Studies on the genetic population structure of *Cooperia oncophora*

Omslag is een kreatie en kadootje van Helga Wellink

Studies on the genetic population structure of *Cooperia oncophora*

Studies naar de genetische populatie structuur van *Cooperia oncophora*
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van
de Rector Magnificus, Prof. Dr. W. H. Gispen
ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen
op donderdag 6 november 2003 des middags om 14.30 uur

door Margreet van der Veer
geboren op 6 juli 1970 te Arnhem

Promotor	Prof. Dr. A. W. C. A. Cornelissen
Co-promotoren	Dr. E. de Vries
	Dr. Ir. H. W. Ploeger

The research described in this thesis was performed at the Department of Infectious Diseases and Immunology, Division of Parasitology and Tropical Veterinary Medicine, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands and supported by the Technology foundation STW, applied science division of NWO and the technology programme of the Ministry of Economic Affairs as part of the project UDG 4889.

ISBN 90-393-3543-5

Voor Pap en Mam

CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

INTRODUCTION

Cooperia oncophora is a parasitic nematode found in moderate and tropical climates. Parasitic nematodes appear worldwide and are considered being one of the most important causes of infectious diseases in ruminants (Perry and Randolph 1999). Although *C. oncophora* on its own is a mild pathogen of the gastrointestinal tract of cattle, it contributes together with other parasitic nematodes to great losses in the dairy and beef industry (Hawkins 1993). Though parasitic gastrointestinal infections can be controlled by anthelmintic treatment, this preventive measurement is threatened by the widespread evolution of drug resistance in parasitic populations (Waller 1997a). Therefore alternative nematode control strategies are being investigated such as the development of vaccines and the selective breeding of resistant hosts. Since it is proven that parasites are capable to adapt to changing environmental conditions like chemotherapeutics the question might be raised whether they can respond and become accustomed to other altered environments like the immunity of the host by either vaccination or selective breeding. It has been demonstrated that the development of anthelmintic resistance has a genetic background (Roos 1990; Grant and Mascord 1996). In addition, the speed by which resistance develops is correlated to the amount of heterogeneity of the population (Le Jambre 1993) and since genetic heterogeneity is essential for parasite populations to respond to changing conditions it is a crucial aspect of the host-parasite interaction. For several strongylid parasitic nematodes is known that they harbour extensive genetic variation within their populations (Blouin *et al.* 1992; Le Jambre 1993; Grant and Whitington 1994). For *C. oncophora* the degree of genetic variation is unknown. The genetic composition of a nematode compasses the nuclear DNA and mitochondrial DNA (mtDNA). A prerequisite for the study of genetic heterogeneity is the development of genetic markers, allowing the qualitative and quantitative analysis of genetic variation and the study of genetic flow within and between different parasite populations. Determining genetic population structures can provide insight whether parasite populations undergo genetic changes when alterations in the environment are met. This knowledge is necessary to help answering the question to what extent and for how long a vaccine or selective host breeding can successfully be applied when parasites are able to change and adapt to their changing environment. In this chapter the background of *C. oncophora* infection as well as some general aspects of parasite genetic diversity and its study with different molecular markers are described.

THE PARASITE; *COOPERIA ONCOPHORA*

Life cycle

C. oncophora has a direct life cycle, without migration in the definite host (Fig. 1). The pre-parasitic phase of larval development is entirely free-living. Eggs are passed in the faeces of infected hosts and hatch in the faecal pat resulting in the first stage larvae (L1). A mould of the L1 larvae results in the L2 larvae which, like the previous stage, feed on soil and faecal bacteria. A second mould of the L2 larvae leads to the infective stage larvae (L3) that cannot feed because they are enclosed by a sheath. The L3 sheath is composed of the retained L2 cuticle and protects the larvae. The ensheathed third stage is capable of migrating from the faecal pat to the grass where it survives by utilizing nutrients stored during the L1 and L2 stages. The host becomes infected after ingesting the L3 while grazing on pasture. During the following parasitic phase the L3 larvae exsheath in the abomasum. Following exsheathment the larvae move to their favourite habitat, the small intestine, where growth and development to adult worms occur. The mature females start producing eggs approximately 2-3 weeks after infection. The eggs are again shed in the faeces of the host and the following cycle commences.

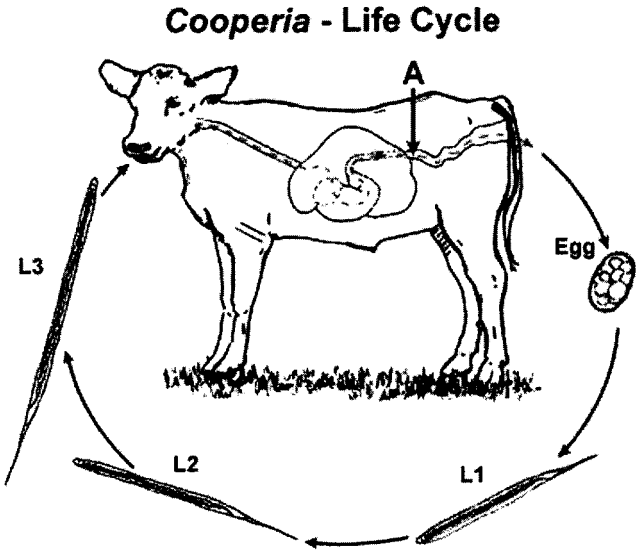

Figure 1. The life cycle of *Cooperia oncophora*. L1, L2 and L3 indicate the different larval stages, A indicates the location of the adult parasites (Source: the online book ‘’Parasites and Parasitic Diseases of Domestic Animals” at http://cal.vet.upenn.edu/merial).

Classification

C. oncophora belongs to the phylum Nematoda order Strongylida and superfamily Trichostrongyloidea. The Strongylida are a diverse and important parasitic group and are found in hosts as diverse as birds and mammals. They are believed to be derived from free-living nematodes which became parasitic about 350 million years ago (Durette-Desset *et al.* 1994). The classification of nematodes relies largely on their morphology combined with additional phenotypic variation like host species, life cycle and habitat.

Molecular phylogenetics based on DNA sequence comparison is increasingly used to interprete nematode classification. Phylogenetic analysis founded on small subunit rRNA gene sequences has suggested that there are five distinct clades in the phylum Nematoda (Blaxter *et al.* 1998; Dorris *et al.* 1999). *C. oncophora* belongs to a clade including other animal parasitic strongylid nematodes like *Ostertagia ostertagi* and *Haemonchus contortus* as well as the free-living nematode *Caenorhabditis elegans*. Nevertheless, the classifications within the phylum are still an issue of research and debate (Gouy de Bellocq *et al.* 2001).

Clinical signs and diagnosis

Although *C. oncophora* may often be the most numerous trichostrongylid parasites present in cattle it usually plays a secondary role in the pathogenesis of parasitic gastroenteritis in these ruminants. *C. oncophora* is a lumen dwelling parasite and considered as a mild pathogen causing minimal damage to the host's intestine (Coop *et al.* 1979; Henriksen 1981). The manifestation of clinical signs depends on the severity of infection as well as the susceptibility of the infected animal. Clinical signs attributed to *C. oncophora* infections include diarrhea, dull hair coat and poor weight gain.

The diagnosis of intestinal parasitism relies mainly on the detection of eggs passed in the faeces. Intestinal parasite infections are quantified as eggs per gram (epg) faeces. The most common technique for determining epg is the McMaster, which is in general applied with a sensitivity of 50 epg (Gordon and Whitlock 1939). The outcome of a faecal examination based on the McMaster is only an indication of presence of infection, since the epg does not correlate very well with the worms present in the intestine (Eysker and Ploeger 2000). Moreover, it prohibits the identification to the genus level since females that belong to the superfamily of the trichostrongyloidea, with the exception of *Nematodirus*, produce morphologically indistinguishable eggs. Although a *C. oncophora* specific ELISA (Poot *et al.* 1997) for accurate establishment of infection level has been developed, the technique is not widely applied since McMaster is cheaper and easier. Given that monospecific infections are very rare, culturing the infective larvae from the faeces must be performed for

determination of the different nematode genera causing infection. Again, like the epg, larval culture is not a precise measure for determining infection levels (Dobson *et al.* 1992). Just recently a PCR was developed for the diagnosis and identification of nematode eggs from a few strongylid species in faecal samples (Schnieder *et al.* 1999; Zarlenga *et al.* 2001). Although the identification to species level is an improvement, it still has the same general disadvantages as the McMaster technique since in both cases diagnosis is based on eggs. Hence, only in case of death of the host a post mortem study of adult parasites recovered from the infected animal will be the exact method for identification of the species and determination of the worm burden.

Epidemiology

The transmission of *C. oncophora* depends on climate, host susceptibility, management and the overwintering of L3 larvae. In the Netherlands, at turnout, susceptible first year grazers are infected with larvae that overwintered on pasture. As a result a rapid increase in epg will occur 2-3 weeks after turnout. Infected animals will contaminate the pasture and subsequently after the initial decrease in pasture infectivity in the spring, an increase will occur after two months. However the seasonal pattern during first year grazing calves shows a decrease in epg following the peak after turnout (Borgsteede 1977). This decrease is caused by the rapid development of immunity against *C. oncophora*. Consequently, the seasonal pattern in second year grazers does not show a peak after turnout and epg remains low during the season. Clearly, anthelmintic treatment and pasture management interfere with the epidemiology of *C. oncophora* and are aimed at reducing pasture contamination and infection levels within the susceptible young animals. For example, epg levels can significantly be reduced when calves are turned out later in the season reducing the uptake of overwintered larvae. Additional factors like using mowed pastures, not grazed before in the same season, contribute to lower epg and thus reduction of pasture infectivity.

Although *C. oncophora* is the most predominant species in first year grazing cattle (Borgsteede 1977), surveys performed in the Netherlands and Belgium in adult dairy cows showed that *Cooperia* spp. are found respectively in 24 and 16% of all adult animals (Agneessens *et al.* 2000; Borgsteede *et al.* 2000).

Treatment and prevention

Pasture management and anthelmintic treatment provide an excellent tool for controlling *C. oncophora*. Part of the nematode life cycle is on pasture and pasture management is designed to reduce the number of infective larvae. Examples of pasture management are the

grazing of less susceptible cattle on more contaminated pastures, rotation between pastures and strategic mowing (Michel 1976; Brunsdon 1980; Eysker *et al.* 1998). Although pasture management can reduce the parasite burden in cattle, the method alone does not result in parasite eradication. Moreover, in many parts of the world pasture management is not feasible and the farmers depend solely on anthelmintics. In case of *C. oncophora* infections the majority of the animals will develop protective immunity within the first grazing season. Since this acquired immunity protects animals against severe infection in the following grazing seasons a low level of parasite infection in young animals should be tolerated and anthelmintic treatment must be applied only when epg reaches a threshold (Vercruysse and Claerebout 2001).

