozygous Null	178 / 178
FrMe13	Homozygous Null	**172** / 176
FrMe14	Homozygous Null	**172** / 178
FrMe15	Homozygous Null	**172 / 172**
FrMe16	Homozygous Null	176 / 176
FrMe17	**253 / 273**	176 / 176
FrMe18	Homozygous Null	176 / 176
FrMe19	Homozygous Null	**172** / 174
FrMe20	Homozygous Null	178 / 178
FrMe21	Homozygous Null	178 / 178
FrMe22	Homozygous Null	**172** / 176
FrMe23	**241 / 241**	178 / 178
FrMe24	Homozygous Null	178 / 178
FrMe25	Homozygous Null	176 / 176
FrMe26	**243 / 255**	176 / 176
FrMe27	Homozygous Null	**172** / 176
FrMe28	Homozygous Null	Homozygous Null
FrMe29	Homozygous Null	**172** / 178
FrMe30	Homozygous Null	**172 / 172**
FrMe31	Homozygous Null	Homozygous Null
FrMe32	Homozygous Null	174 / 174
FrMe33	Homozygous Null	**172 / 172**

Figure 5.9a: Examples of Type I and Type II PCR products amplified using primers flanking the β-tubulin marker

Two 1.5 % agarose gels showing the Type I and Type II products amplified using primers M2 and D7 flanking the β-tubulin marker. Gel A: Lane 1 = Type II genotype, with two within type alleles, Lane 2, 3, and 4 = Type I genotype. Gel B: Lanes 1 and 3 = Type II genotype, showing two within type alleles, Lane 2 = Heterozygous individual with Type I / Type II (faint) genotype, Lane 4 = Type II genotype with one within type allele.

Figure 5.9b: Schematic diagram of rDNA cistron and location of ITS-2 primers

Schematic diagram of the ribosomal DNA cistron adapted from Dorris *et al.* 1999, sizes are not to scale. The rDNA cistron is present in approximately 55 copies of the repeat unit per genome. The repeat unit consists of the small subunit gene (SSU; 18S), the internal transcribed spacer 1 (ITS-1), the 5.8S, internal transcribed spacer 2 (ITS-2), and the large subunit gene (LSU; 28S). The NTS (non-transcribed spacer) separates each cistron. The relative position of the primers NC1 and NC2 are shown. The table shows the size of the actual sequence length amplified by the NC1 and NC2 primers for both *T. circumcincta* 'standard' and *T. circumcincta* 'goat' in relation to the actual size of the ITS-2 sequence itself.

Figure 5.9a: Examples of Type I and Type II PCR products amplified using primers flanking the β-tubulin marker

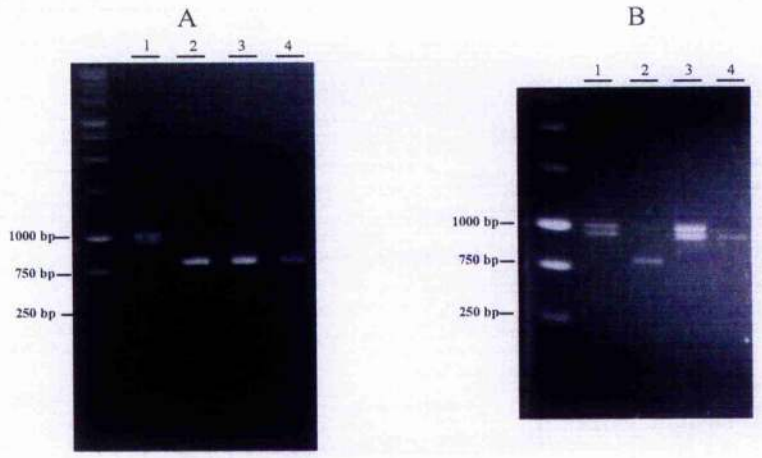
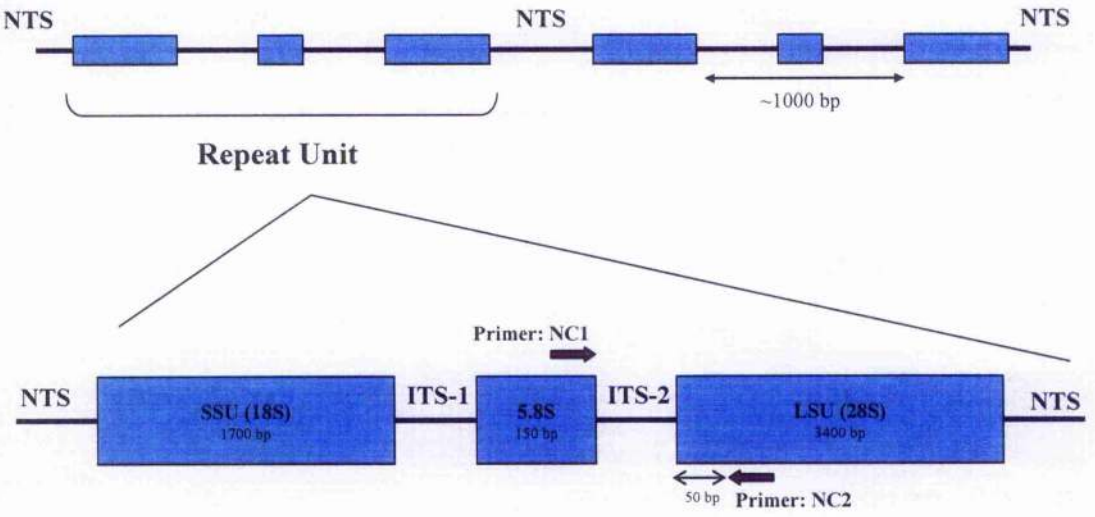


Figure 5.9b: Schematic diagram of rDNA cistron and location of ITS-2 primers



	ITS-2 sequence size	Fragment size amplified by NC1 & NC2
T. circumcincta 'standard' =	246 bp	336 bp
T. circumcincta 'goat' =	252 bp	342 bp

Figure 5.10a: Principle Coordinate Analysis using all five loci for FrMe individuals
Principle Coordinate Analysis using the microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74 with individual genotypes preserved, therefore each data point represents an individual worm. Percentage of variation explained by the first two coordinates is shown on X and Y axis of the graph. The individuals FrMe2, FrMe3, FrMe7, FrMe17, FrMe23 and FrMe26 are individuals from the FrMe population that were genotyped as *T. circumcincta* 'standard' (Type I) ITS-2 genotype. Four of these also had a *T. circumcincta* 'standard' (Type I) β-tubulin genotype (FrMe3, FrMe7, FrMe23 and FrMe26).

Figure 5.10b: Principle Coordinate Analysis using all loci excluding MTG 15 for FrMe individuals
Principle Coordinate Analysis using the microsatellites Hcms 28, MTG 67, MTG 73 and MTG 74 with individual genotype preserved, therefore each data point represents an individual worm. Percentage of variation explained by the first two coordinates is shown on X and Y axis of the graph.

Figure 5.10a

All five microsatellite loci

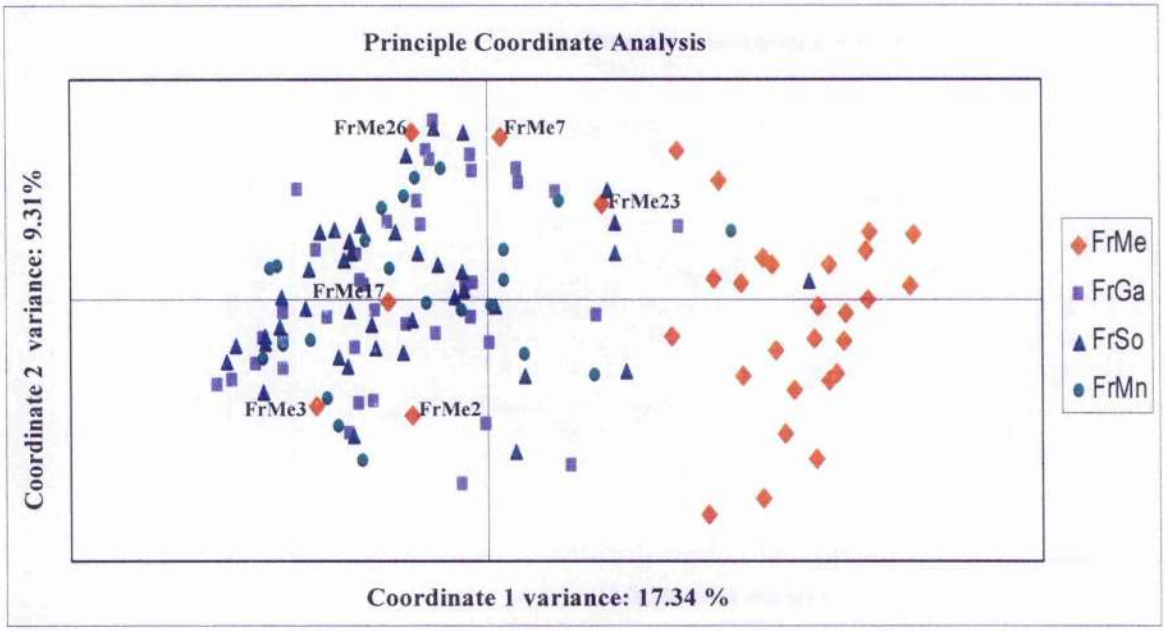


Figure 5.10b

All loci excluding MTG 15

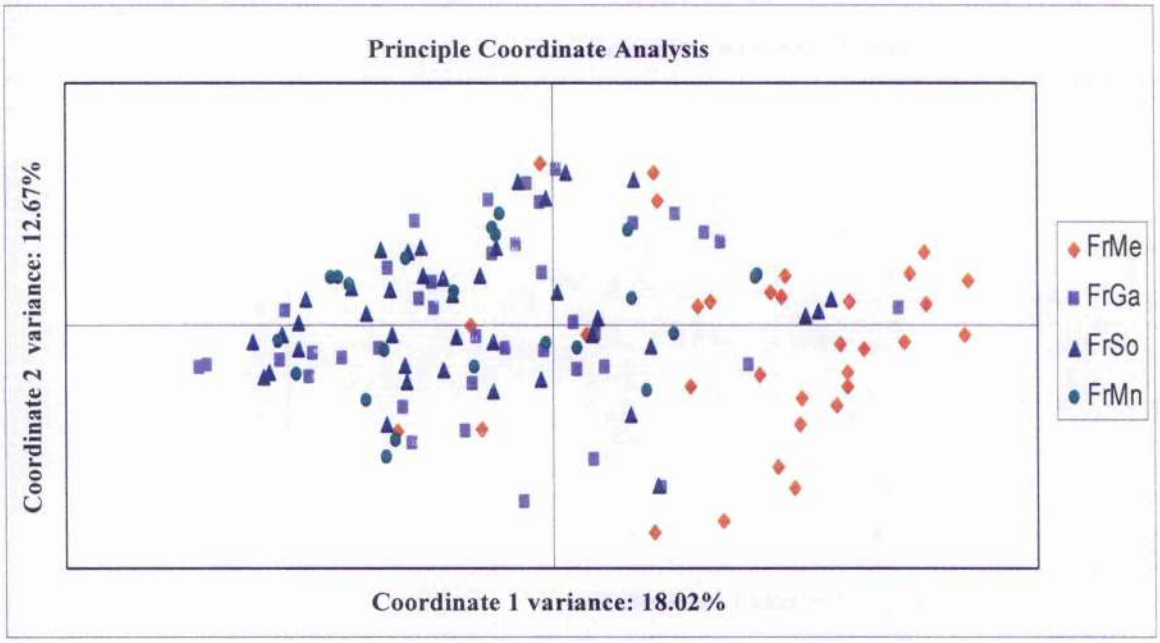
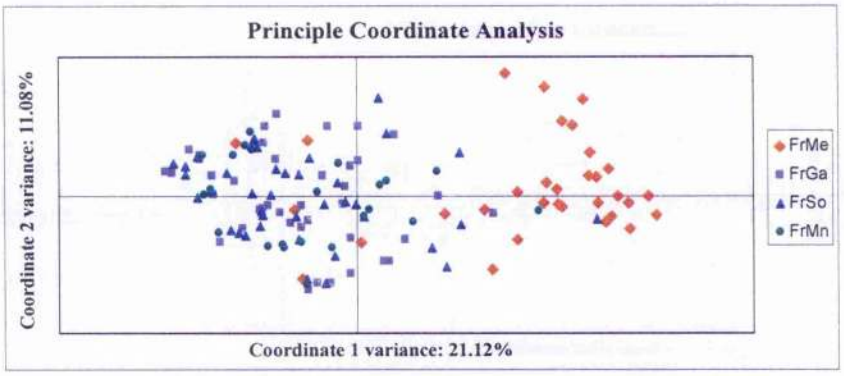


Figure 5.11: Jackknifing Principle Coordinate Analysis

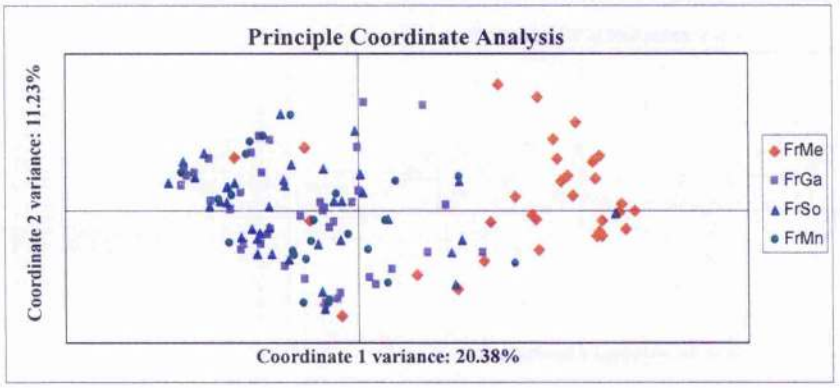
Principle Coordinate Analysis using the microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74 with individual genotype preserved, omitting one locus at a time. Percentage of variation explained by the first two coordinates is shown on the graph.

Figure 5.11: Jackknifing Principle Coordinate Analysis

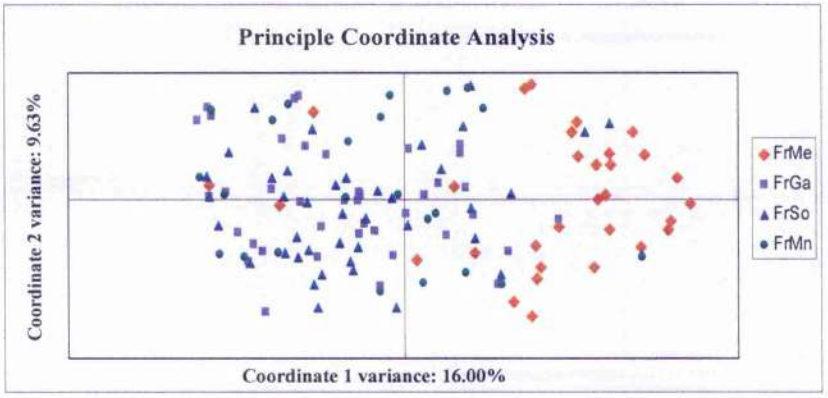
No Hcms 28



No MTG 67



No MTG 73



No MTG 74

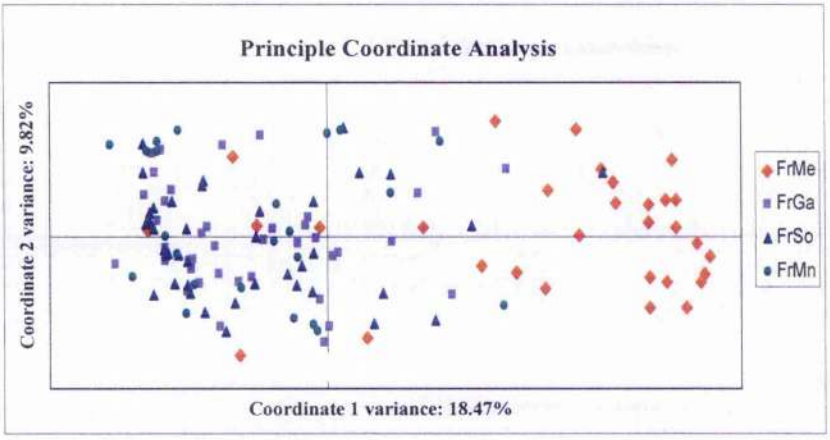


Table 5.9: FrMe genotyping results with MTG 15, MTG 67, β-tubulin and ITS-2 markers

Summary of results for 33 individuals from the FrMe population genotyped with MTG 15, MTG 67, β-tubulin and ITS-2 markers. Individuals shaded in light blue and in bold type highlight those which were typed as *T. circumcincta* 'standard' based on these markers. Results shaded in purple show inconsistent results. The 172 allele of MTG 67 which was unique to the FrMe population is shown in purple font.

FrMe Individuals	MTG 15	MTG 67	ITS-2 (bp)	β-tubulin
FrMe1	Homozygous Null	178 / 178	341	Type II
FrMe2	**253 / 253**	178 / 178	333	**Type II**
FrMe3	**243 / 243**	176 / 178	333	Type I
FrMe4	Homozygous Null	Homozygous Null	341	Type II
FrMe5	Homozygous Null	176 / 178	341	Type II
FrMe6	**258 / 258**	**172 / 172**	**341**	**Type II**
FrMe7	**241 / 263**	178 / 178	333	Type I
FrMe8	Homozygous Null	178 / 178	341	Type II
FrMe9	Homozygous Null	178 / 178	341	Type II
FrMe10	Homozygous Null	**172** / 176	341	Type II
FrMe11	Homozygous Null	**172** / 178	341	Type II
FrMe12	Homozygous Null	178 / 178	341	Type II
FrMe13	Homozygous Null	**172** / 176	341	Type II
FrMe14	Homozygous Null	**172** / 178	341	Type II
FrMe15	Homozygous Null	**172 / 172**	341	Type II
FrMe16	Homozygous Null	176 / 176	341	Type II
FrMe17	**253 / 273**	**176 / 176**	**333/335/337**	**Null**
FrMe18	Homozygous Null	176 / 176	341	Type II
FrMe19	Homozygous Null	**172** / 174	341	Type II
FrMe20	Homozygous Null	178 / 178	341	Type II
FrMe21	Homozygous Null	178 / 178	341	Type II
FrMe22	Homozygous Null	**172** / 176	341	Type II
FrMe23	**241 / 241**	**178 / 178**	**333/335**	**Type I**
FrMe24	Homozygous Null	178 / 178	341	Type II
FrMe25	Homozygous Null	176 / 176	341	Type II
FrMe26	**243 / 255**	**176 / 176**	**333/335**	**Type I / Type II**
FrMe27	Homozygous Null	**172** / 176	341	Type II
FrMe28	Homozygous Null	Homozygous Null	341	Type II
FrMe29	Homozygous Null	**172** / 178	341	Type II
FrMe30	Homozygous Null	**172 / 172**	341	Type II
FrMe31	Homozygous Null	Homozygous Null	341	Type II
FrMe32	Homozygous Null	174 / 174	341	Type II
FrMe33	Homozygous Null	**172 / 172**	341	Type II

Figure 5.12: Genotyping with the ITS-2 marker

Example Genescan chromatograms corresponding to products amplified from four single adult male *T. circumcincta* using the ITS-2 marker. Individual 1 = *T. circumcincta* 'standard' (Type I) and Individual 4 = *T. circumcincta* 'goat' (Type II). Individuals 2 and 3 showing additional polymorphism with multiple amplicons being observed. Repeatability of each ITS-2 chromatogram pattern was tested by genotyping four individuals of each pattern repeated twice. All repeats were identical.

Table 5.10: Summary of ITS-2 genotyping results

Results of nine populations genotyped with the ITS-2 marker showing the number of individuals genotyped for each of the nine PCR product patterns found.

Figure 5.12: Genotyping with the ITS-2 marker

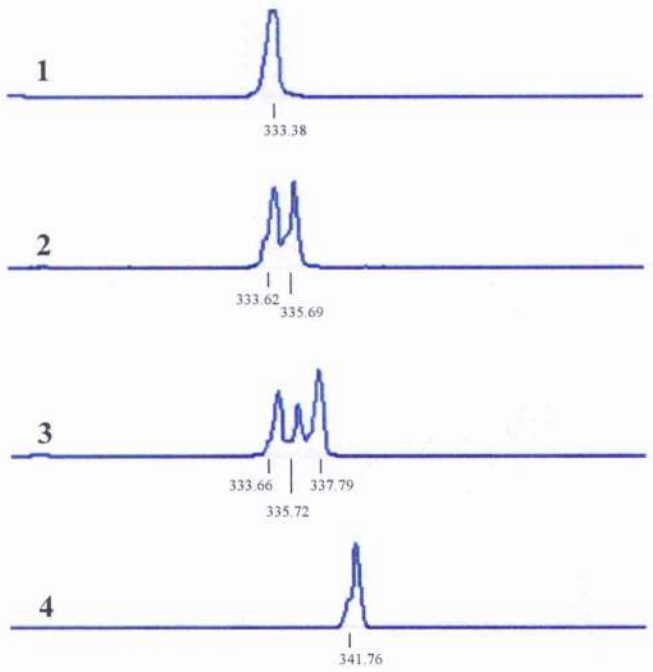


Table 5.10: Summary of ITS-2 genotyping results

Population	No. of samples	No. of individuals with each PCR products pattern for ITS-2 PCR								
		333	335	337	331/333	333/335	335/337	331/333/335	333/335/337	341
FrMe	33	3				2			1	27
FrGa	40	25	1	1		5	1	1	6	
FrSo	40	31				7			1	
FrMn	23	21				2				
ScSo507	21	16				5				
NzWS	48	44				4				
ScKiTD	31	29				2				
ScKiTT	30	23			1	6				
ScKiTC	31	27				4				

Figure 5.13: Allele frequencies for *T. circumcincta*, *T. davtiani* and *T. trifurcata* for each locus

Individual histograms for microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74, showing the allele frequencies for *T. circumcincta*, *T. davtiani* and *T. trifurcata* collected from soay sheep on the island of Hirta.

Figure 5.13: Allele frequencies for *T. circumcincta*, *T. davtiani* and *T. trifurcata* for each locus

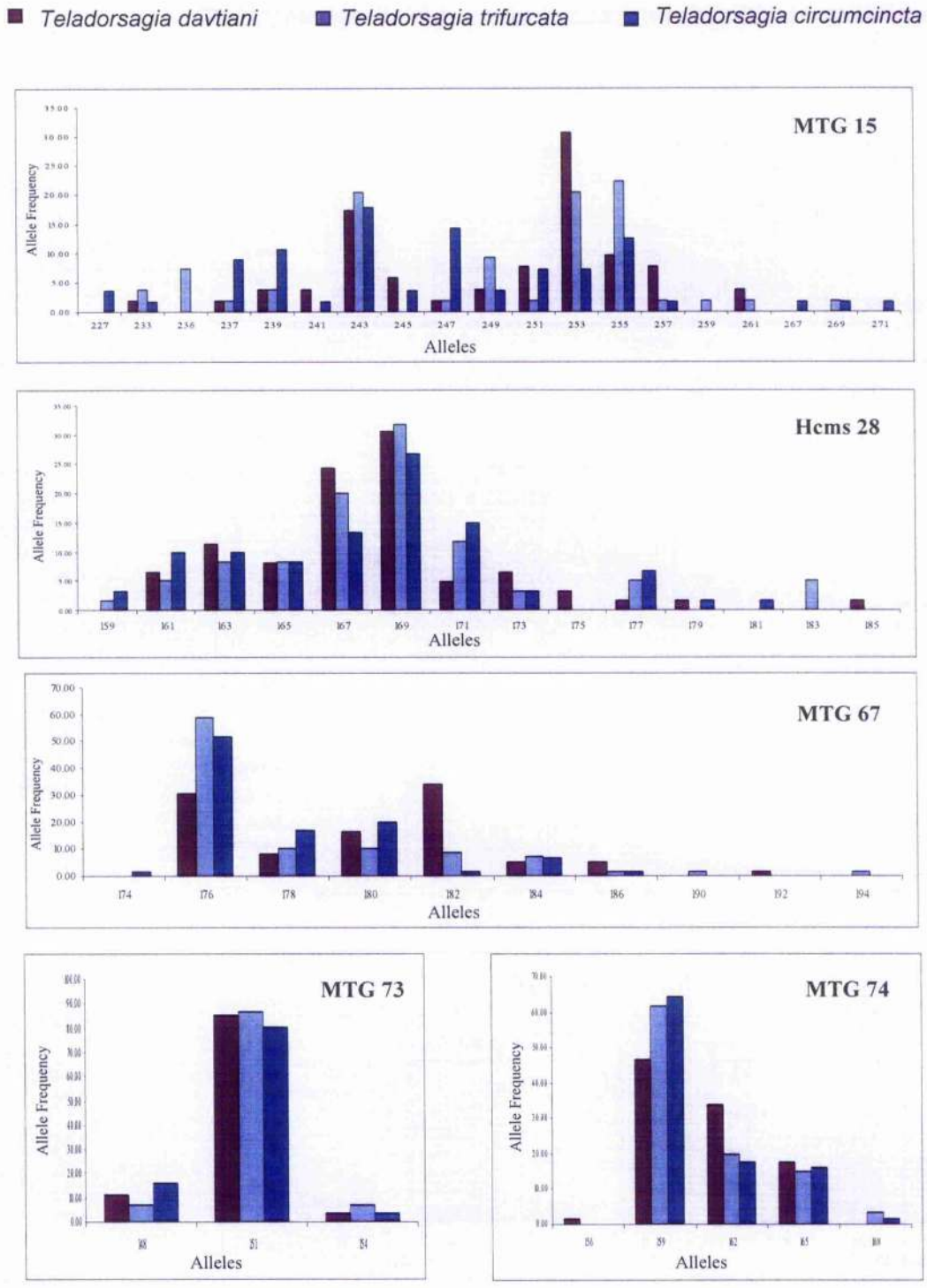


Table 5.11: Pairwise Fst values for *T. circumcincta, T. davtiani* and *T. trifurcata*

Pairwise Fst values for *T. circumcincta, T. davtiani* and *T. trifurcata* using the microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74. Calculations were performed using GDA ver 1.1 (Lewis 2001). Pairwise Fst values found between hosts on the same farm and between populations within the U.K. are also shown for reference.

Figure 5.14: Principle Coordinate Analysis of *T. circumcincta, T. davtiani* and *T. trifurcata* individuals

Principle Coordinate Analysis using the microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74 with individual genotype preserved, therefore each data point represents an individual worm. Percentage of variation explained by the first two coordinates is shown on X and Y axis of the graph.

Table 5.11: Pairwise Fst values for *T. circumcincta, T. davtiani* and *T. trifurcata*

	T. davtiani	T. trifurcata
T. trifurcata	0.0214	
T. circ	0.0250	-0.0068

GDA ver 1.1: Weir, 1990; Weir and Cockerman, 1984

Differentiation on farm between hosts: Fst -0.024 – 0.0066

Differentiation between UK populations: Fst -0.089 – 0.0340

Figure 5.14: Principle Coordinate Analysis of *T. circumcincta, T. davtiani* and *T. trifurcata* individuals

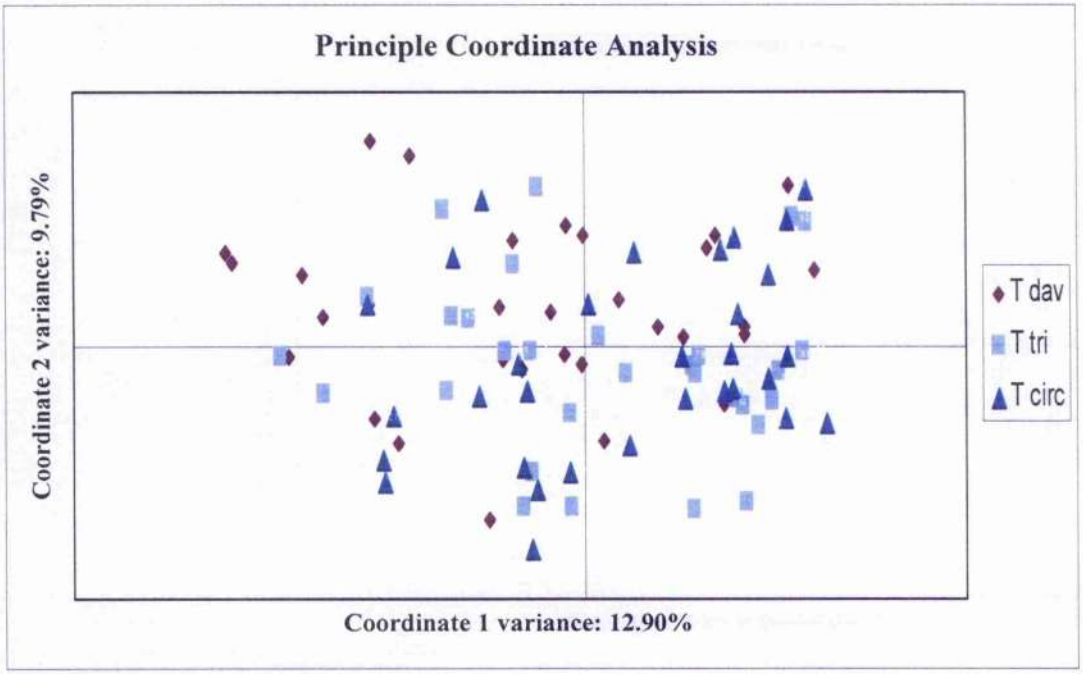


Figure 5.15a: Principle Coordinate Analysis of *T. circumcincta, T. davtiani* and *T. trifurcata* populations

Principle Coordinate Analysis using the microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74 based on Fst values calculated by GDA ver 1.1, with each population representing a data point. The populations NzWs and FrMe have been removed from the analysis to resolve relationships between the remaining populations. Percentage of variation explained by the first two coordinates is shown on X and Y axis of the graph.