Although the use of anthelmintics can be very effective, their use is being threatened by the increase of anthelmintic resistance in parasitic nematodes. The development of vaccines has been investigated but finding a proper vaccine is not easy since parasitic nematodes have a wide range of systems for escaping host immunity (Blaxter *et al.* 1992; Maizels *et al.* 1993). Vaccination using X-irradiated larvae against *H. contortus*, *Trichostrongylus colubriformis* (Jarrett *et al.* 1959; Mulligan *et al.* 1961) and *Dictyocaulus viviparus* (Poynter 1963; Peacock and Poynter 1980) was proven to be successful but with exception of the latter, these vaccines were not practical for commercial use. The combination of drug resistance and the public awareness of the presence of chemical residues in animal products is a stimulus to explore different areas like biological management techniques using fungus control and the supplementation of protein to the cattle diet as alternative options for nematode control (Waller 1997b; Coop and Kyriazakis 2001). An additional approach for controlling helminth infection is selective breeding of genetically resistant hosts or using breeds that have some degree of parasite resistance (Kloosterman *et al.* 1992; Windon 1996). For sheep and cattle the correlation between host genetics and resistance against nematode infections has already been established (Bishop and Stear 2001; Gasbarre *et al.* 2001; Morris *et al.* 2003) but the genes responsible for resistance have not yet been identified. It has been demonstrated that the use of sheep selected for increased resistance has a more persistent effect on egg output and, consequently, reduced pasture infectivity than other non-genetic control strategies (Eady *et al.* 2003).

Anthelmintic resistance

Since control of nematode infections is largely based on anthelmintic treatments, the extensive use of these therapeutics resulted in drug resistance (Sangster 1999; Sangster and Gill 1999). Resistance to anthelmintic compounds has been reported worldwide and is most

prominent in sheep nematodes. Recently, drug resistance in cattle parasites is emerging and cases of ivermectin resistance in *Cooperia* spp. have been reported (Vermunt *et al.* 1995; Stafford and Coles 1999; Fiel *et al.* 2001). The modes of action of anthelmintics are various. For example, the parasite can be paralysed, thereby allowing the host to expel the parasite by hindering the parasites ability to metabolise nutrients or limiting the ability to reproduce (McKellar 1997; Sangster *et al.* 2002). The major chemical groups available for anthelmintic treatment are the benzimidazoles, avermectins and levamisoles.

Resistance to anthelmintics is defined as the loss of sensitivity of a worm population that was sensitive to that same drug before (Shoop *et al.* 1993). During the process of acquiring resistance the anthelmintic treatment removes the worms susceptible to the anthelmintic which is leading to a population with an increased number of resistant individuals. After several treatments the worm population will survive subsequent treatment since the resistant genes accumulated (Kohler 2001). The detection of resistance in an early stage is essential, while it offers the opportunity to change the anthelmintic. Changing the drug for treatment will prevent an increase of resistant individuals in the population and thus may slow down the development of resistance. Different *in vivo* and *in vitro* detection methods exist but all, except for PCR, have rather poor sensitivity. For the faecal egg reduction test and the egg hatch assay detection is only possible when 25% of the population carries the resistance gene (Martin *et al.* 1989). The larval development assay is assumed to have a slightly better sensitivity of 10% (Dobson *et al.* 1996). Unfortunately the most sensitive method, a PCR capable of detecting 1% resistant individuals in a sample of a susceptible population, is only available for detection of benzimidazole resistance genes in some strongylid species (Roos 1995; Elard *et al.* 1999).

INTERACTION WITH THE HOST

Susceptibility and immunity

In general, animals develop a level of immunity when being exposed to *C. oncophora* during the first year grazing season. Immunity is the ability to prevent or limit the establishment or development of parasitic worms. The acquired immunity will subsequently protect the animal in later life against severe parasite infections and thus preventing production losses (Hawkins 1993). The susceptibility to a *C. oncophora* infection is related with age, younger animals are more susceptible than older ones (Kloosterman *et al.* 1991) and additional factors like gender and pregnancy, making bulls and pregnant cows more

susceptible (Armour 1989; Leighton *et al.* 1989). Besides acquired immunity animals possess variable levels of innate immunity, sometimes resulting in a low epg during first year grazing on contaminated pasture. Additionally there are animals that fail to develop a protective immunity and therefore continuously have a relatively high epg (Sonstegard and Gasbarre 2001). Although the latter animals represent only a small part of the cattle population, they are responsible for the majority of pasture contamination.

The differences in the immunological responses between animals become distinguishable when measuring the epg following an experimental infection with, usually, 100,000 *C. oncophora* larvae. In general epg output can be divided in three distinct patterns, which are given in Fig. 2. Egg excretion starts about 14 days past infection (dpi) and the majority of the animals will develop immunity which is characterized by a peak of egg excretion on average 21-28 dpi, after which the developed immunity is visible in the decline in egg excretion. The number of eggs being excreted during the peak depends on the infection dose, for a dose of 100,000 L3 the observed epg values will be between 2,000-10,000. Round 42 dpi the majority of the worm population is expelled from the host which is considered as the end of the parasitic phase. The pattern of egg excretion from animals having an innate immunity is very obvious since it is characterized by a very low egg output. The animals failing to mount an effective immune response have a comparable course in epg as seen for acquired immunity with the difference that after 28 dpi the rapid decline of epg will not occur and the animals remain shedding eggs (Gasbarre *et al.* 2001).

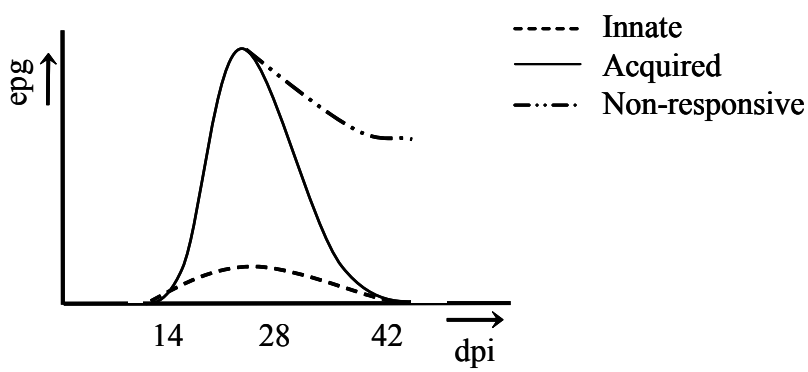

Figure 2. Egg excretion patterns during a primary *C. oncophora* infection in naive animals, showing the differences between innate and acquired immunity or being immunologically non-responsive.

Although protective immunity can be generated by natural or experimental infection, the underlying mechanism for acquiring this immunity is not completely known. While here the outcome of immunity is simplified by epg patterns, immunity to parasite infections includes more criteria like parasite burden and altered morphology of the worms. All these effects result from a very complex immune response to the parasite that involves cellular, humoral and inflammatory processes. The use of rodent parasitic models has made a great contribution to parasite immunology but cannot merely be extrapolated to the situation in the natural host (Balic *et al.* 2000).

Infection and selection

The observation of different egg output patterns following a primary infection with 100,000 L3 indicates the presence of different host immune responses. It has already been demonstrated that egg output is influenced by host genetics and is a heritable trait (Stear *et al.* 1990; Sonstegard and Gasbarre 2001). Obviously, there is a great interest in breeding those animals showing innate immunity against intestinal parasites since these animals hardly have an epg (Gasbarre *et al.* 2001). In addition, if appropriate genetic markers can be identified this may allow prediction of the outcome of an infection for any given animal. Animals that are expected to keep shedding eggs on pasture can be identified and removed from breeding programs or anthelmintic treatment may specifically target these animals.

The use of genetically resistant hosts should permit a reduced frequency of anthelmintic treatment, and hence reduce the rate of development of anthelmintic resistance in the parasite. Ultimately the goal for breeding genetically resistant animals is to reduce the losses caused by gastrointestinal nematode infections (Sonstegard and Gasbarre 2001).

Evidently the use of genetically resistant animals will raise a selection pressure on the parasite population. Whether parasites can evolve to successfully infect resistant animals remains unknown. The growing research in parasite immunology indicates that there is a wide array of mechanisms by which parasites evade or modulate host immunological attack (Balic *et al.* 2000). Since there has been a long history of co-evolution between the parasite and its host, it is imaginable that mechanisms for dealing with host resistance are already within the parasites repertoire and only need to be selected for. Research addressing this question is mostly performed with selection of parasites by repeated passage through uniform and well defined hosts. Some evidence exists for the ability of the parasite population to adapt and suppress the host immune response. The majority of these data were generated with *Heligmosomoides polygyrus* selection lines in mice (Dobson and Tang 1991). Parasites were selected for 30-50 generations in lines of mice which varied in their

levels of resistance (naïve or immune through primary or challenge infection). The selection resulted in different lines of parasites possessing heritable phenotypic changes regarding their fitness when tested in immune animals. Worms selected in resistant mice survived and reproduced better in immune mice compared to worms selected in naïve mice (Su and Dobson 1997). Other forms of selection are demonstrated by the use of alternative hosts for the maintenance of parasites in the laboratory. The worms are adapted to a non natural host by repeated passage through this host and ultimately can be kept in the altered environment of the new host. For example *Necator americanus* a human parasitic nematode can be maintained in hamsters (Sen and Seth 1970) and *Nippostrongylus brasiliensis*, a parasite of rats, is adapted to successfully infect mice (Wescott and Todd 1966). The results suggest that the immunity which normally protects the host against parasite infections, in the face of repeated passage acts as a selective pressure, making the parasites adapt to this immunity in order to survive (Solomon and Haley 1966). On the contrary, selection experiments, performed in the same manner, passing *Haemonchus contortus* in resistant sheep could not establish differences in performance of the parasite (Adams 1988; Albers and Burgess 1988; Woolaston *et al.* 1992). Although no effect was found for *H. contortus* this does not necessarily mean that this parasite is not able to eventually adapt. Further, most passage experiments concentrated on the phenotypic responses to selection, detailed analysis of the genetics of the selection process is lacking but will be necessary to prove whether these phenotypic alterations are genuine genetic adaptations or a consequence of phenotypic plasticity.