Figure 5.15b: Principle Coordinate Analysis of *T. circumcincta, T. davtiani* and *T. trifurcata* populations

Principle Coordinate Analysis using the microsatellites Hcms 28, MTG 67, MTG 73 and MTG 74 based on Fst values calculated by GDA ver 1.1, with each population representing a data point. The populations NzWs and FrMe have been removed from the analysis to resolve relationships between the remaining populations. Percentage of variation explained by the first two coordinates is shown on X and Y axis of the graph.

Figure 5.15a

All five microsatellite loci

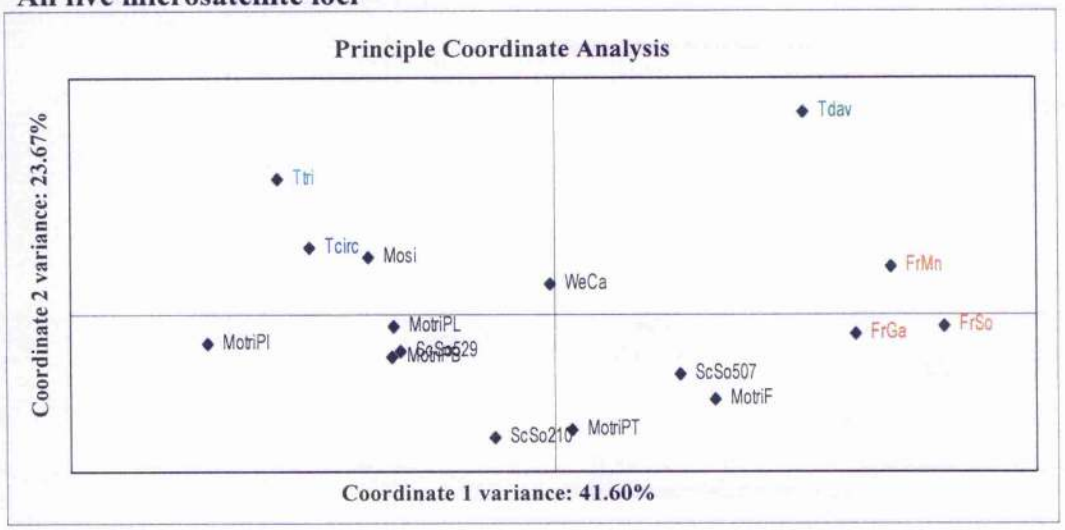


Figure 5.15b

All loci excluding MTG 15

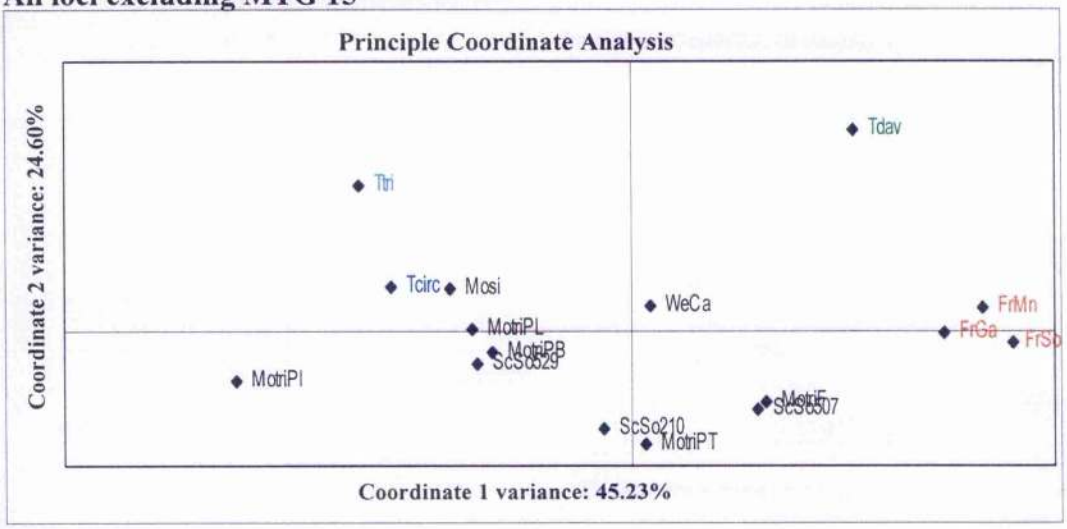
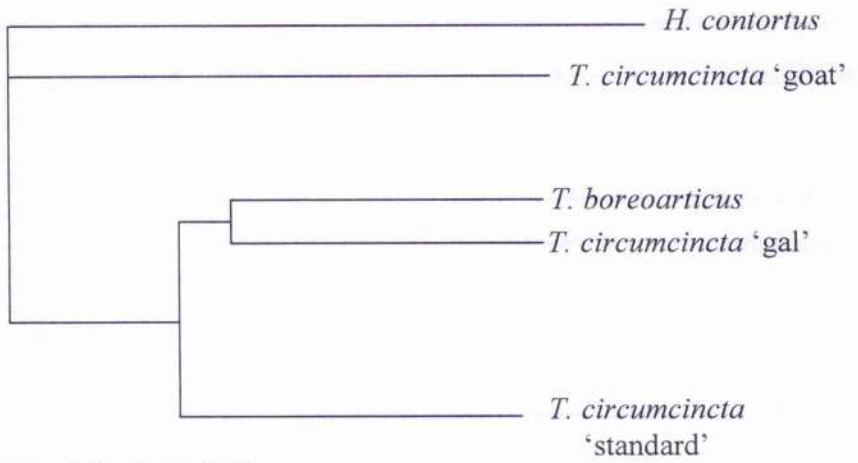


Figure 5.16a: mtDNA ND4 distance tree for *Teladorsagia* species complex
Neighbour-joining tree using mtDNA ND4 sequences. Adapted from Leignel *et al.* (2002).

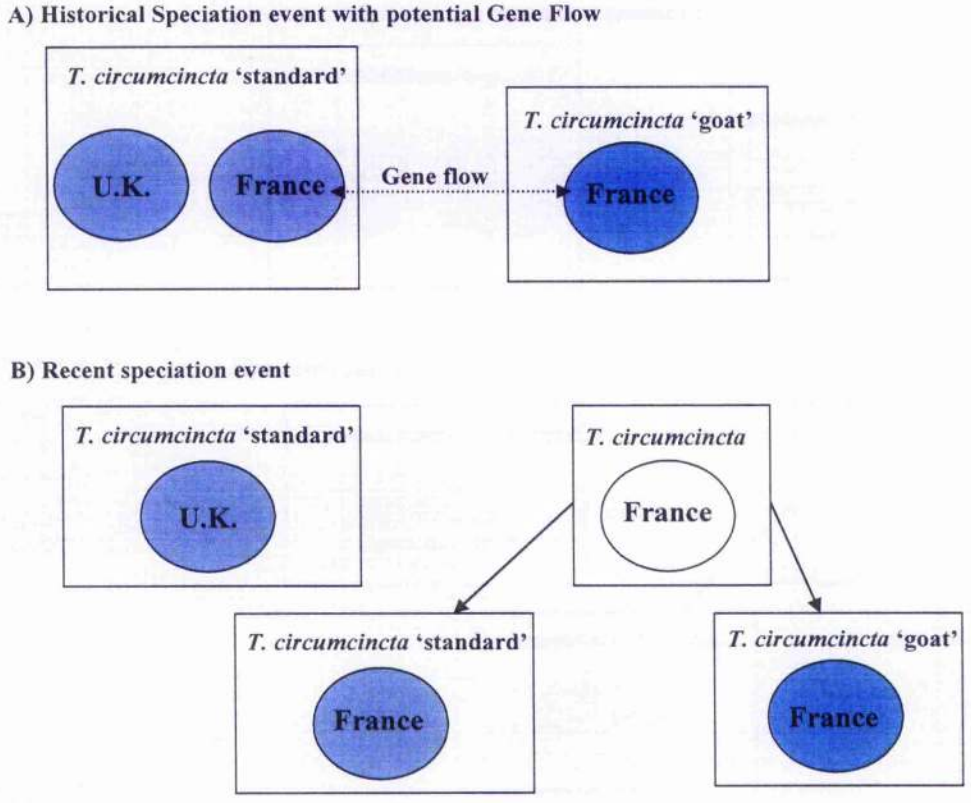
Figure 5.16b: Schematic diagram demonstrating gene flow versus recent speciation
(A) shows *T. circumcincta* 'standard' and *T. circumcincta* 'goat' as separate types with gene flow between French *T. circumcincta* 'standard' and *T. circumcincta* 'goat' due to sympatry. (B) shows a recent speciation event for *T. circumcincta* in France giving rise to *T. circumcincta* 'standard' and *T. circumcincta* 'goat'.

Figure 5.16a: mtDNA ND4 distance tree for *Teladorsagia* species complex



Adapted from Leignel *et al.* 2002

Figure 5.16b: Schematic diagram demonstrating gene flow versus recent speciation



5.4 Discussion:

5.4.1 Heterozygote deficiencies

Deviation from HWE was evident in a number of populations for a number of microsatellite markers, and there was a particularly marked deviation for the microsatellite MTG 15. There are a number of possible reasons for deviation from HWE. Biological factors include inbreeding effects, admixture or sex-linked loci. Inbreeding or admixture would be expected to be manifested as deviation from HWE across all loci, within a population. For example, Picard *et al.* (2004) suggested inbreeding as the most likely cause of HW disequilibrium in plant parasitic nematode *Globodera pallida* populations due to it being apparent at all loci over most populations with no evidence of admixture. However, this was not the case for any of the populations examined in this study. Indeed, in the case of the FrMe population, where the population has been shown to be highly structured - due to the presence of a cryptic species - heterozygote deficiencies were still apparent even after the six individuals with the Type I β-tubulin genotype were excluded from the analysis (data not shown). Hence it appears unlikely that within population sub-structuring due to admixture is an explanation for the heterozygote deficiencies observed. All of the five markers used had heterozygous male individuals and so, assuming an XX / XO sex determination karyotype, none of the loci appear to be sex-linked. Instead a much more likely explanation for the observed heterozygote deficiencies is the presence of null alleles. A number of studies have found evidence of null alleles using microsatellites markers both in population genetics studies (Ball and Chapman 2003;Dumas *et al.* 1998;Morand *et al.* 2002) and in paternity studies (McCoy and Tirard 2002;Mccracken *et al.* 1999;Pemberton *et al.* 1995). The definition of a null allele is any allele at a microsatellite locus which consistently fails to amplify detectable products (Dakin and Avise 2004). Null alleles have been reported for *H. contortus* when individual worms consistently failed to amplify using microsatellite markers (Otsen *et al.* 2000). From a literature survey conducted by Dakin and Avise (2004), 70 % of studies reported evidence of null alleles based purely on observed heterozygote deficiencies. The experiments performed on the inheritance of microsatellite markers in this study have supported the presence of null alleles for these markers in *T. circumcincta* populations.

Null alleles can be apparent in a number of situations. True null alleles are due to non-amplification where there is sequence variation at one or both of the primer annealing sites, preventing primers from annealing and amplifying the locus (Callen *et al.* 1993). However

alleles may fail to amplify for other reasons. For example, there may be size variant bias during amplification; short alleles tend to amplify more efficiently than larger alleles due to the competitive nature of PCR, known as short allele dominance (Dakin and Avise 2004;Wattier *et al.* 1998). Alternatively unreliable DNA template can result in a failure to consistently amplify certain alleles (allelic dropout) (Miller *et al.* 2002). Allelic dropout is most commonly observed when genotyping from museum samples or using non-invasive techniques for DNA isolation, resulting in template amplifying at some loci and not at others or 'false homozygotes' (Miller *et al.* 2002;Morin *et al.* 2001;Taberlet *et al.* 1996). It is unlikely that the null alleles we observe in our populations are the result of allelic dropout due to poor template quality since samples that failed to amplify with a particular marker were often repeatedly genotyped with the same result and amplified well from all other loci. In addition, there was no evidence of short allele dominance, as tested by the Micro-Checker ver 2.2.1. Furthermore, there was evidence of the inheritance of null alleles in the inheritance experiments. Consequently it seems likely that there are "true" null alleles in the *T. circumcincta* populations due to the sequence variation at primer annealing sites preventing amplification of the microsatellite. Although allele sequencing would potentially confirm the type of sequence variation causing the problem, there have been only a handful of studies where this has been done and in most cases the most common method has been re-designing primers which successfully amplify a product, followed by sequencing and comparison of alleles (Callen et al., 1993; Paetkau and Strobeck, 1995; Dakin and Avise, 2004). It is worth noting that failure of primer pairs to amplify products consistently from a high proportion of individual worms was a major reason for not using many of the microsatellite markers originally isolated (Chapter 4). In some cases this was despite designing several primer pairs for each locus which further suggests sequence polymorphism at the primer sites is a common problem for this parasite. One additional factor which has not yet been mentioned is the effect of sample size; deviation from HWE may be due to modest sample sizes in relation to effective population size, which may lower the probability of sampling all possible genotypes at a locus in a population (Weir 1996).

5.4.2 Genetic diversity in *T. circumcincta populations*

The allelic richness found overall was high with a total of 77 alleles isolated in 564 individual *T. circumcincta,* using five microsatellite loci in this study. The allelic richness for each individual *T. circumcincta* population ranged from 5.4 to 9.4, with an average of 7.8 (Table 5.5). The heterozygosities (H_e) for each population based on all five

microsatellites show similar within population diversities (0.628 - 0.728, avg. = 0.685) (Table 5.2 and 5.5). One exception is a slightly lower average number of alleles per locus found for the New Zealand population (5.4), mainly influenced by the lower number of alleles for this population at MTG 15 and Hcms 28. However the H_e for this population was similar to the other populations (0.683). It is difficult to completely compare genetic diversity index results from this microsatellite data to other studies on *T. circumcincta*, as all other studies of population genetics on this parasite species are based on single locus markers and expressed in terms of nucleotide diversity. Moreover, there are very few studies using microsatellites to study the population diversity of other nematodes species. In addition, the polymorphic content of each microsatellite should be considered. This study only used microsatellites which were shown to be polymorphic. Furthermore, the polymorphic content of each marker is potentially dependant on method of isolation. For example, the markers MTG 15 and Hcms 28, have a total of 26 and 18 alleles respectively. Whereas, the microsatellites isolated from EST sequences range from 19 alleles for MTG 67, to 6 alleles for MTG 73 and 8 alleles for MTG 74, potentially resulting from being located in either coding or non-coding transcribed regions. Even considering these points, the results for genetic diversity of each population is still high compared to other studies using microsatellites. Preliminary data from research characterising 30 microsatellites isolated from the closely related parasite, *H. contortus*, found the number of alleles per locus to range from 1 to 7 alleles (Otsen *et al.* 2000). However, Otsen *et al.* (2000) only investigated a total of 8 individual worms for each of the four populations used to characterise the 30 microsatellites. Allelic richness in the potato cyst nematode, *Globodera pallida*, in Peru was found to be higher (63 alleles in 40 individuals) than that found in the same nematode species in Europe (25 alleles in 120 individuals) when genotyped with the same seven microsatellites (Picard *et al.* 2004;Thiery and Mugniergy 2000). Picard *et al.* (2004) suggested the European isolates of *G. pallida* were less diverse due to maintenance of these isolates in the laboratory. Expected heterozygosity (H_e) is a more useful measure of genetic diversity of population, as it takes into account allele frequencies. For example, a population may have 12 alleles at a locus but only one was of high frequency with the other 11 very rare alleles, H_e would be low. The overall gene diversity (H_e) across *G. pallida* populations are slightly lower (0.421 – 0.729) compared to those found for *T. circumcincta*. A recent study investigating the populations of the sugar beet cyst nematode, *Heterodera schachtii*, using five polymorphic microsatellites (number of alleles per locus ranging from 4 - 26) found heterozygosities (H_e) ranging from 0.519 to 0.599 (Plantard and Porte 2004), again

slightly lower than those found here for *T. circumcincta.* An example of low levels of genetic diversity for nematodes using microsatellites was difficult to find from the literature. Therefore, an example to demonstrate low diversity can be found from work carried out on the population structure of *Biomphalaria pfeifferi,* a freshwater snail, found in Madagascar. Charbonnel *et al.*(2002) investigated 30 *B. pfeifferi* populations, genotyping from 11 to 24 individuals per site, and found variability of the seven microsatellite loci to range from 4 to 18 alleles per locus. The allelic richness was low, with the average number of alleles per population ranging from 1 to 3.12 alleles. More striking was the lack of genetic diversity across all populations, with H_e ranging from 0 to 0.39 (Charbonnel *et al.* 2002). The author suggests the lack of genetic diversity seen in *B. pfeifferi* populations was a result of high selfing rates found in this species, rather than a bias result from the microsatellite markers which were used, as previous studies in the same populations using AFLP markers reflect similar low diversities.

The high levels of diversity found for the microsatellite markers in *T. circumcincta* in this study are consistent with previous results using mtDNA markers. When comparing genetic diversities using mtDNA ND4 sequences, Blouin *et al.* (1995;1999) found nucleotide diversities trichostrongylid nematodes populations in the United States, including populations of *T. circumcincta,* to be high (0.019 – 0.028) when compared to the parasitic nematode of soil dwelling insects, *H. marelatus* (0.002). More recently, a population genetic study focusing on the *T. circumcincta* worm populations in Soay sheep from the remote Scottish island of Hirta, found similar results using mtDNA ND4 sequences. Despite this population being historically isolated for over 70 years, the within population diversity was extremely high, with 77 haplotypes identified from 85 individuals (Braisher *et al.* 2004). Similar results were found for *T. circumcincta* populations investigated in France and Morocco; the within population diversity ranging from 0.017 to 0.029 (Leignel and Humbert 2001). In contrast, the nucleotide diversities found for the parasitic nematode of wood mouse, *Longistriata caudabullata,* based on the mtDNA ND4 gene were almost half that found in trichostrongylids by Blouin *et al.* (1995) (0.012). Therefore, it seems that *T. circumcincta* has been shown to have high levels of within population diversity using both microsatellites markers used in this study and using mtDNA ND4 sequences.

5.4.2.1 Cause of high levels of genetic diversity: effective population size and mutation rate

The extent of genetic diversity of populations depends on a number of different factors. Under a model of drift-mutation equilibrium, the within population sequence diversity, π, is a simple function of sequence mutation rate, μ, and effective population size, N_e (for mitochondrial gene $\pi = N_e\mu$, while for nuclear genes $\pi = 4\ N_e\mu$) (Anderson *et al.* 1998). The very high levels of diversity within populations is likely to be due to either a very large effective population size of these parasites and / or a very high mutation rate (Beech *et al.* 1994). However it is difficult to estimate effective population size (N_e) for parasitic nematode populations, especially when models for estimating N_e do not consider the complications of these organisms' life cycles (Nadler 1995). Post-mortem worm counts, for example, may not accurately estimate adult worm populations since the adult worm population will be dispersed unevenly between each host's infra-population. In addition, it is unclear to what extent the larval population on the pasture contributes to the effective population size; it is very large in total but it is unclear what proportion contributes to the next generation. Indeed this is very likely to vary considerably from year to year and farm to farm. Blouin *et al.* (1995) suggested that the effective population sizes on each farm were not large enough to cause the extreme population diversities seen, based on mtDNA ND4 sequences. Consequently, it was suggested that gene flow facilitated by human transport of the host makes the separate trichostrongylid populations in North America into one large meta-population. This one huge population would then have a large enough N_e to sustain the high levels of diversity seen on each farm. This was supported by the lack of genetic sub-structuring between different populations. However, this hypothesis did not explain why the deer parasite in North America, *Mazamastrongylus odocoilei*, had some of the highest nucleotide diversities but had high levels of genetic sub-structuring between populations suggesting little gene flow (Blouin *et al.* 1995). Although it is not possible to accurately calculate N_e, our knowledge of the biology of *T. circumcincta* and other trichostrongylid parasites suggests N_e is likely to be very large. Individual hosts can contain 1000s to 10,000s of adult parasites each producing 1000s of eggs each day (Anderson 1988;Eysker and Kooyman 1993;Urquhart *et al.* 1996). Hence a single field with a flock of sheep will be contaminating the pasture with numbers of eggs in the orders of magnitude between 10^7 to 10^9. Therefore, a large N_e seems likely to be a major contributory cause of the high levels of genetic diversity in *T. circumcincta*.

Phylogenetic trees of mtDNA genes comparing numerous taxa, have been shown to have increased branch lengths for nematodes species, consistent with an accelerated rate of evolution relative to other phyla (Hoeh *et al.* 1996;Okimoto *et al.* 1992). Accelerated rates of mtDNA gene evolution suggest high mutation rates, potentially caused by increased metabolic rates or short generation times (Hoeh *et al.* 1996). *C. elegans* has been shown to have increased mutation rates for both nuclear and mitochondrial DNA over one generation (Denver *et al.* 2000;Denver *et al.* 2004). Denver *et al.*(2000) estimated mtDNA mutations rates of 8.9 per nucleotide site per million years for *C. elegans,* two orders of magnitude higher than human phylogenetic mutation rate estimates (0.02 – 0.26 per site per million years). Recently the parasitic nematode, *Heligmosomoides polygyrus*, was shown to have a evolutionary mtDNA mutation rate 1.5 - fold higher than its specific host the wood mouse (*Apodemus sylvaticus*) using orthologous mtDNA *cyt b* gene (3.5 - 3.7 % kimura two parameter (K_2P) distance per million years vs 2.6 - 2.85 % K_2P distance per million years) (Nieberding *et al.* 2004). These data point towards increased mutation rates being a potential contributory factor to high levels of genetic diversity in nematodes.

This leads to the question as to what is the most important contributory factor: effective population size or mutation rate? It is important to note that not all parasitic nematode populations have levels of genetic diversity as high as that described for *T. circumcincta.* For example, *A. suum* and the trichostrongylid parasite *D. viviparous* have comparatively much lower levels of mtDNA nucleotide diversity, 0.016 and 0.006, respectively (Anderson 2001;Hoglund *et al.* 2004), compared to 0.024 estimated for *T. circumcincta* (Blouin *et al.* 1995). Interestingly these parasites would be expected to have effective population sizes that are much smaller than for *T. circumcincta* based on the number of adult worms in each host. Similarly, it is interesting to note that the nucleotide diversity found in *Teladorsagia boreoarcticus* was five times lower than the diversity seen in *T. circumcincta* populations in North America (Hoberg *et al.* 1999). The author argued that *T. boreoarcticus* has a much lower long-term effective population size, due to small dispersed host populations, different winter and summer foraging zones and short summer periods for larval development (Hoberg *et al.* 1999). Hence, it could be argued from these studies that the level of genetic diversity broadly reflects the population size of the particular parasite suggesting N_e is the critical factor in determining the level of genetic diversity.

5.4.2.2 Genetic diversity of laboratory isolates

The laboratory populations genotyped in this study (MOSI, MotriF, MotriPT, MotriPB, MotriPI, MotriPL, and NzWs) are as equally diverse as the field populations (Table 5.5). These populations cannot rely on larval stages on the pasture to maintain diversity, but instead go through potential bottlenecks during passage through sheep. For example, for the lab strain Tci1 (MOSI) from Moredun has been passaged by experimental infection of sheep for > 20 years and the routine dose used is 5,000 - 10,000 larvae. Given an expected establishment rate of approximately 30 - 50 % (Leathwick *et al.* 1999) this would mean an effective adult population size ranging from 1,500 to 5,000 adults. However, this is a very rough estimate, as a number of factors may effect establishment of adults including host age, breed, immune status, and resistance status, in addition to potential unknown selection factors. Since this strain has a similar level of genetic diversity to field populations, these numbers appear to have been sufficient to maintain genetic diversity of the original population. These findings based on the analysis of microsatellite markers from *T. circumcincta* are similar to those previously reported for other trichostrongylid nematodes using different techniques. A laboratory strain of *Cooperia oncophora* which had been passaged for > 40 years was found to be equally polymorphic when compared to a field isolate based on PCR-RFLP polymorphism of the mtDNA gene, cytochrome oxidase c subunit 1 (van der Veer *et al.* 2003). Similarly, one *T. colubriformis* population maintained by experimental passage for > 40 years was as diverse as the field population isolate using RFLP analysis (Grant and Whitington 1994). Although both of these studies are only comparing one laboratory isolate to one field isolate, the results suggest no loss of genetic diversity in the lab strains. A more recent study was conducted by Otsen *et al.* (2001) investigating the genetic diversity within and between populations of *H. contortus*, during consecutive stages of increased benzimidazole (BZ) and levamisole (LEV) resistance. Six consecutive *H. contortus* populations starting with a susceptible population were selected for, firstly BZ resistance by using drug concentration whereby only 20 % of the original susceptible eggs hatch and independently for LEV resistance using a drug concentration at which 50 % of susceptible eggs hatch. Using amplified fragment length polymorphism (AFLP), the BZ selected population showed no loss of within population diversity, suggesting lab propagation did not effect within population diversity. However, a loss of within population diversity was found between the consecutive populations of LEV selection, yet was generally still high. This loss of genetic diversity could have been due to lab propagation, LEV selection or the originating susceptible strain (which differed in origin

to the BZ susceptible population). However, the microsatellite results presented here support previous studies and suggest that laboratory strains of trichostrongylid nematodes maintain the high level of diversity, similar to field populations, for many years using standard protocols of experimental passage through the host.

5.4.3 Genetic differentiation of *T. circumcincta* populations

5.4.3.1 Sub-structuring of populations between different host species

There is evidence to suggest genetic variation in the resistance of different host species to specific parasite infections. For example, studies using fibre goats in comparison to sheep, suggest that goats may lack the ability to regulate establishment and rejection of established populations of gastrointestinal parasites (Huntley *et al.* 1995). This difference in the susceptibility to nematodes between host species may in turn affect the genetic variation of parasitic genotypes. Host species may differ in a number of ways which will in turn affect parasite genotypes, however there has been relatively little research investigating the genetic structuring of parasite populations between different host species. One recent study by Brant and Orti (2003) found no genetic differentiation between the parasitic nematode, *Longistriata caudabullata*, populations isolated from an area, in central North America, between two host species (*Blarina brevicauda* and *B. hylophaga*). The authors sequenced the mtDNA ND4 gene from 8 - 12 *L. caudabullata* individuals of each host species and found no evidence of genetic differentiation between parasite populations of the two host species, suggesting no co-speciation event took place. However, evidence of host specialization of parasite nematodes has been demonstrated to the extent of warranting specific species classification. For example, the morphologically identical parasitic nematodes, *Ascaris lumbricoides* and *A. suum,* have been shown to specifically infect humans and pigs, respectively, using seven nuclear genes, including the ITS region of rDNA (Anderson 2001;Anderson and Jaenike 1997). A total of 140 worms were collected from both humans and pigs from two geographical locations in Guatemala. The multilocus nuclear data clustered the parasites into two distinct groups, with nematodes collected from pigs in one group and nematodes collected from humans in the second group.

Evidence of genetic differentiation between host species was investigated here. The *T. circumcincta* populations in this study include populations collected from goats (ScSo210, ScSo529, ScSo507) and sheep (WeCa, ScKiTC, ScKiTT, and ScKiTD). Pairwise Fst values were all low, indicating little genetic differentiation among any of these populations

(Table 5.6a). All the populations clustered together equally in all PCA plots (Figure 5.15a and 5.15b). This provides evidence that generally *T. circumcincta* populations do not show genetic variation partitioned between these two host species in the U.K. However, the FrMe may represent a marked example of sub-structuring between host species consistent with previous suggestions of a goat specific cryptic species of *T. circumcincta* in France (Gasnier *et al.* 1997;Leignel *et al.* 2002). This is discussed in further detail later in this discussion.