GENETIC VARIATION

Genetic variation is the driving force of evolution and thus essential for a species to adapt to changing environmental conditions. An example of the adaptation of the parasite population is the development of resistance against anthelmintics. The altered resistance gene either arises from a mutation or is already present in a heterogeneous population and accumulates in the population because it is beneficial for the parasite. For the development of anthelmintic resistance in parasite populations the latter is suggested since it has been demonstrated that the amount of genetic variation in a population is correlated to the rate at which resistance develops (Le Jambre 1993). The level of polymorphism within *C. oncophora* populations is not known but is likely to be considerable since other related strongylids have been shown to contain large within population variation (Blouin *et al.*

1992; McManus and Bowles 1996; Thompson and Lymbery 1996; Wakelin and Goyal 1996). Knowledge on the distribution of genetic variation within and between populations will provide tools to investigate host parasite interactions. Depending on the research question, different genomic regions located on the mitochondrial (mt) genome or nuclear genome can serve as a target region for the analysis of genetic variability. Both accumulate mutations over time but the rate with which this happens varies. Whereas non-coding regions evolve more rapidly, genes associated with a particular function are less likely to accumulate a spontaneous mutation since this may influence the gene function. An exception to the latter are the genes encoded by the mt genome, which evolve independently and more rapidly compared to the nuclear genome (Wolstenholme 1992b). In general nuclear sequences harbour less within species than between species variation and are therefore suited as genetic markers for the identification of species (Chilton *et al.* 1995; Hoste *et al.* 1998; Newton *et al.* 1998). In contrast, the maternal mode of inheritance in combination with the higher evolutionary rate of the mt genome, causing sufficient within species variation, has made mt sequences a popular marker for analysing the variation within and among parasite populations for establishing the genetic structure (Anderson *et al.* 1993; Blouin *et al.* 1995; Anderson and Jaenike 1997).

The nuclear genome

If the genome size and coding capacity of *C. oncophora* resembles that of *C. elegans* it will be in the order of 100 million base pairs (Mb) containing approximately 20,000 protein coding genes (The *C. elegans* Sequencing Consortium 1998). *C. elegans* not only shares morphological similarities with parasitic nematodes but also processes like biochemical adaptation to extreme conditions. The complete sequence of *C. elegans* facilitates, by comparing the expressed sequence tags (ESTs) from parasitic nematodes, identification of genes that are nematode and/or parasitic nematode specific. While some genes will have no homologues, they might therefore have a role in specific parasitic features like establishment in the host (Lizotte-Waniewski *et al.* 2000). Extensive research of gene function in *C. elegans* resulted in the development of efficient techniques but difficulties to maintain parasites make that most techniques cannot easily be extrapolated. Therefore, although gene function studies can be very informative it has only recently found its way into the parasitic field (Brooks and Isaac 2002). A very successful technique for studying gene function in *C. elegans* is the use of double stranded (ds) RNA mediated interference (RNAi). The gene of interest is knocked down by injecting dsRNA into the gonad, feeding of dsRNA expressing bacteria or soaking the worms in dsRNA. For some parasites that can

be maintained and manipulated in the laboratory the RNAi technique has been applied successfully (Hussein *et al.* 2002; Aboobaker and Blaxter 2003). An alternative option for studying parasitic gene function is to transform *C. elegans* with the parasite gene of interest.

Studies on the nuclear encoded ribosomal RNA (rRNA) genes of parasites have mainly been performed for species identification and establishing phylogenetic relations between parasitic worms. rRNA sequences are highly conserved, while the internal transcribed spacer (ITS) sequences situated in between show extensive variation (Hillis and Dixon 1991). These features allow the design of primers based on the conserved regions for amplification of the variable ITS region from multiple nematode species. The variable ITS region can subsequently be used for the identification of the parasite to the species level and in particular has been very useful for distinguishing closely related species from the strongylida order (Dame *et al.* 1991; Nadler 1992; Newton *et al.* 1998).

Genetic linkage maps of the nuclear genome would be a powerful tool in the characterization of genetic responses to selective pressures on parasite populations. When selection is involved a genetic map allows the localization of the loci even if they are distributed throughout the genome. For the construction of a genetic linkage map association between phenotypes and molecular markers are needed and crosses between phenotypically distinct individuals are the most powerful tool to establish genetic linkage. However, for most parasitic nematodes the controlled crossing is not feasible. An alternative is to genotype large numbers of individuals at many loci and subsequently search for association of a marker with the phenotype under examination. Although the screening of many individuals may not be the obstacle, the generation of a sufficient number of markers that cover the complete nuclear genome is complicated and therefore the application of genetic maps in parasitic nematodes has not yet been applied in parasite genetics.

The mitochondrial genome

The mitochondrial (mt) genome of related nematodes like *C. elegans*, *Ascaris suum* (Okimoto *et al.* 1992), *Necator americanus*, *Ancylostoma duodenale* (Hu *et al.* 2002a) and *Onchocerca volvulus* (Keddie *et al.* 1998) is a circular molecule of about 14 thousand base pairs (kb). It is compact compared to the nuclear genome, with no introns and very little non-coding regions. The mt genome from parasitic nematodes usually encodes for 12 proteins of the oxidative respiration cascade, 22 transfer RNAs (tRNA) and the large and small subunit ribosomal RNAs (rRNA). Additionally it contains a hypervariable control region for regulation of transcription and translation of the mt genome (Clayton 1982). Nucleotide composition and the use of altered amino acid codons like ATA for methionine,

TGA for tryptophan and the codons AGA and AGG for serine instead of arginine differ from those in the nuclear genome (Wolstenholme 1992a; Hyman and Azevedo 1996).

In general, genetic markers localized on the mt genome of parasitic nematodes are considered as neutral, i.e. they are not under selection. Therefore, these mt markers are not useful in determining a trait under selection but can be used to establish the amount of genetic variation within a population and in addition how this variation is structured within and between populations. The mt genome has two important characteristics that facilitate the determination of the genetic population structure. First, the maternal inheritance of the mt genome results in a simple linear evolutionary line of mt sequences. Second the mt genome has, compared with the nuclear genome, a higher evolution rate which, combined with the lack of recombination, makes it a suitable population genetic marker (Avise *et al.* 1992). The use of coding sequences of the different mt genes have been used to elucidate population structures and phylogenetic relations of the parasitic nematodes (Tarrant *et al.* 1992; Rollinson *et al.* 1997; Blaxter *et al.* 1998; Blouin *et al.* 1999; Hu *et al.* 2002b; Hu *et al.* 2002c; Mes 2003). Although no linkage exists with loci under selection in the nuclear genome the markers localized on the mt genome can be used to monitor genetic changes within a population that can provide insight into which circumstances influence the population structure.

MOLECULAR ANALYSIS

After introduction of the PCR method (Saiki *et al.* 1988), molecular techniques have become widely used for detecting variation in populations. Depending on the research question several techniques can be applied. In the following section some common techniques are described. Since for the analysis of individual worms the amount of DNA is limited only PCR based technologies are mentioned.

Amplified fragment length polymorphism

The amplified fragment length polymorphism (AFLP) technique is a fingerprinting technique having the advantage that no sequence information of the organism to be studied is required (Vos *et al.* 1995; Janssen *et al.* 1996). The method combines the use of restriction enzymes with PCR amplification of fragments and detects fragment length polymorphisms. The first step in the generation of AFLPs is the double digest of genomic DNA with two different restriction enzymes (Fig. 3). Next, short DNA oligos (adapters) are

ligated to the generated restriction fragments. Besides that these adapters are designed to fit the created restriction fragments they disrupt the recognition sequence of the restriction enzyme after ligation. The restriction fragments are amplified with primers that anneal to the adapters. The primers contain selective bases at their 3`-end enabling selective amplification of a subset of the fragments. Only those fragments of which the selective bases are complementary to nucleotides flanking the restriction sites are amplified. The number of selective bases can vary to reduce or increase the number of fragments amplified. Differences in fingerprints reflect mutation in the restriction sites or sites adjacent to the restriction sites, which cause the AFLP primers to mispair at the 3' end, thus preventing amplification. In addition, deletions, insertions and rearrangements affecting the presence or size of restriction fragments will also result in polymorphisms detected by AFLP.

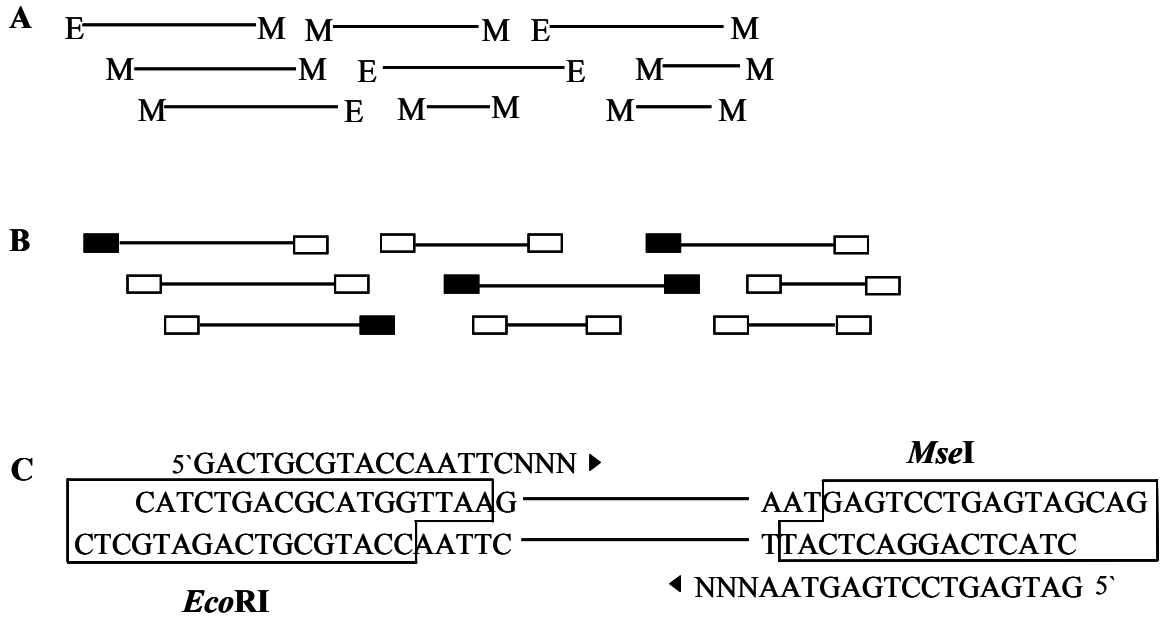

Figure 3. Schematic representation of the AFLP. A; Total DNA is restricted with two restriction enzymes, which usually have a 4 bp (i.e. *Mse*I (M)) and a 6 bp (i.e. *Eco*RI (E)) recognition sequence resulting in three kinds of fragments. Each restriction fragment contains either a *Mse*I (M-M) or *Eco*RI (E-E) site on each end or one of each (M-E). B; Adapters compatible to the two restriction sites (open *Mse*I and filled *Eco*RI boxes) are ligated to the restriction fragments. C; Primers for amplification anneal to the adapters (boxed) and contain up to three selective bases (NNN) at their 3`-end. Only perfect matching primers will be elongated. Because only the *Eco*RI primer is labelled, the *Mse*I-*Mse*I fragments will not be visible after amplification. Fragments containing two *Eco*RI adapters are very rare since *Mse*I cuts more frequently. The *Mse*I-*Eco*RI fragments of which the selective bases annealed with the target sequence can be visualized on a gel.

The AFLP generates multiple polymorphic markers from a single PCR reaction, based on the presence or absence of restriction fragments and is thus suited to determine genetic variation within a population. AFLP fragments correspond with unique positions on the genome and can therefore be used to construct a genetic map (Mueller and Wolfenbarger 1999). An additional advantage of the AFLP is that it allows recovery of markers from the gel that subsequently can be cloned and characterized. Although the AFLP technique detects a large number of loci, reveals a great deal of polymorphism and produces complex fingerprints which can be used in population genetic studies, the method is technically difficult and expensive to set up. The latter may be a reason that it has not found widespread application in veterinary parasite genetics.

Microsatellite analysis

Microsatellites are the most common polymorphic regions of vertebrate genomes. A microsatellite is a simple repeat that is flanked by an unique DNA sequence (Tautz 1989). The repeats vary in length between individuals which is assumed to be caused by slippage of DNA polymerases (Levinson and Gutman 1987). Amplification of the variable repeats by primers annealing to the unique flanking sequences of the repeats followed by separation on a polyacrylamide gel, results in unique fingerprinting patterns (Fig. 4). Microsatellite markers can be used for the construction of genetic maps which allows the detection of genetic loci which are under selection. As the AFLP, microsatellite markers can also be used for diversity studies and determination of population genetic structures. The disadvantage of microsatellite analysis is that the search for repeats requires a lot of effort, involving the construction of a genomic library, screening the library for clones containing repeats, sequencing the positive clones and finally constructing PCR primers to amplify the microsatellite repeat. Whereas the AFLP examines the complete genome and multiple loci in one reaction, the microsatellite marker detects only different alleles from one locus. To study the diversity present in the complete genome multiple microsatellite markers have to be identified.

Like the AFLP technique the use of microsatellite analysis has not been used commonly in parasitological research. For *Strongyloides ratti* microsatellite markers were identified but amplification of the repeats, with the exception of one microsatellite locus, was not successful (Fisher and Viney 1996). For *H. contortus* two independent studies characterized 13 and 59 microsatellites but these were all imperfect (Hoekstra *et al.* 1997; Otsen *et al.* 2000) which implies that the perfect repeat stretches are interrupted by non repeated sequences reducing the length of the repeat. Since the length of the repeat is correlated with

its polymorphic content the number of alleles established was low. In addition several of the microsatellites were null alleles or not polymorphic when tested in different populations.

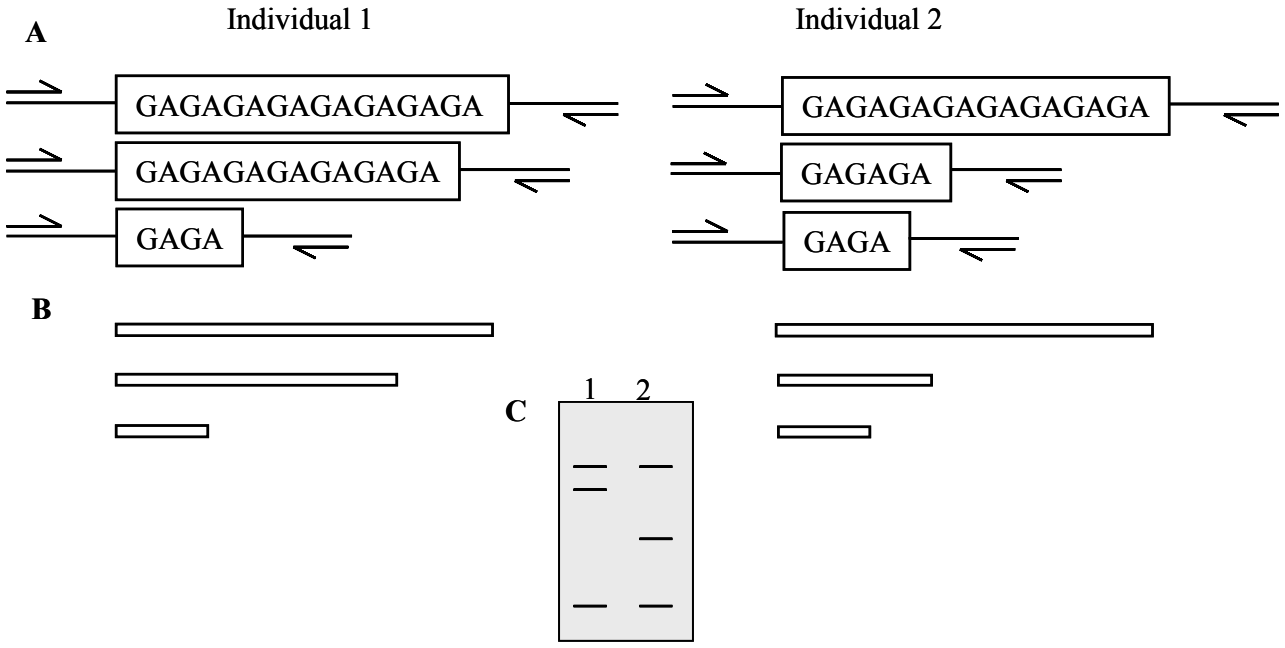

Figure 4. Schematic representation of the microsatellite technique. A; Amplification of the DNA sequence of two individuals containing three different microsatellite markers (within boxes). B; The length of one microsatellite differs between the individuals, which results in different lengths of the amplified fragments. C; The separation of the different fragments on gel results in different patterns for the two individuals.

Restriction fragment length polymorphism

Analysis based on restriction fragment length polymorphism (RFLP) identifies DNA polymorphisms that, due to a change in nucleotide, create or disrupt the recognition sequence, thus altering the length of the DNA fragment. The RFLP is in general used in combination with an amplification of the target DNA by PCR. Besides that the amount of DNA required is greatly reduced by the initial amplification, it additionally allows the detection of the restriction fragments after staining the gel (Fig. 5).

The PCR-RFLP can be used to study population structures but are less suited for reconstruction of molecular evolutionary processes. Nucleotide substitutions can lead to the disruption of a restriction site. In addition, the insertion or deletion of nucleotides within or between restriction sites also alters the length of the restriction fragment. Whether the polymorphism is caused by insertions, deletions or substitutions is not revealed by the technique. As comparable with microsatellite markers the RFLP is normally used on one

locus. However, with microsatellite markers multiple alleles can be detected within a single locus, whereas the use of one restriction enzyme during the RFLP generates only two states, i.e. presence or absence of the restriction site. Therefore the variation detected by the RFLP is considered to be lower than detected with the AFLP or microsatellite markers. Although PCR-RFLP has been used in studying parasite population structures (Anderson *et al.* 1995; Sorensen *et al.* 1999; Le *et al.* 2000), it is mostly applied within the taxonomy and phylogeny of parasitic nematodes (Hoste *et al.* 1998; Chilton *et al.* 2001; Gasser *et al.* 2001).

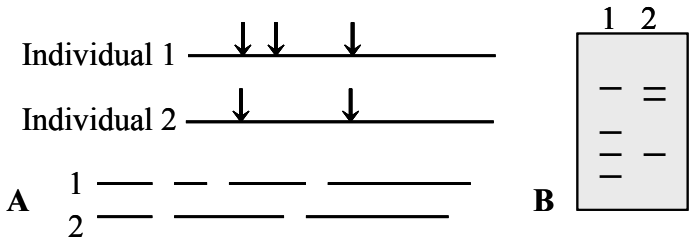

Figure 5. Schematic representation of the restriction fragment length polymorphism technique. The arrows indicate restriction sites in the DNA sequence of two individuals. A; Cutting the DNA sequence results in different fragments for each individual. B; Fragments are separated by size on a gel and the difference between the two individuals is visualised after staining the gel.

Sequencing

Of all techniques to study genetic variation, determination of the nucleotide sequence is the most informative. The most difficult problem for analyzing the genetic diversity, as with the RFLP, lies in the selection of the appropriate region of the genome. The DNA sequence needs to have allelic variation and has to allow efficient amplification. Although the primer sequences can often be determined from flanking sequences of the region in closely related species, for parasitic nematodes the knowledge of variable sequences within the genome is restricted to the previously mentioned ITS and mt sequences. Once primers are designed the PCR products can be sequenced which allows the identification of single nucleotide polymorphisms (SNPs) that in general are overlooked when using the above described techniques. Besides determination of the degree of allelic variation in a given sequence, it has the advantage of providing additional information on the genetic composition like nucleotide bias and transition/transversion ratios of which the latter can be helpful in determining selection pressures on the DNA sequence under study. The use of DNA sequences for determining population structures can be applied on nuclear and

mitochondrial genes but the use of sequencing as a tool in determination of diversity in parasite population genetics has been applied using predominantly mitochondrial DNA sequences. The mt sequence data from different strongylid parasites from domesticated ruminants (*O. ostertagi*, *H. placei*, *T. circumcincta* and *H. contortus*) were used to compare the genetic structures with those from a parasite of a wild ruminant (*Mazamastrongylus odocoilei*) (Blouin *et al.* 1995). The parasite population structure of the wild deer was structured by isolation indicating the existence of distinct separated subpopulations. In contrast, the parasite population structures found in cattle and sheep showed little structure among populations. The high gene flow between the domesticated ruminants was suggested to be correlated with the movement of the hosts which is substantially higher than for the deer.

OUTLINE OF THE THESIS

It is known that gastrointestinal parasites are capable of rapid adjustment to anthelmintics most likely by selection of a resistance gene already present in a heterogeneous population. Therefore, research has focused on alternative control strategies to eliminate dependence on chemotherapy. Major interest lays in the selective breeding for host resistance. However, the question remains whether parasites are able to change and adapt to this selective pressure from host immunity. The efficacy of selectively breeding resistant hosts would benefit from knowledge concerning parasite adaptation. For genetic adaptation genetic variation is necessary. Before genetic adaptation can be studied knowledge on the amount of genetic heterogeneity within the parasite population is required. Since for *C. oncophora* practically no genetic data is available, the majority of this thesis is focused on the characterization of genetic markers for determination of population diversity using some of the molecular techniques described in the preceding sections.