5.4.3.2 Sub-structuring between field and laboratory isolates

There was no evidence of sub-structuring between U.K. *T. circumcincta* field (WeCa, ScSo210, ScSo529, ScSo507, ScKiTC, ScKiTT and ScKiTD) and laboratory strains (MOSl, MotriF, MotriPT, MotriPT, MotriPI, and MotriPB). Pairwise Fst values were all low, indicating little genetic differentiation among any of these populations (Table5.6a). All the U.K. populations clustered together and were equally separated from each other in all PCA plots (Figure 5.15a and 5.15b). There was no evidence of clustering of either the lab isolates or the field isolates. Furthermore, AMOVA analysis indicated only 0.90 % of the overall genetic variation was partitioned between populations within the U.K. (Table 5.7a and 5.7b). In contrast, genetic differentiation of laboratory strains has been shown for other trichostrongylid nematodes, including *T. colubriformis* (Grant and Whitington 1994) and *C. oncophora* (van der Veer *et al.* 2003) for which laboratory strains had been maintained for > 40 years in both studies. The within isolate genetic diversity of these populations was maintained and therefore not the cause of this differentiation. However, only one lab isolate was compared to one field isolate in both studies. Furthermore, geographical location differed between the field and lab isolates, in both studies and consequently it is difficult to know if the differences seen were due to a difference in laboratory versus field populations per se.

5.4.3.3 Geographical sub-structuring.

i) Little differentiation between *T. circumcincta* populations isolated from different hosts on the same farm

One preliminary question was: are infra-populations genetically distinct or can a sample population from one host represent the farm as a whole? Three infra-populations were sampled (21 - 30 individual male worms each – populations ScSo529, ScSo210 and ScSo507), from three goats of the same age, raised under identical management conditions, were genotyped using the five microsatellite markers. The distribution of allele frequencies

for each of the three loci are broadly similar for each of the three populations (Figure 5.5) and low Fst values (ranging from 0.0024 - 0.0066) suggest very little genetic differentiation between host species (Table 5.6a and 5.6b, highlighted grey). AMOVA of these three populations showed no genetic partitioning between host infra-populations (data not shown). This agrees with the results for *T. circumcincta* populations conducted using five polymorphic allozyme loci (Gasnier *et al.* 1996;Gasnier and Cabaret 1998). Gasnier *et al.* (1996) collected a total of 998 individual *T. circumcincta* from four and three infected lambs on the same pasture in 1990 and 1992, respectively. The allelic frequencies between infra-population isolated from hosts on the same pasture in 1990 were very similar (Fst = 0.007), as was the result for the three hosts in 1992 (Fst = 0.12). This study also found no genetic differences between the years 1990 and 1992 (Fst = 0.000) nor between autumn and spring infections (Fst = 0.01). The later study found a similar lack of genetic differentiation between two infra-populations taken from goats on the same farm which found similarly low Fst values of 0.008 (Gasnier and Cabaret 1998). Similar results were found by Braisher *et al.* (2004) who investigated between host variation of *T. circumcincta* at three locations in the U.K. Braisher *et al.* (2004) collected an average of seven worms from 4 - 6 hosts, from two isolated islands, Lundy and Hirta, and a mainland population from Norfolk. No between host sub-structure was found in any of the three locations using mtDNA ND4 sequences. The results obtained in this study and from previous studies suggest that sampling from one host is representative of the parasite population of a group of co-grazing animals on a farm.

ii) Little differentiation between British and French *T. circumcincta* populations.

There are three main studies investigating the geographical sub-structure of *T. circumcincta* populations, all of which used mtDNA ND4 sequences to measure the degree and partitioning of genetic differentiation. In North America there was no population structure found in the trichostrongylid nematodes of domesticated hosts, whereas the deer parasite, *M. odocoilei* did have population structure based on geography (Figure 5.2) (Blouin *et al.* 1995). There are two alternative explanations for these results: a) that parasitic nematodes of North American livestock where derived from the same parasite population several hundreds of years ago and genetic drift has occurred too slowly to allow these populations to have differentiated between geographical locations over this time or b) high levels of contemporary gene flow due to livestock movement allows geographically separated parasite populations to behave as one large population, whereas wild deer parasite

populations are much less mobile than domestic animals. Blouin *et al.* (1995) concluded the latter hypothesis explained the lack of subdivision in North America.

A more recent study was conducted by Braisher *et al.* (2004) investigating the genetic diversity of *T. circumcincta* populations within the U.K. from three sheep populations, two populations collected from the isolated islands of Lundy and Hirta, and one population isolated from a mainland population. Similar to the results found by Blouin *et al.* (1995), very little population sub-structuring was seen between the three populations (Braisher *et al.* 2004). However, unlike Blouin's work, the population collected from the island of Hirta has been isolated since 1932; therefore recent gene flow between populations could not explain the lack of genetic differentiation. Braisher *et al.* (2004) estimated the effective population size of *T. circumcincta* on Hirta to be in the region of 260,000 million adult females; based on 1000 females per host, 1 - 2 generations per year and 260 soay sheep. Based on these calculations, the author estimates that it would take 180,000 years to get fixation or loss of haplotypes by genetic drift. Consequently they argued that it was not surprising that genetic differentiation was not apparent between U.K. mainland parasites and those on Hirta even though they had been separated for over 70 years. Hence based on these studies one might not expect to find geographical sub-structuring of *T. circumcincta* populations when using neutral genetic markers due to their enormous effective population size and consequently very low rate of genetic drift. However this expectation relies upon on a greatly oversimplified view of parasite population dynamics and there is one study which provides experimental evidence that the situation is more complex. Leignel and Humbert (2001) found significant genetic differentiation among populations of *T. circumcincta* in central and southern France and Morocco, isolated for approximately 50 years. There are a number of possibilities to explain the conflicting results presented by Braisher *et al.*(2004) and Leignel and Humbert (2001). Firstly, there could be differences in the effective population size of *T. circumcincta* populations on the island of Hirta compared to the population represented on each farm in France. However, each author estimated the respective effective population sizes to be similar. Therefore, it appears unlikely that differences in population effective size are a major factor. The conflicting data may be explained by either population bottlenecks or differential selection pressures or both. Population bottlenecks caused by anthelmintic treatment in the French populations may have caused the effective population size to become small enough to lose alleles or change allele frequencies. The Soay sheep populations have never been treated with anthelmintics as a whole, whereas the farms in France have been. However, population bottlenecks may also occur whenever there is a

severe soay sheep population crash. Furthermore, population bottlenecks would tend to decrease population diversity (Frankham *et al.* 2002), which is not apparent in either population. However, anthelmintic treatment may have a selective effect on the populations in France, promoting genetic differentiation. This selection pressure may have been increased by the fact that these populations are effectively closed, with no new migrants. Environmental conditions may also have a significant influence on parasite populations since if the infra-population size (i.e. the worms in the host) goes through a severe bottleneck, the larval population on the pasture can act as a reservoir of genotypes buffering the bottleneck effect. However, in extreme climate conditions, such as extreme cold or heat, or drought, larval stage reservoir may be lost. On farms this situation may be artificially created when host animals are treated with anthelmintics and then placed on 'clean' pasture (void of animals > 2 yrs and therefore void of the larval reservoir). Further selection pressures also need to be considered including husbandry and cultural practices, host immune status, as well biochemical and physical conditions existing in host species. For example, in the human hookworm species, *N. americanus*, cultural practices can affect the movement of the parasites: one *N. americanus* population in China was shown to be genetically distinct compared to three others, yet this was not a function of geographical distance (Hawdon *et al.* 2001). Instead, this is suggested to have been result of a population bottleneck caused by transmission occurring only within a single family whereas in the other areas, wider transmission of the parasite was facilitated by communal latrines, which promotes genetic mixing of populations. Hence different population dynamics in different regions can produce different population structures in this parasite.

The results from the work presented in this thesis broadly supports the results found by Blouin *et al.* (1995) and Braisher *et al.* (2004) and are consistent with a model of large population size and little genetic drift. The levels of genetic differentiation between populations within the U.K. and between the U.K. and France (not including FrMe) are very low, based on the pairwise Fst values found (Table 5.6a and 5.6b, highlighted with purple shading). The PCA plots graphically demonstrate that these populations cluster together and are equally separated. In addition, AMOVA analysis shows little partitioning of genetic variation among populations in the U.K. Furthermore there is direct support for Braisher's work, as the *T. circumcincta* populations collected from Soay sheep on Hirta in this study (ScKiTC, ScKiTD, and ScKiTT) are not genetically distinct from any of the other U.K. populations. However it is worth noting that the microsatellite work presented here provides some evidence that the French populations (excluding FrMe) seem to be more closely

related to each other (Fst = -0.0004) than they are to the U.K. populations (Fst = -0.021) and than the U.K. populations are to each other, including the Hirta population (Fst = 0.013). This is a small effect but is worth consideration. All the French farms are from central France and all have been closed for approximately 20 - 25 years and the livestock are all thought to have originated from same stock herd. It could be suggested that the above result is due to the fact that these French populations have a more recent origin or common ancestor than the U.K. populations, with minimal genetic drift having occurred in the intervening 20+ years. Hence the U.K. populations are more temporally removed from their common ancestor, allowing a greater time period over which genetic drift could occur, potentially creating relatively more genetic structure. However, as the Fst values are so low, this has to be a tentative hypothesis. In order to investigate this further, more *T. circumcincta* populations from different regions of France and the U.K. need to be tested. For example, populations studied by Leignel and Humbert (2001) could be compared using the microsatellites isolated in this study. In addition, more closed herds in which the stock was founded from single herd at a known time could be examined and compared to herds that routinely import livestock. Such comparisons from the two countries would enable genetic differences to be confirmed and the relative importance of geography versus husbandry factors to be assessed.

In summary, the general results from population genetic analysis of the populations in U.K. and France (not including FrMe) show a lack of genetic differentiation, consistent with large effective population size preventing genetic drift and genetic differentiation. On a worldwide level, there were two populations, NzWs (from New Zealand) and FrMe (from France), which were found to be markedly genetic differentiated from the other populations genotyped. These will each be discussed in turn in the following sections.

iii) Marked differentiation of New Zealand population

In this study, the New Zealand population showed moderate genetic differentiation from the other populations based on pairwise Fst values (Table 5.6a and 5.6b) being clearly separated from all other populations in PCA plots (Figure 5.6a and 5.6b). Additional AMOVA analysis indicated that the NzWs population, when removed from the analysis, had contributed to the genetic variation partitioned among countries. The history of animal imports to Australia and New Zealand suggest many diseases were prevented from entering these countries firstly by unavoidable quarantines imposed by long sea journeys and secondly by the increasing restrictions to imports imposed by quarantine (Pierce 1975). New

Zealand has a strict policy of quarantine for livestock entering the country, and in addition to the advent of effective anthelmintics, it is unlikely that nematodes have been imported with their host species for at least 80 years (Dr Stewart Bisset, pers. comm.) Therefore *T. circumcincta* populations in New Zealand have been isolated for a minimum of 80 years. Early imports to NZ were mainly from Australia, with imports to Australia thought to primarily come from the U.K. but have also been documented from India and South Africa (Pierce 1975). There are a number of reasons for the moderate levels of genetic differentiation of the New Zealand population (NzWs). Firstly, this could be due to the period for which this population has been isolated (>80 years), or potentially longer considering the historical accounts of sheep in New Zealand. However, the populations collected from Hirta (ScKiTC, ScKiTT, and ScKiTD) have been isolated for approximately 70 years, but do not show any genetic differentiation from the rest of the U.K. Perhaps this could be explained by different establishing *T. circumcincta* populations, with the origin of *T. circumcincta* in Hirta being similar to those in the rest of the U.K., and the origins of the NZ populations from elsewhere. The NzWs population does not have any unique alleles for any of the loci in this study, which might have been evidence for different origins. The second possible explanation for the divergence of the NzWs population could be the result of this being a laboratory isolate from the 1950's. The long term passage of this isolate (> 60 years) in a relatively constant environment may have provided the constant selection pressure required for genetic divergence. Little genetic differentiation was evident between U.K. lab and field populations, as discussed in section 5.4.3.2, suggesting that this not the reason for NzWs differentiation. In comparison to other populations, the NzWs population did have a slightly lower average number of alleles per locus (5.4) (Table 5.5), mainly influenced by the lower number of alleles for this population at MTG 15 and Hcms 28. However, the average expected heterozygosity for this population is similar to those found in all the other populations (Table 5.5), suggesting this population is no less diverse and not inbred. Although laboratory passage techniques tend to follow the same standards worldwide, as seen by the similar infecting doses for both the U.K. and New Zealand lab strains (Table 2.2), it is difficult to be sure of the precise history of any particular laboratory strain and any unrecorded bottlenecks and selections that might have occurred. A third explanation is that the New Zealand population may represent a cryptic species. Although genotyping with the β-tubulin and rRNA ITS-2 loci did not provide any supporting evidence for this it remains a possibility that requires further investigation.

iv) Extreme differentiation of FrMe population

The French population, FrMe, was found to be markedly genetically distinct from the other *T. circumcincta* populations in the study. Pairwise Fst values between FrMe and the U.K. population and the three other French populations were very high (Table 5.6a and 5.6b) and when used to calculate the PCA plots demonstrated clear separation of this population from all the others (Figures 5.6a - 5.7). AMOVA analysis supported genetic distinction and demonstrated that the FrMe population is a main contributor to the genetic variation partitioned between populations (Table 5.7a and 5.7b). The extreme genetic differentiation of the FrMe population is discussed in the next section.

5.4.4 Microsatellite genotyping supports hypothesis that *T. circumcincta* is a species complex

The results from the microsatellite genotyping of the FrMe population clearly showed that this population was genetically distinct from the other *T. circumcincta* populations. This was based on pairwise Fst values, PCA plots and AMOVA analysis (section 5.4.3 iv). When the microsatellite genotyping results were examined in further detail, the markers MTG 15 and MTG 67 were found to further support the differentiation of this population. From the 33 individuals genotyped by MTG 15, only 7 worms produced amplicons, with the remaining 27 individuals consistently failing to be amplified. Additional primers sets were tested for the MTG 15 marker, which consistently only amplified from the same 7 individuals (Figure 5.8). It was considered that the 27 individuals contained polymorphisms within the flanking regions of this locus, preventing primer annealing. Hence this constitutes a null allele present at relatively high frequency in the FrMe population which is not the case in the other populations supporting the genetic distinction of this isolate. The FrMe population was also found to contain a unique MTG 67 allele (172) at a frequency of 0.26. The *T. circumcincta* 'goat' cryptic species, as defined by Leignel *et al.* (2002), had been previously reported as being present on the farm from which the FrMe population sample genotyped in this thesis had been recovered (Leignel *et al.* 2002). In order to see if any *T. circumcincta* 'goat' individuals were present in this population of worms, all 33 individuals were genotyped with the isotype 1 β-tubulin marker and the rDNA ITS-2 marker described by Leignel *et al.* (2002). The result of the isotype 1 β-tubulin and the rDNA ITS-2 genotyping showed the *T. circumcincta* 'goat' cryptic species made up the majority of worms in the FrMe population (26 out of 33 worms). The PCA analysis of the microsatellite

genotyping independently identified the same individuals from this population as being genetically distinct from 'standard' *T. circumcincta* (Figure 5.9a - 5.10).