In chapter 2 the use of a technique for which no knowledge of the genetic composition of the study item is necessary was tested. This technique, the AFLP, was examined for identifying genetic markers spread over the complete genome of *C. oncophora*. In chapter 3 a conserved gene of the mt genome was studied for the presence of genetic variation. The identified variation was subsequently compared for a laboratory maintained population and a field population of *C. oncophora*. The useful polymorphisms found in the mt gene studied in chapter 3 led to the decision to characterize the complete mt genome of *C. oncophora*. The variation found in the complete mt genome of *C. oncophora* is described in chapter 4.

A selection of the genetic diversity found in chapter 4 was used for large scale population analysis of two populations of *C. oncophora* in an attempt to characterize the effect of host immunity on the parasite population. One population was the laboratory maintained population, the other was a population derived from a serial passage experiment for nine generations in primed animals. The results of this study are presented in chapter 5. Finally in chapter 6 the findings for application of the different markers is summarized and discussed.

REFERENCES

Aboobaker A.A. and Blaxter M.L., (2003) Use of RNA interference to investigate gene function in the human filarial nematode parasite *Brugia malayi*. *Mol Biochem Parasitol*, 129; 41-51.

Adams D.B., (1988) Infection with *Haemonchus contortus* in sheep and the role of adaptive immunity in selection of the parasite. *Int J Parasitol*, 18; 1071-1075.

Agneessens J., Claerebout E., Dorny P., Borgsteede F.H.M. and Vercruysse J., (2000) Nematode parasitism in adult dairy cows in Belgium. *Vet Parasitol*, 90; 83-92.

Albers G.A. and Burgess S.K., (1988) Serial passage of *Haemonchus contortus* in resistant and susceptible sheep. *Vet Parasitol*, 28; 303-306.

Anderson T.J., Romero-Abal M.E. and Jaenike J., (1993) Genetic structure and epidemiology of *Ascaris* populations: patterns of host affiliation in Guatemala. *Parasitology*, 107; 319-334.

Anderson T.J., Romero-Abal M.E. and Jaenike J., (1995) Mitochondrial DNA and *Ascaris* microepidemiology: the composition of parasite populations from individual hosts, families and villages. *Parasitology*, 110; 221-229.

Anderson T.J. and Jaenike J., (1997) Host specificity, evolutionary relationships and macrogeographic differentiation among *Ascaris* populations from humans and pigs. *Parasitology*, 115; 325-342.

Armour J., (1989) The influence of host immunity on the epidemiology of trichostrongyle infections in cattle. *Vet Parasitol*, 32; 5-19.

Avise J.C., Bowen B.W., Lamb T., Meylan A.B. and Bermingham E., (1992) Mitochondrial DNA evolution at a turtle's pace: evidence for low genetic variability and reduced microevolutionary rate in the Testudines. *Mol Biol Evol*, 9; 457-473.

Balic A., Bowles V.M. and Meeusen E.N., (2000) The immunobiology of gastrointestinal nematode infections in ruminants. *Adv Parasitol*, 45; 181-241.

Bishop S.C. and Stear M.J., (2001) Inheritance of faecal egg counts during early lactation in Scottish Blackface ewes facing mixed, natural nematode infections. *Anim Sci*, 73; 389-395.

Blaxter M.L., Page A.P., Rudin W. and Maizels R.M., (1992) Nematode surface coats: actively evading immunity. *Parasitol Today*, 8; 243-247.

Blaxter M.L., De Ley P., Garey J.R., Liu L.X., Scheldeman P., Vierstraete A., Vanfleteren J.R., Mackey L.Y., Dorris M., Frisse L.M., Vida J.T. and Thomas W.K., (1998) A molecular evolutionary framework for the phylum Nematoda. *Nature*, 392; 71-75.

Blouin M.S., Dame J.B., Tarrant C.A. and Courtney C.H., (1992) Unusual population genetics of a parasitic nematode; mtDNA variation within and among populations. *Evolution*, 46; 470-476.

Blouin M.S., Yowell C.A., Courtney C.H. and Dame J.B., (1995) Host movement and the genetic structure of populations of parasitic nematodes. *Genetics*, 141; 1007-1014.

Blouin M.S., Liu J. and Berry R.E., (1999) Life cycle variation and the genetic structure of nematode populations. *Heredity*, 83; 253-259.

Borgsteede F.H.M., (1977) The epidemiology of gastro-intestinal helminth infections in young cattle in the Netherlands. *PhD Thesis, Utrecht, The Netherlands*,

Borgsteede F.H.M., Tibben J., Cornelissen J.B., Agneessens J. and Gaasenbeek C.P., (2000) Nematode parasites of adult dairy cattle in the Netherlands. *Vet Parasitol*, 89; 287-296.

Brooks D.R. and Isaac R.E., (2002) Functional genomics of parasitic worms: the dawn of a new era. *Parasitol Int*, 51; 319-325.

Brunsdon R.V., (1980) Principles of helminth control. *Vet Parasitol*, 6; 185-215.

Chilton N.B., Gasser R.B. and Beveridge I., (1995) Differences in a ribosomal DNA sequence of morphologically indistinguishable species within the *Hypodontus macropi* complex (Nematoda: Strongyloidea). *Int J Parasitol*, 25; 647-651.

Chilton N.B., Newton L.A., Beveridge I. and Gasser R.B., (2001) Evolutionary relationships of trichostrongyloid nematodes (Strongylida) inferred from ribosomal DNA sequence data. *Mol Phylogenet Evol*, 19; 367-386.

Clayton D.A., (1982) Replication of animal mitochondrial DNA. *Cell*, 28; 693-705.

Coop R.L., Sykes A.R. and Angus K.W., (1979) The pathogenicity of daily intake of *Cooperia oncophora* larvae in growing calves. *Vet Parasitol*, 5; 261-269.

Coop R.L. and Kyriazakis I., (2001) Influence of host nutrition on the development and consequences of nematode parasitism in ruminants. *Trends Parasitol*, 17; 325-330.

Dame J.B., Yowell C.A., Courtney C.H. and Lindgren W.G., (1991) Cloning and characterization of the ribosomal RNA gene repeat from *Ostertagia ostertagi*. *Mol Biochem Parasitol*, 45; 275-280.

Dobson C. and Tang J.M., (1991) Genetic variation and host-parasite relations: *Nematospiroides dubius* in mice. *J Parasitol*, 77; 884-889.

Dobson R.J., Barnes E.H., Birclijin S.D. and Gill J.H., (1992) The survival of *Ostertagia circumcincta* and *Trichostrongylus colubriformis* in faecal culture as a source of bias in apportioning egg counts to worm species. *Int J Parasitol*, 22; 1005-1008.

Dobson R.J., LeJambre L. and Gill J.H., (1996) Management of anthelmintic resistance: inheritance of resistance and selection with persistent drugs. *Int J Parasitol*, 26; 993-1000.

Dorris M., De Ley P. and Blaxter M.L., (1999) Molecular analysis of nematode diversity and the evolution of parasitism. *Parasitol Today*, 15; 188-193.

Durette-Desset M.C., Beveridge I. and Spratt D.M., (1994) The origins and evolutionary expansion of the Strongylida (Nematoda). *Int J Parasitol*, 24; 1139-1165.

Eady S.J., Woolaston R.R. and Barger I.A., (2003) Comparison of genetic and nongenetic strategies for control of gastrointestinal nematodes of sheep. *Livest Prod Sci*, 81; 11-23.

Elard L., Cabaret J. and Humbert J.F., (1999) PCR diagnosis of benzimidazole-susceptibility or -resistance in natural populations of the small ruminant parasite, *Teladorsagia circumcincta*. *Vet Parasitol*, 80; 231-237.

Eysker M., van der Aar W.M., Boersema J.H., Githiori J.B. and Kooyman F.N., (1998) The effect of repeated moves to clean pasture on the build up of gastrointestinal nematode infections in calves. *Vet Parasitol*, 76; 81-94.

Eysker M. and Ploeger H.W., (2000) Value of present diagnostic methods for gastrointestinal nematode infections in ruminants. *Parasitology*, 120 Suppl; S109-119.

Fiel C.A., Saumell C.A., Steffan P.E. and Rodriguez E.M., (2001) Resistance of *Cooperia* to ivermectin treatments in grazing cattle of the Humid Pampa, Argentina. *Vet Parasitol*, 97; 211-217.

Fisher M.C. and Viney M.E., (1996) Microsatellites of the parasitic nematode *Strongyloides ratti*. *Mol Biochem Parasitol*, 80; 221-224.

Gasbarre L.C., Leighton E.A. and Sonstegard T., (2001) Role of the bovine immune system and genome in resistance to gastrointestinal nematodes. *Vet Parasitol*, 98; 51-64.

Gasser R.B., Zhu X., Beveridge I. and Chilton N., (2001) Mutation scanning analysis of sequence heterogeneity in the second internal transcribed spacer (rDNA) within some members of the *Hypodontus macropi* (Nematoda: Strongyloidea) complex. *Electrophoresis*, 22; 1076-1085.

Gordon H.M. and Whitlock A.V., (1939) A new technique for counting nematode eggs in sheep faeces. *J Coun Sci Ind Res Aust*, 12; 50-52.

Gouy de Bellocq J., Ferte H., Depaquit J., Justine J.L., Tillier A. and Durette-Desset M.C., (2001) Phylogeny of the Trichostrongylina (Nematoda) inferred from 28S rDNA sequences. *Mol Phylogenet Evol*, 19; 430-442.

Grant W.N. and Whitington G.E., (1994) Extensive DNA polymorphism within and between two strains of *Trichostrongylus colubriformis*. *Int J Parasitol*, 24; 719-725.

Grant W.N. and Mascord L.J., (1996) Beta-tubulin gene polymorphism and benzimidazole resistance in *Trichostrongylus colubriformis*. *Int J Parasitol*, 26; 71-77.

Hawkins J.A., (1993) Economic benefits of parasite control in cattle. *Vet Parasitol*, 46; 159-173.

Henriksen S.A., (1981) Infections with *Cooperia oncophora* in calves. In: Current topics in veterinary medicine and animal science, P. Nansen, R. J. Jorgensen and E. J. L. Soulsby (eds), 9; 547-555.

Hillis D.M. and Dixon M.T., (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol*, 66; 411-453.

Hoekstra R., Criado-Fornelio A., Fakkeldij J., Bergman J. and Roos M.H., (1997) Microsatellites of the parasitic nematode *Haemonchus contortus*: polymorphism and linkage with a direct repeat. *Mol Biochem Parasitol*, 89; 97-107.

Hoste H., Chilton N.B., Beveridge I. and Gasser R.B., (1998) A comparison of the first internal transcribed spacer of ribosomal DNA in seven species of Trichostrongylus (Nematoda: Trichostrongylidae). *Int J Parasitol*, 28; 1251-1260.

Hu M., Chilton N.B. and Gasser R.B., (2002a) The mitochondrial genomes of the human hookworms, *Ancylostoma duodenale* and *Necator americanus* (Nematoda: Secernentea). *Int J Parasitol*, 32; 145-158.

Hu M., Chilton N.B., Zhu X. and Gasser R.B., (2002b) Single-strand conformation polymorphism-based analysis of mitochondrial cytochrome c oxidase subunit 1 reveals significant substructuring in hookworm populations. *Electrophoresis*, 23; 27-34.

Hu M., Hoglund J., Chilton N.B., Zhu X. and Gasser R.B., (2002c) Mutation scanning analysis of mitochondrial cytochrome c oxidase subunit 1 reveals limited gene flow among bovine lungworm subpopulations in Sweden. *Electrophoresis*, 23; 3357-3363.

Hussein A.S., Kichenin K. and Selkirk M.E., (2002) Suppression of secreted acetylcholinesterase expression in *Nippostrongylus brasiliensis* by RNA interference. *Mol Biochem Parasitol*, 122; 91-94.

Hyman B.C. and Azevedo J.L., (1996) Similar evolutionary patterning among repeated and single copy nematode mitochondrial genes. *Mol Biol Evol*, 13; 221-232.

Janssen P., Coopman R., Huys G., Swings J., Bleeker M., Vos P., Zabeau M. and Kersters K., (1996) Evaluation of the DNA fingerprinting method AFLP as an new tool in bacterial taxonomy. *Microbiology*, 142; 1881-1893.

Jarrett W.F.H., Jennings F.W., McIntyre W.I.M., Mulligan W. and Sharp N.C.C., (1959) Studies on immunity to *Haemonchus contortus* infection-vaccination of sheep using a single dose of X-irradiated larvae. *Am J Vet Res*, 20; 527-531.

Keddie E.M., Higazi T. and Unnasch T.R., (1998) The mitochondrial genome of *Onchocerca volvulus*: sequence, structure and phylogenetic analysis. *Mol Biochem Parasitol*, 95; 111-127.

Kloosterman A., Ploeger H.W. and Frankena K., (1991) Age resistance in calves to *Ostertagia ostertagi* and *Cooperia oncophora*. *Vet Parasitol*, 39; 101-113.

Kloosterman A., Parmentier H.K. and Ploeger H.W., (1992) Breeding cattle and sheep for resistance to gastrointestinal nematodes. *Parasitol Today*, 8; 330-335.

Kohler P., (2001) The biochemical basis of anthelmintic action and resistance. *Int J Parasitol*, 31; 336-345.

Le Jambre L.F., (1993) Molecular variation in trichostrongylid nematodes from sheep and cattle. *Acta Trop*, 53; 331-343.

Le T.H., Blair D. and McManus D.P., (2000) Mitochondrial genomes of human helminths and their use as markers in population genetics and phylogeny. *Acta Trop*, 77; 243-256.

Leighton E.A., Murrell K.D. and Gasbarre L.C., (1989) Evidence for genetic control of nematode egg-shedding rates in calves. *J Parasitol*, 75; 498-504.

Levinson G. and Gutman G.A., (1987) Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Mol Biol Evol*, 4; 203-221.

Lizotte-Waniewski M., Tawe W., Guiliano D.B., Lu W., Liu J., Williams S.A. and Lustigman S., (2000) Identification of potential vaccine and drug target candidates by expressed sequence tag analysis and immunoscreening of *Onchocerca volvulus* larval cDNA libraries. *Infect Immun*, 68; 3491-3501.

Maizels R.M., Bundy D.A., Selkirk M.E., Smith D.F. and Anderson R.M., (1993) Immunological modulation and evasion by helminth parasites in human populations. *Nature*, 365; 797-805.

Martin P.J., Anderson N. and Jarrett R.G., (1989) Detecting benzimidazole resistance with faecal egg count reduction tests and in vitro assays. *Aust Vet J*, 66; 236-240.

McKellar Q.A., (1997) Ecotoxicology and residues of anthelmintic compounds. *Vet Parasitol*, 72; 413-426; discussion 426-435.

McManus D.P. and Bowles J., (1996) Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. *Int J Parasitol*, 26; 687-704.

Mes T.H., (2003) Demographic expansion of parasitic nematodes of livestock based on mitochondrial DNA regions that conflict with the infinite-sites model. *Mol Ecol*, 12; 1555-1566.

Michel J.F., (1976) The epidemiology and control of some nematode infections in grazing animals. *Adv Parasitol*, 14; 355-397.

Morris C.A., Green R.S., Cullen N.G. and Hickey S.M., (2003) Genetic and phenotypic relationships among faecal egg count, anti-nematode antibody level and live weight in Angus cattle. *Anim Sci*, 76; 167-174.

Mueller U.G. and Wolfenbarger L.L., (1999) AFLP genotyping and fingerprinting. *Trends Ecol Evol*, 14; 389-394.

Mulligan W., Gordon H.M., Stewart D.F. and Wagland B.M., (1961) The use of irradiated larvae as immunising agents in *Haemonchus contortus* and *Trichostrongylus colubriformis* infections of sheep. *Aust J Agr Res*, 12; 1175-1187.

Nadler S.A., (1992) Phylogeny of some ascaridoid nematodes, inferred from comparison of 18S and 28S rRNA sequences. *Mol Biol Evol*, 9; 932-944.

Newton L.A., Chilton N.B., Beveridge I., Hoste H., Nansen P. and Gasser R.B., (1998) Genetic markers for strongylid nematodes of livestock defined by PCR-based restriction analysis of spacer rDNA. *Acta Trop*, 69; 1-15.

Okimoto R., Macfarlane J.L., Clary D.O. and Wolstenholme D.R., (1992) The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. *Genetics*, 130; 471-498.

Otsen M., Plas M.E., Lenstra J.A., Roos M.H. and Hoekstra R., (2000) Microsatellite diversity of isolates of the parasitic nematode *Haemonchus contortus*. *Mol Biochem Parasitol*, 110; 69-77.

Peacock R. and Poynter D., (1980) Field experience with a bovine lungworm vaccine. *Symp Brit Soc Parasitol*, 18; 141.

Perry B.D. and Randolph T.F., (1999) Improving the assessment of the economic impact of parasitic diseases and of their control in production animals. *Vet Parasitol*, 84; 145-168.

Poot J., Kooyman F.N., Dop P.Y., Schallig H.D., Eysker M. and Cornelissen A.W., (1997) Use of cloned excretory/secretory low-molecular-weight proteins of *Cooperia oncophora* in a serological assay. *J Clin Microbiol*, 35; 1728-1733.

Poynter D., (1963) Parasitic bronchitis. *Adv Parasitol*, 1; 179-212.

Rollinson D., Kaukas A., Johnston D.A., Simpson A.J. and Tanaka M., (1997) Some molecular insights into schistosome evolution. *Int J Parasitol*, 27; 11-28.

Roos M.H., (1990) The molecular nature of benzimidazole resistance in helminths. *Parasitol Today*, 6; 125-127.

Roos M.H., (1995) New genetic and practical implications of selection for anthelmintic resistance in parasitic nematodes. *Parasitol Today*, 11; 148-150.

Saiki R.K., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., Horn G.T., Mullis K.B. and Erlich H.A., (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239; 487-491.

Sangster N.C., (1999) Anthelmintic resistance: past, present and future. *Int J Parasitol*, 29; 115-124; discussion 137-138.

Sangster N.C. and Gill J., (1999) Pharmacology of anthelmintic resistance. *Parasitol Today*, 15; 141-146.

Sangster N.C., Batterham P., Chapman H.D., Duraisingh M., Le Jambre L., Shirley M., Upcroft J. and Upcroft P., (2002) Resistance to antiparasitic drugs: the role of molecular diagnosis. *Int J Parasitol*, 32; 637-653.

Schnieder T., Heise M. and Epe C., (1999) Genus-specific PCR for the differentiation of eggs or larvae from gastrointestinal nematodes of ruminants. *Parasitol Res*, 85; 895-898.

Sen H.G. and Seth D., (1970) Development of *Necator americanus* in golden hamsters *Mesocricetus auratus*. *Indian J Med Res*, 58; 1356-1360.

Shoop W.L., Haines H.W., Michael B.F. and Eary C.H., (1993) Mutual resistance to avermectins and milbemycins: oral activity of ivermectin and moxidectin against ivermectin-resistant and susceptible nematodes. *Vet Rec*, 133; 445-447.

Solomon G.B. and Haley A.J., (1966) Biology of the rat nematode *Nippostrongylus brasiliensis* (Travassos, 1914) V. Charateristics of *N. brasiliensis* after serial passage in the laboratory mouse. *J Parasitol*, 52; 237-241.

Sonstegard T.S. and Gasbarre L.C., (2001) Genomic tools to improve parasite resistance. *Vet Parasitol*, 101; 387-403.

Sorensen E., Bogh H.O., Johansen M.V. and McManus D.P., (1999) PCR-based identification of individuals of *Schistosoma japonicum* representing different subpopulations using a genetic marker in mitochondrial DNA. *Int J Parasitol*, 29; 1121-1128.

Stafford K. and Coles G.C., (1999) Nematode control practices and anthelmintic resistance in dairy calves in the south west of England. *Vet Rec*, 144; 659-661.

Stear M.J., Hetzel D.J., Brown S.C., Gershwin L.J., Mackinnon M.J. and Nicholas F.W., (1990) The relationships among ecto- and endoparasite levels, class I antigens of the bovine major histocompatibility system, immunoglobulin E levels and weight gain. *Vet Parasitol*, 34; 303-321.

Su Z. and Dobson C., (1997) Genetic and immunological adaptation of *Heligmosomoides polygyrus* in mice. *Int J Parasitol*, 27; 653-663.

Tarrant C.A., Blouin M.S., Yowell C.A. and Dame J.B., (1992) Suitability of mitochondrial DNA for assaying interindividual genetic variation in small helminths. *J Parasitol*, 78; 374-378.

Tautz D., (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res*, 17; 6463-6471.

The *C. elegans* Sequencing Consortium, (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science*, 282; 2012-2018.

Thompson R.C. and Lymbery A.J., (1996) Genetic variability in parasites and host-parasite interactions. *Parasitology*, 112 Suppl; S7-22.

Vercruysse J. and Claerebout E., (2001) Treatment vs non-treatment of helminth infections in cattle: defining the threshold. *Vet Parasitol*, 98; 195-214.

Vermunt J.J., West D.M. and Pomroy W.E., (1995) Multiple resistance to ivermectin and oxfendazole in *Cooperia* species of cattle in New Zealand. *Vet Rec*, 137; 43-45.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J. and Kuiper M., (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res*, 23; 4407-4414.