The previous results of Leignel *et al.* (2002) combined with the microsatellite data presented in this thesis strongly suggests that *T. circumcincta* is indeed a species complex with at least two separate species existing in sympatry in goats in France. The results found for the FrMe populations are particularly interesting as they show this population of *T. circumcincta* 'goat', which co-exists with *T. circumcincta* 'standard' in goats in France, to be highly genetically distinct. The results therefore suggest that *T. circumcincta* 'goat' is potentially a separate species, living in sympatry with *T. circumcincta* 'standard'. Defining a new species is not easy, as there are numerous species concepts in use; for example species may be defined on the basis of diagnostic traits (Cracraft, 1982 referenced by (Kunz 2002), cladistic classification or biological concepts (Dobzhansky referenced by Anderson and Jaenike (1997). For parasitic nematodes, species classification has traditionally used morphological measurements for cladistic taxonomy (Durette-Desset *et al.* 1999;Gouy de Bellocq *et al.* 2001). However due to nematodes having a relatively small number of distinctive morphological characteristics, numerous debates over speciation have arisen. Based on traditional morphological characteristics of adult males, *T. circumcincta* 'goat' and *T. circumcincta* 'standard' are identical. However, Gasnier *et al.* (1997) suggested the two *T. circumcincta* types, as defined by the MDH isoenzyme genotyping, could be distinguished by combining a number of morphometric measurements of the dorsal rays in male specimens. Morphometric measurements were performed on the male tails of the 33 FrMe individuals investigated here that had been removed prior to DNA preparation (data not shown, Dr J. Cabaret, INRA France). However the results were inconclusive. This could have been due to the poor condition of the tails after removal from the rest of the body and their preservation in fixative (Denke solution; Appendix D) or may simply reflect the difficulties of separating the two *T. circumcincta* 'types' on morphological grounds.

In spite of a lack of reliable morphological evidence of the presence of separate species, the molecular genetic analysis is very persuasive. Evidence for two species is supported by the cladistic species concept based on phylogenetic analysis because two distinct monophyletic groups based on ITS-2 and mtDNA ND4 sequences were found to correspond to *T. circumcincta* 'goat' and *T. circumcincta* 'standard' (Leignel *et al.* 2002). The phylogenetic species concept was further supported on the basis of parsimony and neighbour-joining analysis of the mtDNA ND4 sequences, grouping the previously

described new species, *T. boreoarcticus*, (Hoberg *et al.* 1999) with individuals typed as *T. circumcincta* 'standard' and the *T. circumcincta* 'goat' individuals forming a separate monophyletic group (Leignel *et al.* 2002) (Figure 5.16a). The theory that *T. circumcincta* 'standard' and *T. circumcincta* 'goat' types represent two species was further supported by the divergence of the ITS-2 sequences between the two types being of the same magnitude (4.4 %) as found between difference species of *Trichostrongylus* species (1.3 % - 7.6 %) (Hoste *et al.* 1995;Leignel *et al.* 2002).

The results presented within this thesis strongly support *T. circumcincta* 'goat' and *T. circumcincta* 'standard' as separate species on the basis of the biological species concept as there is evidence of reproductive isolation. Investigations by Leignel *et al.* (2002) showed the MDH-2 and β-tubulin markers revealed strong heterozygote deficiency, with a distinct lack of MDH-2 A / C and B / C worms in addition very few Type I / Type II heterozygotes. A lack of heterozygotes was also apparent in the results for the FrMe population presented in this thesis with only one individual identified as heterozygous for the Type I / Type II alleles of the β-tubulin marker (FrMe26, Table 5.10). As discussed above, the microsatellite data, based on 5 independent loci, strongly supports a lack of gene flow between the two *T. circumcincta* types. This demonstrates that there is a significant reproductive barrier between *T. circumcincta* 'standard' and *T. circumcincta* 'goat' worms that coexist in the same abomasum of the same goat. Hence the two *T. circumcincta* types fulfil the basic criteria for separate species status on the basis of the biological species concept.

If these two *T. circumcincta* 'types' represent two separate species, how are the heterozygous individuals in the study by Leignel *et al.* (2002) explained. There were 18 MDH-2 A / C and B / C heterozygous individuals out of 712 individuals genotyped with MDH-2 marker and three heterozygous Type I / Type II out of 190 individuals genotyped with the β-tubulin marker. Of the 33 individuals genotyped here from the FrMe population, one individual (FrMe26) was heterozygous Type I / Type II for the β-tubulin marker (Table 5.10). Furthermore, there are three individuals which do not follow the pattern of being *T. circumcincta* 'standard' or *T. circumcincta* 'goat' across all distinguishing loci used (FrMe2, FrMe17 and FrMe26 for the β-tubulin marker, FrMe6 for the MTG 15 locus) (Table 5.10).

There are three potential explanations for these relatively uncommon "hybrid genotypes". Firstly, the two *T. circumcincta* 'types' are genuinely separate species but these hybrid individuals are potentially the result from interspecies hybridisation. In this case, these

hybrid individuals would not be fertile if the biological species concept is to be fulfilled. Although there are few well documented cases of species hybridisation occurring between nematode species in field populations, it seems likely that they do exist. For example, *H. contortus* and *H. placei* are now widely considered to be separate species based on morphological and molecular evidence (Blouin *et al.* 1997;Stevenson *et al.* 1995). However, female *H. contortus* and male *H. placei* can be hybridised by experimental co-infection and since these species co-exist in the field, it seems likely that hybrid genotypes will occur (Achi *et al.* 2003). Potential evidence of hybridisation between the two strongyloid nematode species, *Paramacropostrongylus iugali* and. *Paramacropostrongylus typicus* has been found in eastern (*Macropus gigantus*) and western (*M. fuliginosus*) grey kangaroos using four enzyme loci (Chilton *et al.* (1997).

The second explanation is similar to the first, but if hybrid genotypes were fertile then the two *T. circumcincta* 'types' would not be genuinely separate species based on the biological species concept. This possibility is clearly difficult to distinguish from the first based on population genetic studies.

The third explanation, as favoured by Leignel *et al.* (2002), is that the two 'types' are genuinely separate species, with no gene flow between them, but the alleles concerned are not unique to each species but are instead present at dramatically different frequencies, e.g. the C allele of the MDH-2 locus is present at low frequency in the *T. circumcincta* 'standard' population and so A / C or B / C genotypes are not 'hybrid' but are simply rare heterozygotes within the *T. circumcincta* 'standard' population. This is commonly referred to as "ancestral polymorphism". This may occur when there has been recent divergence and the effective population size has been large enough to prevent genetic drift leading to fixation of alleles (Muir and Schlotterer 2005). As discussed earlier, trichostrongylid nematodes show extreme levels of population diversity, due to large effective population sizes; therefore genetic drift is proposed to be slow.

It is extremely difficult to determine whether there is a low level of gene flow or no gene flow between the two populations of worms. However it is worth noting that Fst calculations based on the five microsatellite loci suggest the FrMe population is less differentiated from the French populations (Fst = 0.0875 ± 0.005) than it is from the U.K. populations (Fst = 0.140 ± 0.005) (p-value ≤ 0.001). This could be taken as evidence of some gene flow between French *T. circumcincta* 'standard' and French *T. circumcincta* 'goat' populations reducing the genetic differentiation between them (Figure 5.16a) The

only alternative, and much less likely, explanation would be a very recent speciation event in which the *T. circumcincta* 'goat' separated from *T. circumcincta* 'standard' in French populations (Figure 5.16b).

One additional result of note was that from the ITS-2 genotyping. The FrGa population was distinctly different based on the ITS-2 patterns obtained (Table 5.10). This may suggest the presence of another distinct sub-population of *T. circumcincta* in French goats. Interestingly, Leignel *et al.* (2002), found a population of worms from the same farm to have a distinct mtDNA ND4 sequences, grouping them separately from *T. circumcincta* 'standard' (Figure 5.16a; *T. circ* Gal). However, this population was indistinguishable from other *T. circumcincta* populations based on β-tubulin locus, ITS-2 sequence and microsatellite genotypes.

5.4.5 The relationship between *T. circumcincta, T. trifurcata* and *T. davtiani*

Teladorsagia davtiani, *Teladorsagia trifurcata*, and *Teladorsagia circumcincta* have been traditionally considered as three separate species based on the morphology of the male spicules (Lichtenfels *et al.* 1988;Lichtenfels and Hoberg 1993;Urquhart *et al.* 1996) (Figure 5.1). This is well illustrated by the fact that many commercial anthelmintic products list these as separate species in their spectrum of activity claims (NOAH 2004). However, the validity of this distinction has been questioned by a number of authors. For example, *T. trifurcata* and *T. davtiani* were proposed to be morphological variants of *T. circumcincta* based on a number of observations including the observation that male morphological variants always occurred together, with one (*T. circumcincta*) constituting a major proportion of the population (Lichtenfels *et al.* 1988). A number of studies which were described in more detail in the introduction of this Chapter (section 5.1.2) have failed to show genetic differentiation between *T. davtiani*, *T. trifurcata*, and *T. circumcincta* (Andrews and Beveridge 1990;Gasnier *et al.* 1993;Stevenson *et al.* 1996). Although no fixed differences in the ITS-2 sequences were found between *T. davtiani*, *T. trifurcata*, and *T. circumcincta*, it should be noted that this was only based on the sequence of 7 individual worms in total (Stevenson *et al.* 1996). The population genetic analysis, using microsatellite markers, presented in this chapter sheds further light on this issue. A total of 31 *T. davtiani* (ScKiTD), 30 *T. trifurcata* (ScKiTT), and 31 *T. circumcincta* (ScKiTC) were collected and morphologically characterised by Dr B. Craig, from soay sheep on the isolated island of Hirta. Equal numbers were, as far as possible, collected from a total of twelve soay sheep to

avoid any host bias (Table 2.3). These 92 individual worms were genotyped using the five microsatellite markers. Similar levels of polymorphism and diversity were found for the *Teladorsagia davtiani*, *Teladorsagia trifurcata*, and *Teladorsagia circumcincta* populations (Table 5.5). Visual inspection of histograms of the allele frequencies for each marker revealed little variation between the allele frequencies. There were some minor allele frequency differences noted for markers MTG 15 and MTG 67 between *T. davtiani* and the other two morphological types (Figure 5.13). However the numbers of worms genotyped for each allele in question was very low making it difficult to interpret these differences by qualitative inspection. Quantitative analysis of the data using pairwise Fst values showed very low levels of genetic differentiation (Fst ranging from -0.0068 to 0.025) and this was of a comparable scale to the low level of genetic differentiation found between host infra-populations grazed on the same pasture (Table 5.11). Furthermore these Fst values were far lower than those seen for the NzWs and FrMe population. These results were supported by the PCA analysis based on individual worm genotypes, which showed *T. davtiani* (ScKiTD), *T. trifurcata* (ScKiTT), and *T. circumcincta* (ScKiTC) to be evenly mixed, with no clustering of any one type (Figure 5.14). The *T. davtiani* population was separated slightly from the *T. circumcincta* and *T. trifurcata* populations from the same hosts on the PCA plots based on pairwise Fst values, but no more so than some of the other U.K. *T. circumcincta* populations (Figure 5.15a and 5.15b). Hence the microsatellite population genetic analysis strongly supports the suggestion that *Teladorsagia davtiani*, *Teladorsagia trifurcata*, and *Teladorsagia circumcincta* are unlikely to be separate species but instead more likely to be morphological variants of the same species.

5.4.6 Inheritance of microsatellite markers demonstrates multiple paternity in *T. circumcincta*

In many species females mate with more than one male (polyandrous), including insects (Baer and Schmid-Hempel 1999;Bretman and Tregenza 2005), amphibians (Myers and Zamudio 2004) and birds (Griffith *et al.* 2002). The mating behaviour of trichostrongylid nematodes has never been investigated before and so the inheritance studies of the 5 microsatellite markers was of additional interest in this respect. The genotypes of five female worms and their progeny was determined with all 5 markers. The results showed that one of these female worms contained the progeny of at least 3 male worms and the remaining 4 female worms contained the progeny of at least 2 male worms. Hence there is unequivocal evidence of multiple paternity. The estimation of the number of male worms that had mated with each female was very simple and conservative; simply the minimum

number of males that could have contributed all the paternal alleles detected in the brood. This would be expected to underestimate the number of males since some males may have been homozygotes and so only contributed one allele and different males may have contributed the same allele (Bretman and Tregenza 2005). Hence it may well mean that a larger number of males may have mated with each female than the minimum numbers presented here. There are a number of computer programs which use population allele frequencies to estimate the number of potential fathers. However, programs such as PARENTAGE assume no inbreeding and all males to have equal probability of mating with a female (Bretman and Tregenza 2005). Furthermore, these programs do not account for null alleles. The best way to improve the accuracy of the multiple parentage estimates would be to use a larger numbers of markers and particularly ones which have a large number of relatively frequent alleles. There is no published data on the mating strategy of trichostrongylid nematodes but similar results have recently been obtained for *H. contortus* (Dr J. Gilleard and Dr E. Packard, pers. comm.) and *Trichostrongylus tenuis* (Dr P. Johnson, pers. comm.). Although we only examined five female worms and broods, polyandry was demonstrated in all cases suggesting that this is probably the case for most, if not all female *T. circumcincta*.

It is interesting to speculate on the relative costs and benefits of polyandry to *T. circumcincta*. It has been shown that in species where time and energy are required to find a mate, then the benefits of multiple mating may be outweighed by the energy costs (Yasui 1998). On the other hand multiple paternity may be a benefit in nematodes where females are not be able to choose between high and low quality males either due to inability to distinguish or where encounters are random (Watson *et al.* 1991 referenced from McCoy *et al.* (2002)). It is difficult to know where the balance lies for *T. circumcincta* since in some hosts worm population (infra-population) densities will be very high (10,000s of worms in a single abomasum) whereas in many other cases, due to the over dispersed nature of parasite populations, infra-population densities will be very low. It is interesting to note that the worms genotyped for the inheritance study presented here were from a host with several thousand adult worms present in the abomasum. It may be the case that polyandrous behaviour would decline at lower worm burdens. Nevertheless there are a number of studies that show there are direct benefits of polyandrous behaviour to generating genetic diversity (Yasui 1998). Offspring from females which have multiply mated will potentially have increased overall fitness and be more likely to survive the variable conditions from hatching through to reproduction themselves. For example Baer and Schmid-Hempel (1999) found

increased fitness in the offspring of bumbles-bees which were inseminated with high diversity sperm, with offspring having lower parasite loads and greater reproductive success.

Whatever the benefits to the parasite in their natural environment, polyandrous mating behaviour has practical implications for experimental work in these organisms. Of particular importance is the impact on the ability to generate inbred lines for genetic and molecular studies. The standard approach to this has been to infect single host with larvae derived from eggs recovered from a single female worm. Polyandrous mating will clearly increase the genetic diversity of a single brood and consequently limit the ability to develop inbred lines using these approaches. Indeed in the *Haemonchus contortus* inbred lines ISE and IRE which have been developed by multiple rounds of inbreeding (Roos *et al.* 2004) still have high levels of genetic diversity as determined by microsatellite genotyping (Dr E. Packard and Dr J. Gilleard, pers. comm.).

Chapter 6: General Discussion

The parasitic nematode *T. circumcincta* infects sheep and goats worldwide, and is of major economic importance for countries in temperate regions, including the U.K. The control of parasitic nematodes, in both domestic livestock and man, is dependent upon the strategic use of anthelmintic drugs. However, for parasitic nematodes of sheep and goats, resistance to these drugs is becoming increasingly common. In order to address these issues, an understanding of the population genetic structure is required, yet very few authors have investigated this area. The main aim of my thesis was to investigate the population genetic structure of the economically import parasitic nematode *T. circumcincta* found in sheep and goats. This involved isolating and characterising microsatellite markers prior to investigating a number of basic questions regarding the population genetic structure. In addition, a survey of U.K. goat farms was conducted, as the current prevalence of anthelmintic resistance in U.K. goats is unknown. The survey was conducted to investigate the efficacy of parasitic control methods and identify resistant populations of *T. circumcincta*.

There have been a limited number of surveys conducted in the U.K. investigating the parasitic control strategies used by goat farmers. A survey of U.K. goat farms was carried out to investigate parasitic control strategies and the efficacy of anthelmintic treatments. The most reliable tests which could be used to conduct this survey by post were the Drench Efficacy Test and the Egg Hatch Assay (EHA). However, there are number of the inherent problems associated with both of these tests, as discussed in Chapter 3, and consequently only very general conclusions could be made from this survey. The Drench Efficacy Test demonstrated that the majority of anthelmintic treatments used by goat farmers in the survey were of poor efficacy. There are two possible reasons the poor efficacies seen; it is either due to the presence of anthelmintic resistant parasites or alternatively inappropriate administration or dosing with anthelmintic. The presence of benzimidazole resistant strains was supported by the results from the egg hatch assays for three farms for which benzimidazole resistance was detected. In order to accurately assess the extent of anthelmintic resistance, further investigations of farms showing poor drench efficacies could be followed up. This would best be achieved by performing Faecal Egg Count Reduction Tests on the farms concerned. This is very labour intensive requiring at least two farm visits and a high degree of cooperation from the farmers. Furthermore, the Faecal Egg Count Reduction Test is of limited sensitivity with approximately 25 % of the parasite population

needing to be phenotypically resistant before anthelmintic resistance can be detected. Hence the results in chapter 3 clearly illustrate the problems of diagnosing and surveying for anthelmintic resistance using the currently available techniques. There is an urgent need for improved diagnostic tests, in particular, molecular based tests that can accurately identify the proportion of resistant genotypes in parasite populations.

Molecular based tests to identify species of larvae and eggs present in faecal samples would be a valuable tool both to improve the accuracy of anthelmintic resistance diagnosis and aid epidemiological / population genetic studies. The standard techniques currently used to identify nematode species found in faecal samples are based on larval culture and morphological identification which are time consuming and require an experienced operator. Therefore in order to quickly and efficiently identify *T. circumcincta* in faecal samples which contain mixed nematode infections, a new molecular technique for identifying *T. circumcincta* individuals was developed. Primers designed to amplify the non-transcribed spacer region of the rDNA cistron were found to produce a consistent and unique banding pattern from *T. circumcincta, H. contortus, H. placei* and *N. battus* L3s. This new molecular method has the advantage of being a one step molecular technique which is rapid, easy and relatively inexpensive and has been extremely useful in the course of this project when used in conjunction with the molecular speciation technique described by Wimmer *et al.* (2004). It has also been useful for the identification of *H. contortus and H. placei* in other projects (Dr J. Gilleard and Dr E. Packard, pers. comm.). However the utility of the technique could be greatly improved by further work to increase the number of species which can be identified.

The main focus of this project was to develop microsatellite markers in order to investigate the population genetic structure of *T. circumcincta* populations. However there were very few genomic resources available to aid in marker identification and so three different approaches were used to isolate microsatellites from *T. circumcincta*. The first approach involved screening a small insert *T. circumcincta* genomic library, using a $(CA)_{25}$ oligonucleotide probe, which yielded 45 putative positive plasmids, one (MTG 15) of which was found to be useful as a population genetic marker. The high attrition rate is not uncommon to screening genomic libraries for microsatellites in nematodes. One of the main reasons for discarding putative positives was due to twenty-eight CA / GT microsatellites being associated with the tandem repetitive sequence 146 bp TecRep, as this additional repetitive sequence may have increased the complexity of polymorphisms found. This was

confirmed by sequencing individual alleles from the microsatellite MTG 1a. Work carried out by Hoekstra *et al.* (1997) and Otsen *et al.* (2000) for *H. contortus* and Callaghan and Beh (1994) for the *T. colubriformis* found similar repetitive elements associated with $(GT)_n$ microsatellites showing it to be a common feature of trichostrongylid nematode genomes. The second approach used primers designed to the sequence flanking 19 microsatellites previously isolated from the closely related nematode, *H. contortus.* One *H. contortus* microsatellite primer set (Hcms 28) was found to be suitable for population genetic analysis. This suggests the sequence flanking most *H. contortus* microsatellites is insufficiently conserved to allow amplification from *T. circumcincta,* which probably reflects the evolutionary distance between these parasite species. The third approach of searching the *T. circumcincta* EST database for repetitive sequences was the most productive, with three out of fourteen markers found to be suitable for population genetic analysis (MTG 67, MTG 73 and MTG 74). The five microsatellite markers which were used for population genetic analysis were all found to be polymorphic. Hence a panel of five microsatellites was developed for population genetic studies. Ideally, more microsatellite markers should be isolated, which would increase the power of *T. circumcincta* population genetic analysis. However, isolation by screening small insert or enriched genomic libraries was deemed too labour intensive with a low return in usable markers. As searching the EST database was the most efficient, it seems that the best approach would be to search new *T. circumcincta* ESTs for future microsatellite isolation and characterisation. Once larger numbers of suitable microsatellite markers have been isolated, more detailed population genetic analysis can be carried out. In addition microsatellite markers could be used for gene mapping approaches, by linkage analysis on populations with particular traits, such as resistance.

The microsatellites MTG 15, Hcms 28, MTG 67, MTG 73 and MTG 74 were used to genotype single adults, L3 stages or eggs from eighteen *T. circumcincta* populations from Scotland, France and New Zealand. Overall genetic diversity of these populations was found to be high corresponding to previous data for this nematode using mtDNA markers (Blouin *et al.* 1995;Braisher *et al.* 2004;Leignel and Humbert 2001). The majority of diversity seen was within rather than between populations, and was similar for both field and laboratory strains. These results suggest that the high levels of polymorphism broadly reflect the larger population sizes of this parasite. Future work could investigate the effects of population bottlenecks on *T. circumcincta* populations by studying the genetic diversity of populations on different farms, in different regions, at different times of year and following different anthelmintic treatments.

Population genetic analysis of *T. circumcincta* populations found little evidence of sub-structuring among U.K. populations, regardless of the host species, geographical location or whether or not they were a field or lab isolate. On a more global scale, little genetic differentiation was found between population in the U.K. and France (excluding FrMe). However, two populations were found to be genetically distinct, NzWs and FrMe. The moderate genetic differentiation of the NzWs population could be due to geographical isolation for more than 80 years or due to the fact that it is a laboratory strain or it could be another example of cryptic speciation. In order to investigate this further, more *T. circumcincta* populations need to be analysed from New Zealand, including both field and laboratory strains.

Population genetic analysis with the microsatellites markers found the French population, FrMe, to be highly distinct from both the other French populations and U.K. and New Zealand populations. This supports the hypothesis that a cryptic species of *T. circumcincta* exists in French goats which has been termed *T. circumcincta* 'goat' (Leignel *et al.* 2002). β-tubulin and ITS-2 genetic markers were used to genotype the population and confirmed the presence of 27 *T. circumcincta* 'goat' individuals in this population. The results of the microsatellite genotyping presented in this thesis strongly support that these *T. circumcincta* 'goat' individuals belong to a separate species compared to the more widely distributed 'standard' *T. circumcincta* present in both sheep and goats. It is an interesting discussion point as to whether this new species should be differentiated from *T. circumcincta* by the designation of a new species name. Interestingly, the FrMe populations was less genetically differentiated from the other French populations than it was from the U.K. populations which could be suggestive of gene flow or retention of common alleles. Further population genetic analysis of the populations within France and the U.K. using additional microsatellite markers is necessary to clarify this issue. More immediate work could include genotyping more populations from France, using the five microsatellite markers within this thesis, which have already been characterised by Leignel *et al.* (2002). In addition, investigations using these microsatellites could include populations of *T. boreoarcticus* and North American *T. circumcincta* populations. No study as yet has directly compared these populations to *T. circumcincta* populations in Europe using a multilocus approach. *T. boreoarcticus* is predicted to have diverged prior to the introduction of *T. circumcincta* into North America over 200 - 300 years ago (Adams 1998;Hoberg *et al.* 1999). A number of questions are immediately apparent: Is *T. boreoarcticus* equally genetically differentiated from European and Northern American *T. circumcincta* populations? Does gene flow occur

between *T. circumcincta* populations of North America and Europe? Are there additional cryptic species present in *T. circumcincta* populations?

Population genetic analysis using the microsatellites showed no genetic differentiation between *T. davtiani, T. circumcincta* and *T. trifurcata*. These were traditionally defined as separate species but these results support more recent suggestions that these may be morphological variants of the same species. Hence the genetic analysis presented in this thesis suggests morphological variation of the spicule of the male tail is a poor parameter for defining species in the *Teladorsagia* genus. In the case of the FrMe population, worms with indistinguishable spicules are genetically distinct whereas for *T. trifurcata* and *T. davtiani* dramatic differences in spicule morphology do not reflect genetic differentiation.

In conclusion, the results from this work support previous studies showing *T. circumcincta* is a highly diverse parasitic nematode and is part of a species complex. However, additional knowledge is required to understand the population genetic relationship between *Teladorsagia* species and populations worldwide.

Appendix A: Anthelmintic Resistance Questionnaire

ALL DATA WILL BE TREATED AS CONFIDENTIAL

Please tick the appropriate box or boxes unless otherwise stated

1. Name and address?			
2. Post code?			
3. Total area of pasture used by goats?	.....Acres or .....Hectares		
4. How much is permanent pasture?	.....Acres or .....Hectares		
5. Are the goats housed during the year?	All year round ☐ Seasonally ☐ How many months?:..... From:.....To:.....		
6. How many goats do you have?	Does:	Kids:	Billies:
7. What breed of goat do you have?			
8. When are your kidding times?	State which months:		
9. Do you have sheep or cattle?	Sheep ☐	Cattle ☐	Other:
10. Do you co-graze, rotationally graze or graze your animals separately?	Graze separately: ☐	Rotationally graze: Goats & Cattle ☐ Sheep & Goats ☐	Co-graze: Goats & Cattle ☐ Goats & Sheep ☐
11. Do you treat your animals with anthelmintic? If not what do you do instead?	Yes: ☐	No: Organic ☐ Grazing management ☐ Other (please state) ☐	
12. How often do you drench - numbers of times and which months of the year?	Number of times per year:		
If you drench more than 6+ times per year, please could you state the exact number of times per year here.....	Does: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 6+☐ Kids: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 6+☐ Billies: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 6+☐		
	Months - Circle the appropriate month(s).		
	Does: J F M A M J J A S O N D Kids: J F M A M J J A S O N D Billes: J F M A M J J A S O N D		
13. How do you drench your animals? (Tick all that apply)		Does	Kids / Billies
	Follow a set drench program	☐	☐ ☐
	At sign of disease	☐	☐ ☐
	At housing	☐	☐ ☐
	When docking/hoof trimming	☐	☐ ☐
	Weaning	☐	☐
	At turning out	☐	☐ ☐
	Pre tupping	☐	☐
	Pre/post Kidding	☐	

Continued Overleaf

14. What anthelmintic(s) did you use this year?	Class I: Benzimidazole ☐ Class II: Levamisole/ Morantel ☐ Class III: Macrocyclic Lactone /Avermectin ☐ Other:.....					
15. How long have you been using present drug?		1 Year	2 Yrs	3 Yrs	4 Yrs	5 Yrs	5+Yrs
	Does:	☐	☐	☐	☐	☐	☐
	Kids:	☐	☐	☐	☐	☐	☐
16. What anthelmintics have you used in the past 5 years?		**2001**	**2000**	**1999**	**1998**	**1997**
	Class I	☐	☐	☐	☐	☐
	Class II	☐	☐	☐	☐	☐
	Class III	☐	☐	☐	☐	☐
17. How often do you change your anthelmintics?	Never: ☐		Each time you use drench ☐ Every 3 months ☐ Every 6 months ☐ Annually ☐ Longer ☐			
18. Do you drench all of your does/kids/billies or do you drench selectively?	Drench selectively: ☐		Yes, all: Does ☐ Kids ☐ Billies ☐			
19. Do you buy in replacement stock?	No: ☐		Yes: ☐ % per year:.....			
20. Do you bring in a billy for breeding?	No: ☐		Yes: ☐			
21. Do you drench animals brought onto the farm?	No: ☐		Yes, with: Class I ☐ Class II ☐ Class III ☐			
22. Do you keep animals separate from the rest of the stock when brought onto the farm?	No: ☐		Yes: ☐ How long for:.....			
23. How do you determine amount of drench to use?	Estimate weight of individuals and dose accordingly ☐ Weigh individual animals and dose accordingly ☐ Weigh individuals and dose according to heaviest animal ☐ Dose to average weight of group ☐					
24. What form of anthelmintic do you use to treat you sheep/goats? (Tick all that apply)	Drenches: ☐	Injectables: ☐	In feed: ☐			
25. Do you believe the efficiency of drenches are more, less or unaltered compared to previous years?	More: ☐	Less: ☐	Unaltered: ☐			
26. Do you have any confirmed anthelmintic resistance?	No: ☐	Yes, to which family: Class I ☐ Class II ☐ Class III ☐				
27. Do you move your animals to clean pasture after treatment?	No: ☐	Yes: ☐				

28. Do you have a problem with any other parasites?	No: ☐	Yes: Liver fluke ☐ Tapeworm ☐ Scab/Lice ☐ Blowfly ☐ Coccidia ☐
29. Do you use ectoparasitic treatments?	No: ☐	Yes: ☐ When:..... What:.....