Wakelin D. and Goyal P.K., (1996) *Trichinella* isolates: parasite variability and host responses. *Int J Parasitol*, 26; 471-481.

Waller P.J., (1997a) Anthelmintic resistance. *Vet Parasitol*, 72; 391-405; discussion 405-412.

Waller P.J., (1997b) Sustainable helminth control of ruminants in developing countries. *Vet Parasitol*, 71; 195-207.

Wescott R.B. and Todd A.C., (1966) Adaptation of *Nippostrongylus brasiliensis* to the mouse. *J Parasitol*, 52; 233-236.

Windon R.G., (1996) Genetic control of resistance to helminths in sheep. *Vet Immunol Immunopathol*, 54; 245-254.

Wolstenholme D.R., (1992a) Genetic novelties in mitochondrial genomes of multicellular animals. *Curr Opin Genet Dev*, 2; 918-925.

Wolstenholme D.R., (1992b) Animal mitochondrial DNA: structure and evolution. *Int Rev Cytol*, 141; 173-216.

Woolaston R.R., Elwin R.L. and Barger I.A., (1992) No adaptation of *Haemonchus contortus* to genetically resistant sheep. *Int J Parasitol*, 22; 377-380.

Zarlenga D.S., Barry Chute M., Gasbarre L.C. and Boyd P.C., (2001) A multiplex PCR assay for differentiating economically important gastrointestinal nematodes of cattle. *Vet Parasitol*, 97; 199-209.

CHAPTER TWO

GENETIC INTRA POPULATION VARIATION, REVEALED BY AMPLIFIED FRAGMENT LENGTH POLYMORPHISM, WITHIN A POPULATION OF THE TRICHOSTRONGYLID NEMATODE *COOPERIA ONCOPHORA*

Reprinted from Experimental parasitology, Vol 104, Van der Veer and de Vries, pp 70-73,
Copyright 2003, with permission from Elsevier

Cooperia oncophora, a parasitic gastrointestinal nematode of cattle, belongs to the superfamily of Trichostrongyloidea. Extensive genetic variation, detected within different trichostrongylid nematode populations (Nadler 1990; Thompson and Lymbery 1990), is an important parameter in the ability of a population to respond to selective pressure and the changing conditions that are often met by parasitic nematodes (Le Jambre 1993; Grant 1994). For instance, the development of benzimidazole resistance has been shown to be linked to genetic variation (Roos 1990; Kwa *et al.* 1994).

In our laboratory we are selecting parasite lines, by repeated passage through the host, to analyse the effect of host immunity on the genetic structure of the *C. oncophora* population. Genetic marker systems are needed to analyse selection. AFLP is a fingerprinting technique based on the detection of restriction fragments by selective PCR amplification (Vos *et al.* 1995) and retrieves information on DNA regions distributed randomly throughout the genome without the need for prior knowledge of the target genome (Vos *et al.* 1995; Janssen *et al.* 1996). In *Haemonchus contortus* the AFLP technique proved to be successful in determining the level of polymorphism (Otsen *et al.* 2001). Here the technique was applied in a first attempt to characterize the population genetics of *C. oncophora*.

Fig. 1 shows the AFLP fingerprinting patterns from four individuals with five different primer combinations. Digestion with *Eco*RI and *Mse*I, ligation and preamplification yielded DNA fragments in a size range from 50-2000 bp on an agarose gel.

Assuming a genome size for *C. oncophora* of 100 Mb and an AT content of 64% like for *Caenorhabditis elegans* (The *C. elegans* Sequencing Consortium 1998) predicts about 34,000 *Eco*RI restriction sites. Thus the selective nucleotides used (1 in pre-amplification and 2 in second amplification for all primers) expected to amplify 13 to 41 labelled fragments. A higher number of bands was actually observed (Fig. 1), ranging from 29 in lane 1 to 68 bands in lane 16 (Table 1), which is comparable to the approximately 25 bands that were found in a smaller selection of the gel of an AFLP performed on *H. contortus* under equally selective conditions (Otsen *et al.* 2001).

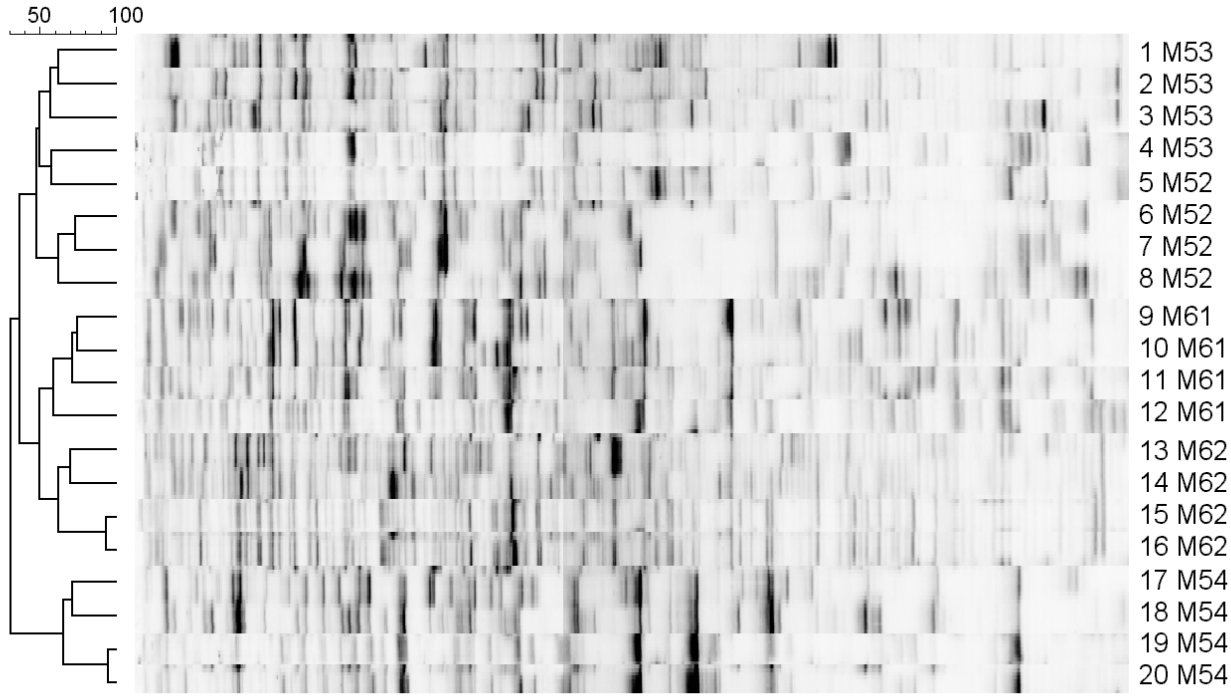

Figure 1. Dendrogram based upon representative 100-500bp part of AFLP fingerprinting patterns from four *Cooperia oncophora* individuals using the same *Eco*RI primer E34 in combination with different *Mse*I primers. The mean Pearson correlation coefficient is expressed as percentage similarity.

Adult *C. oncophora* individuals were obtained from the intestine of a three months old Holstein Friesian calf 34 days after infection with 100,000 infective (L_3) larvae. A 200 ng sample of genomic DNA from individual worms, or cDNA from 10 pooled individuals, was restricted with 5 U *Eco*RI (Life technologies) and 5 U *Mse*I (New England Biolabs Ltd.) for 2h at 37 °C. Specific *Eco*RI adapters (5' CTCGTAGACTGCGTACC 3' and 5' AATTGGTACGCAGTCTAC 3') and *Mse*I adapters (5' GACGATGAGTCCTGAG 3' and 5' TACTCAGGACTCAT 3'), were added and ligated to the restriction fragments by 1 U T4 DNA ligase (Life technologies) for an additional 3h at 37 °C. The first step of the two-step selective amplification is selective amplification of the adapter ligated restriction fragments with the specific *Eco*RI and *Mse*I primers E01 5' AGACTGCGTACCAATTCA 3' and M02 5' GATGAGTCCTGAGTAAC 3' having one single selective nucleotide (underlined) each. PCR conditions (annealing temperature, number of cycles, template concentration) of experiments shown in Fig. 1 to 3 were experimentally optimized for maximal reproducibility. Performing the AFLP protocol on each DNA or cDNA sample in duplo usually resulted in equal banding patterns. The following cycling conditions were used: 30 cycles each of 30s denaturating at 94 °C, 60s annealing at 56°C and 60s extension at 72 °C. 5 µl of the amplicon was checked on an 1% agarose gel. The remaining amplicon was diluted 1:9 in double-distilled water of which 5 µl was used as template in the second selective amplification. This amplification was performed with an [γ-^{33}P]ATP (3,000 Ci/mmol, Amersham) labelled *Eco*RI primer (E34 5' GACTGCGTACCAATTCAAT 3' or E38 5' GACTGCGTACCAATTCACT 3') having two additional selective nucleotides, in combination with an unlabeled *Mse*I primer, having two selective nucleotides, M52 (5' GATGAGTCCTGAGTAACCC 3'), M53 (5'

GATGAGTCCTGAGTAACCG 3'), M54 (5' GATGAGTCCTGAGTAACCT 3'), M61 (5' GATGAGTCCTGAGTAACTG 3') or M62 (5' GATGAGTCCTGAGTAACTT 3'). The following cycling conditions were used: 13 cycles each of 30s at 94°C, 30s at 65°C lowering each cycle with 0.7°C and 60s at 72°C followed by 23 cycles of 30s at 94°C, 30s at 56°C and 60s at 72°C. The amplicon was mixed 1:1 with formamide dye and denaturated 5 min at 95°C. 3 µl of the sample was loaded on a 6% polyacrylamide gel and electrophoresed in 1x TBE buffer at 80W for 2,5 hour. The gel was dried and exposed for at least 24 h to an x-ray film (Fuji). The AFLP fingerprint patterns were analyzed using the curve-based protocol of the Pearson product-moment correlation coefficient (*r*) allowing direct comparison of whole densitometric curves hereby indicating the relationship between two ordered sets of numbers. It indicates both how the two sets are related and the strength of that relationship (Hane *et al.* 1993). In terms of fingerprints the value 1 indicates a perfect correlation (100%) and the value 0 indicates no correlation at all. Grouping of the AFLP patterns by the Pearson correlation cluster analysis were performed using the Bionumerics software (Applied Maths BVBA, Kortrijk, Belgium). The relationships between individual worms were visualized as dendrograms.

Table 1. Number of unique bands and total bands for each AFLP fingerprint pattern for all used primer combinations and the average (percentage) unique bands for each combination.