Please write any additional information you feel is relavant to your anthelmintic treatment in the box below:

Any additional information regarding the origin of your original stock, your replacement stock and any other movement of stock onto or off your farm will provide useful information.

Thank you for completing this questionnaire

Appendix B: Directions sent to each Goat Farmer

Department of Veterinary Parasitology
University of Glasgow
Veterinary School
Bearsden Road
Glasgow, Scotland
G61 1QH
Tel: 0141 330 6923
e-mail: vlg1r@udcf.gla.ac.uk

How To Collect and Submit Faecal Samples

- Please sample from 10-15 goats. If you have fewer goats, please sample from as many as possible. Samples from individuals should be mixed together as described below.
- Decide on the best time to collect your samples. The best results are often obtained from goats that have been grazing on dirty pasture. Samples should be taken at least four weeks after the animals last oral drench or eight weeks after treatment with a persistent anthelmintic such as doramectin or moxidectin (Cydectin, Dectomax) for the pre-dosing sample.
-

Timing of pre and post dosing sample is crucial to the interpretation of results.

The Pre-dosing Sample: must be taken on the same day as your next dosing before administration of dose.

- Collect fresh faecal samples from the animals selected for the trial. For the storage system to work properly, the dung samples must have been observed to be freshly passed.
- Mix the dung samples from separate goats by kneading together in the plastic bag provided. Expel any air and seal. Place inside second clean bag.
- Please fill in the sample information sheet

Post dosing Sample: must be taken 7-10 days post dosing

- Collect fresh faecal samples from the animals selected for the trial as done for the pre-dosing sample.
- Mix the dung samples from separate goats by kneading together in the plastic bag provided. Expel any air and seal. Place inside second clean bag.
- Please fill in the sample information sheet bags if different sample have been taken
-

Do not refrigerate! Keep samples at room temperature and return to the Department of Veterinary Parasitology, University of Glasgow, using the pre-paid labelled box, as soon as possible.

If you have any questions regarding the procedures above, please do not hesitate to contact Tiggy at the above address.

Recommendations for dosing your animals

Goats require higher doses than sheep. Please use the drug list provided to see what class of anthelmintic you are using and adjust using the guidelines below.

Benzimidazoles:	2 x sheep dosage
Levamisoles:	1 ½ x sheep dosage
Ivermections:	same as sheep dosage

All animals being treated with anthelmintic should be given the recommended dose require for their individual weight or based on the weight of the heaviest animal. Underdosing may predispose to the development of resistance.

All orally administered drugs should be given over the back of the tongue, preventing the oesophageal bypass system. All ruminants, including sheep and goats have this reflex which can be triggered by placing liquid in the mouth but not over the back of the tongue. Rumenal by-pass reduces the absorption of the drug and therefore reduces its efficacy.

Appendix C: Microsatellite Primers

Microsatellite	Primer A/C 5'-3'	Primer B/D 5'-3'
MTG 1a	ATT GAA AAT TGA CTG CGG CAA CC	ATG TTT CCG TCC AAC CCC TAC AC
MTG 1b a / b	TTT AGC CTG TAT CCC ATT GAC CC	ATA TGT TTA TTC CAA TGA GTT CC
MTG 1b c / d	GAT CCT TAA CAT CAA ATG GTT ACC	CCT GCG AGC TAC CCG ATT TTG TTA GC
MTG 1c	ATG CTT TCA TTG ACG TGA TAA GG	TCA GCC GTT ATC AAT TTG GGT CC
MTG 3	GTG GCG CTT TTA CGT CAT GGA ATG	CCT CGT TCC CGC CAA ATA TGT TCC
MTG 5 a / b	GCC ATG ACA ATT TAT GAA AGT CC	TAA AGT GAG CCG TGA GCG CAA GC
MTG 5 c / d	GAT GGT CGT ATT CAT TCC CTT CAC C	GGA ATA ATT AGG GTG GCA TTG ACG C
MTG 6	TGT TAT TGC CAT CGT GGT CGC TC	CGC CTC TTA TCA GCC CTC ATC GG
MTG 7	AAT GAT TAA GTG CTT CTG CTG AAC	CAT GAG CCG TTA TCA GGT GTT ATC
MTG 8 a / b	ACG TAT TGG TCA GGG ACT GCC AC	TGG AGC AGG TGA GCC CGT ACA AC
MTG 8 c / d	CCA AGT AGG GAG TAC GTA TTG G	CGC CTC TTC CCT ATT TTT AGA GG
MTG 11	CAA CCC AGT CAG TCA GCC CTA CC	GTG TTG GAC CTG GGT TTG TAC GGC
MTG 14	ATT CAG AAC GGT ATT CTG AGC CGC	CAC TTG TTG AAA GCA ACG CCT CAC
MTG 15	TGC AAG GAA ACT GCT AAG AAG GAG	ATC ATG GAA CCT TGA TAC CGC AAG
MTG 16 a / b	AAT GTA AAG GTC ACT CGA AAC CAG	TGG GTG TAG TCA ATT CCG ATC GAC
MTG 16 c / d	AAG GGA CAG AGG GTG GTC ACA AAT G	ATC ACT TGT TGG AAC CGT CGC TCC
MTG 17 a / b	GCT CAC CAT CAC TAG GAG TCC TAC	TTC AAT AAC TTA ATT CCG TGC GTG
MTG 17 c / d	CAT CCG TGT GGC TGA AAC AGA AAC	GTG CGT GAA ATT TTC ATG CCG CC
MTG 18	GTT GAA AG CAG AGG TCC GAA TG	GTT TTG ACG CCT CTT ATC AGC TC
MTG 38 a / b	CAA CCC ACC CAG TAA GAA TCC	CAT AAT TTT CCG TAT CTG TCA TTT GG
MTG 38 c / d	GGT GGG TCA TTC TTA GGA TG	CTG CGG AAG GAG CGG TTT AAA C
MTG 39	GTG AAA TGA TCA CAA TTC CTA G	GAT TAC AAC GTG TCC AGA AC
MTG 41	CCT GTG GAT CAC AAA TCT CAC G	CTA TCG TTC AAT CTG GAA ACG CG
MTG 61	CCA TCG TTT CGC TGG CAA CAT GC	CTT ATC TTT ACC CTC AGA TCC
MTG 62 a / b	GGC TTC GGT GGT GGC TTT GGA GG	GTC AAA TGT ATT TTA TTA GTG C
MTG 62 c / d	GTG ATG GAG AGT GGT GGT TTT GG	TAT TAG TGC AGT TGA ACA TTA TC
MTG 63	GTA TCA GAA TCA GCA GCC ACA GTG G	CCA TGA ACT GGG ACT TCC TCT GC
MTG 64 a / b	GCA GAG TCG GCA CGA GGC CTC G	GAG TCC TGG CAT CTC ATT CTC TGC
MTG 64 c / d	CGC GGC TGC AGA GTC GGC ACG AGG	GAC CAT ACT TCT TAC GCA TCG C
MTG 65	CAT GAA GTA CGC ACT TCC GCT GC	CCT CCG CCT GCT CCT CCA TAC G
MTG 66	CTA GGT ATC AAG CCC GTT ATT CG	GTT GAC GAA CGC GGC CAT TCA GTG C
MTG 67	CAA GTC GTT TAG GCA CGT CTG G	CAG GGC GGA ACC CAA TTG ATC G
MTG 68 a / b	CTG CTA CGT CAA GCA CAG GAG	CGA GAT TGA GCA CCA GAT C
MTG 68 c / d	ATC ACC AGG CGG CTG CTA CG	CGA AAA GTA GAG TAT GAG C
MTG 69	CCA GCC CAC CTG CCA AAC C	GTC AGG AGT GTA GCT CGA CG
MTG 70	GGC CGG GAG TCT AGT GGC	GTA GGC CAC GAA GCG GTA GG
MTG 71 a / b	GCA AGT TGT TAT TGA GGA GC	AAA TTG CTT TCA CTA CGG CC
MTG 71 c / d	CAC AAC GTG CAG CGT CGG C	GCT GAA CAT TAA TAA ATT GC
MTG 73 a / b	CCT TGT ATA AAT TCG AAG C	GAT TAA CTT CCG TCA TCA TCC
MTG 73 c / d	TGT TCC TTG TAT AAA TTC G	GTA GTA GTG ATT AAC TTC CG
MTG 74 a / b	GTA CAG CGA AGA GTA CTA GGC	TCA ATA ACG AAC TAA ATA TG
MTG 74 c / d	GAT GGA CTC GTG GTA CAG CG	AAT TTA ATC AAT AAC GAA C

Appendix D: Solutions

Hybridisation and Southern Solutions

1) Denaturation Solution: 1 L

0.5 M NaOH	20.0 g
1.5 M NaC	187.66 g

Make up to 1L with dH2O and AUTOCLAVE if storing

2) Detection Buffer: 500 ml

100 mM Tris (pH 9.5 with HCl)	6.7 g
100 mM NaCl	2.922 g
50 mM MgCl2	10.16 g

Make up to 500 ml with dH2O
DO NOT autoclave

3) 20 x SSC: 1 L

3 M NaCl	175.32 g
300 mM Na Citrate (pH 7.0)	88.23 g

Make up to 1 L with dH2O and AUTOCLAVE

4) Maleic Acid Buffer: 1 L **pH 7.5**

0.1 M Maleic Acid	11.61 g
0.15 M NaCl	8.66 g

pH 7.5 using NaOH pellets
Make up to 1 L with dH2O and AUTOCLAVE if storing

5) Neutralisation Solution: 1 L (500 ml) **pH 7.5**

1.0 M Tris (pH 7.5 with HCl)	134 g	(67 g)
1.5 M NaCl	87.66 g	(43.83 g)

Make up to 1 L (500 ml) with dH2O and AUTOCLAVE if storing

6) Southern Neutralisation Solution: 1 L **pH 7.5**

0.5 M Tris (pH 7.5 with HCl)	67 g
3 M NaCl	175.32 g

Make up to 1 L (500 ml) with dH2O and AUTOCLAVE if storing

7) SDS 10%: 1 L

10% weight/volume in dH2O	
SDS	100 g

DO NOT autoclave

8) Washing Buffer: 500 ml

Maleic Acid Buffer	498.5 ml
0.3% Tween 20	1.5 ml

DO NOT autoclave

9) 0.2 M NaOH / 0.1% SDS: 100 ml

NaOH dissolve in dH2O	0.8 g
10% SDS	1 ml

Make up to 100 ml and DO NOT autoclave

Mini Prep Solutions

10) Cell Resuspension Solution 1:

dH2O	92 mls
1 M Tris (pH 7.5)	5 mls
0.5 M EDTA (pH 8)	2 mls

AUTOCLAVE
Add Rnase to small aliquot when ready to use. Rnase kept @ -20°C.
Use Rnase (10mg/ml) at a 1:100

11) Cell Lysis Solution 2:

dH2O	91 mls
20% SDS /10% SDS	5 mls
5M NaOH	4 mls

AUTOCLAVE

12) Neutralising Solution 3: pH 4.8

dH2O	120 mls
Potassium acetate	24.11 g
Glacial acetic acid (pH4.8)	x mls - add to correct pH

Make up to 200 mls with dH2O

Additional Solutions

13) Ampicillin (100 mg/ml)
Add 1 g Ampicillin to 10 ml dH2O
Filter sterilise
Store in aliquots at -20°C

14) Denke Solution

Formalin (37% Formaldehyde)	15ml
Acetic Acid	5ml
Glycerol	10ml
Ethylic Acid (ethanol 100%)	24ml
Distilled Water	42ml

15) Gel loading Buffer: 40 % sucrose / 0.25 % Bromophenol Blue

Sucrose	4 g
dH2O	10 ml
Bromophenol Blue	0.025 g

16) Helminthological iodine

Potassium Iodine	250 g
Resublimed Iodine	50 g

Dissolve in 500 mls tap water

17) IPTG 0.0238 g

Make up with 1 ml dH2O. Store at -20°C

18) LB Agar

Tryptone	5 g
Yeast Extract	2.5 g
NaCl	2.5 g
BACTO agar	7.5 g
5 M NaOH	100 µl

Make up to a volume of 500 mls and Autoclave

19) LB Broth

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
5 M NaOH	200 µl

Make up to a volume of 1L and Autoclave

20) M9

KH2PO4	3 g
Na2HPO4	50 mM
NaCl	85 mM
MgSO4	1 mM

21) Protinase K:

Reconstitute as 10 mg/ml concentration
Make up with a solution of 50/50 dH2O and glycerol. Store at -20 C

22) X- gal (40 mg/ml)

X-gal	0.4 g
Dimethylformamide	10 ml

Make up under hood and store at -20°C in a light proof container

23) 50 x TAE:

Tris	242 g
Glacial Acetic Acid	57.1 ml
0.5 M EDTA pH 8.0	100 ml

Make up to 1 L and AUTOCLAVE. Store at room temperature

24) 0.5 M EDTA pH 8 (1 L)

EDTA	186.12 g EDTA

Adjust to correct pH using NaOH pellets. Make up to 1 L and AUTOCLAVE

25) 11.1 x PCR Buffer

Reagent	Stock concentration	Volume (µl)	Final concentration
Tris HCl (pH 8.8)	2 M	334	45 mM
Ammonium Sulphate	1 M	166	11 mM
MgCl2	1 M	67	4.5 mM
2-mercaptoethanol	100 %	7.2	0.005 %
EDTA (pH 8.0)	10 mM	6.8	4.4 µM
dATP	100 mM	150	1 mM
dCTP	100 mM	150	1 mM
dGTP	100 mM	150	1 m
dTTP	100 mM	150	1 mM
BSA	10 mg/ml	170	113 µg/ml
Total volume:		**1352**	

Store at -20°C and always made up using PCR pipettes

26) DNA Lysis Buffer (Williams, 1995)

Reagent	Stock concentration	Volume (µl)	Final concentration
KCl	1 M	250	50 mM
Tris (pH 8.3)	1 M	50	10 mM
MgCl2	1 M	12.5	2.5 mM
Nonidet p-40	n/a	22.5	0.45 %
Tween - 20	n/a	22.5	0.45 %
Gelatin	2 %	25	0.01 %

Make up to 5ml with dH2O

Store at -20°C and always made up using PCR pipettes

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