	Primer combination										
Lane		1	2	3	4	Average	%				
Unique	E34/M53	15	15	14	19	15.75	40.13				
Total bands		29	35	41	42	39.25					
Lane		5	6	7	8						
Unique	E34/M52	17	19	17	20	18.25	42.69				
Total bands		42	42	39	48	42.75					
Lane		9	10	11	12						
Unique	E34/M61	13	12	13	27	16.25	31.10				
Total bands		54	46	52	57	52.25					
Lane		13	14	15	16						
Unique	E34/M62	13	17	9	12	12.75	44.21				
Total bands		56	56	53	68	58.25					
Lane		17	18	19	20						
Unique	E34/M54	22	15	13	14	16	38.79				
Total bands		46	46	30	43	41.25					
Lane		1	2	3	4	5	6	7	8	Average	%
Unique	E38/M52	14	8	10	15	12	7	1	16	12.13	30.90
Total bands		42	33	36	38	42	35	5	42	39.25	
Unique	E38/M54	6	8	10	7	14	11	1	9	9.75	20
Total bands		37	35	48	59	51	55	3	50	48.75	
Dilution (Fig.3)		-	1:1	1:2	1:9	Average	%				
Unique	E34/M52	4	4	3	4	3.75	16.30				
Total bands	(female)	26	26	20	20	23					
Unique	E34/M52	1	2	4	5	3	11.77				
Total bands	(male)	21	24	28	29	25.5					

The number of bands unique to an individual worm as compared to the band patterns obtained with the same specific primer combination on other individuals (Table 1) indicated a high degree of variation within the population. However, scoring of unique bands by eye in the complex patterns obtained, introduces a degree of subjectivity that is less suitable for quantitative analysis. Therefore, a method was used in which full densitometric curves of each lane were corrected for background and shifted to obtain maximal similarity contour pairs (Hane *et al.* 1993). Dendrograms based on these correlations show that percentages of similarity between individuals with the same primer combination had a high degree of variation within the population. For example, in Fig. 1 the E34/M54 (lane 17-20) primer combination gave the closest clustering results. Although the similarity between individuals in lane 19 and 20 is 94%, between lane 19 and 18 is only 58%. Although Pearson correlation in most cases clustered together the individuals that were tested with the same primer combination, in one occasion (lanes 4 and 5), due to low overall similarities, this distinction could not be made.

The use of a more selective primer set (E38 with M52 or M54) on eight different individuals resulted in patterns showed in Fig. 2. Although the amount of unique and total bands was lower than in Fig.1 (Table 1) the obtained patterns had less similarity among individuals as compared to Fig. 1. For the E38/M52 primer combination variation ranged from 29 to 73%, while for E38/M54 variation ranged from 31 to 68%.

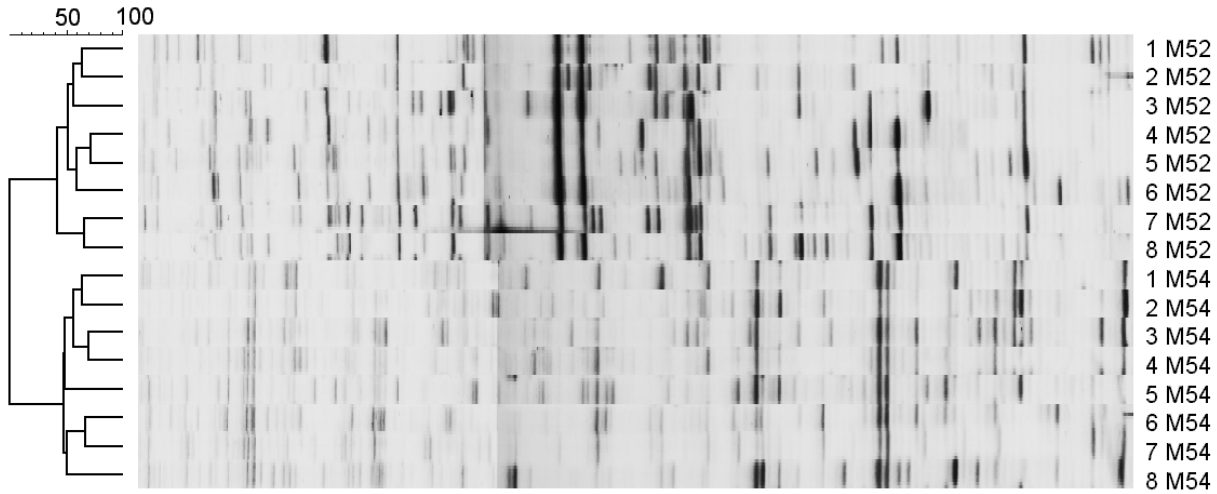

Figure 2. Dendrogram based upon representative 100-500bp part of AFLP fingerprinting patterns from eight *Cooperia oncophora* individuals (ln 1-8) using the same *Eco*RI primer E38 in combination with two different *Mse*I primers M52 and M54. The Pearson correlation coefficient is expressed as percentage.

Recently, the use of AFLP has been recommended as the prime method for the genetic analysis of animal parasitic nematodes (Grant 2001). However, the observed high degree of polymorphism between *C. oncophora* individuals within the same population makes the use of pooled individuals not suited for comparing different populations of *C. oncophora*. Whereas the use of single worms would require the analysis of an unacceptable high number of individuals. It was demonstrated (Otsen *et al.* 2001) that despite selection for drug resistance in the gastrointestinal nematode *H. contortus*, no decrease of genetic diversity was seen. The variation found within isolates was as high as between related mammalian species. AFLP was also performed on cDNA as the coding regions might be less variable but are supposed to be under a higher selective pressure. The use of cDNA-AFLP has already been successfully applied in plants, identifying differentially expressed genes (Bachem *et al.* 1996; Money *et al.* 1996). The cDNA-AFLP of *C. oncophora* showed indeed less variability (Fig. 3) but is less efficient and therefore requiring groups of ten individuals to be used.

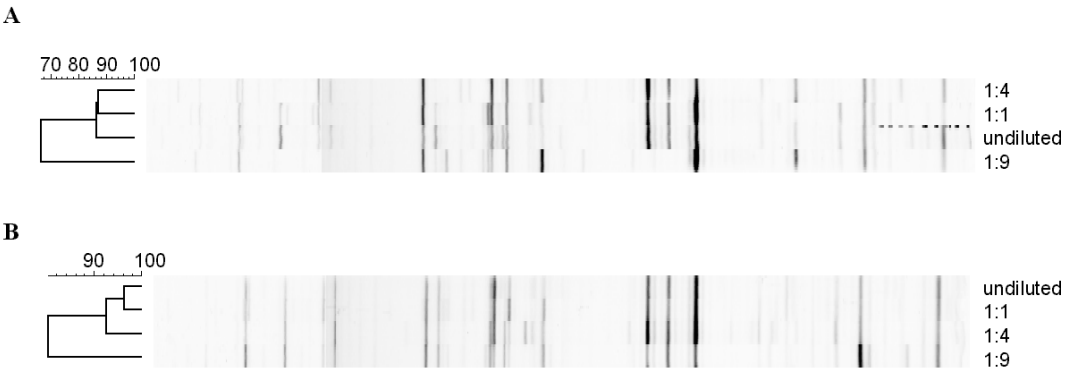

Figure 3. Dendrogram based upon representative 100-500bp part of cDNA-AFLP patterns (primer combination E34 and M52) from a group of 10 individuals with different dilutions of the digestion/ligation sample. The Pearson correlation coefficient is expressed as percentage. Panel A: group of 10 female, panel B: group of 10 male adult worms. Dilution is given on the right next to each lane. RNA used for cDNA-AFLP was extracted with the RNAzol RNA isolation kit (Campro), according to the supplier's protocol. First and second strand cDNA synthesis was carried out according to standard protocols (Sambrook *et al.* 1989). The resulting cDNA was phenol-extracted, ethanol precipitated, taken up in 20 µl double-distilled water, and used in the AFLP protocol similar as described at Fig. 1.

A major problem, encountered in repeated experiments, of the cDNA-AFLP is the dependence of the banding patterns on the amount of mRNA used in the selective amplification. Pearson correlation analysis demonstrated that for both experiments (male and female) the most diluted sample (1:9) had the least similarity with the other samples. The remaining three dilutions tested established an overall variation in banding pattern of 20% for the female group and 15% for the male group. AFLP has been shown to be sensitive to excessive template dilutions (Vos *et al.* 1995). Because it is impossible to standardise the amount of mRNA from a small pool of worms, the inconsistent banding pattern of the cDNA-AFLP may be due to variation of the amount of mRNA used. Nevertheless when comparing the undiluted sample from both groups it is clear that the banding patterns differ in only one band and that the percentage unique bands has dropped to a comprehensive extent (Table 1). Therefore, this approach will be useful in identifying polymorphisms in expressed sequences between different *C. oncophora* populations using for instance the experimentally laboratory selected populations.

Since it was not possible to identify AFLP markers for typing individual *C. oncophora* worms we are now concentrating on a more definite region of the genetic subset. The mitochondrial genome will be studied to determine its usefulness as a basis for molecular genotyping *C. oncophora* individuals.

ACKNOWLEDGEMENTS

We are grateful to Dr. L.M. Schouls for analysing the AFLP patterns. We thank Dr. M. Otsen for technical support and for providing the AFLP oligo's. Prof. Dr. A.W.C.A. Cornelissen, Dr. M. Eysker and Dr. H.W. Ploeger are thanked for careful reading of the manuscript. This research was supported by the Technology Foundation STW, applied science division of NWO and the technology programme of Ministry of Economic Affairs (UDG 4889).

REFERENCES

Bachem C.W., van der Hoeven R.S., de Bruijn S.M., Vreugdenhil D., Zabeau M. and Visser R.G., (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. *Plant J*, 9; 745-753.

Grant W.N., (1994) Genetic variation in parasitic nematodes and its implications. *Int J Parasitol*, 24; 821-830.

Grant W.N., (2001) Population genetics and drug resistance in nematode. *Trends Parasitol*, 17; 410.

Hane B.G., Jager K. and Drexler H.G., (1993) The Pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. *Electrophoresis*, 14; 967-972.

Janssen P., Coopman R., Huys G., Swings J., Bleeker M., Vos P., Zabeau M. and Kersters K., (1996) Evaluation of the DNA fingerprinting method AFLP as an new tool in bacterial taxonomy. *Microbiology*, 142; 1881-1893.

Kwa M.S., Veenstra J.G. and Roos M.H., (1994) Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. *Mol Biochem Parasitol*, 63; 299-303.

Le Jambre L.F., (1993) Molecular variation in trichostrongylid nematodes from sheep and cattle. *Acta Trop*, 53; 331-343.

Money T., Reader S., Qu L.J., Dunford R.P. and Moore G., (1996) AFLP-based mRNA fingerprinting. *Nucleic Acids Res*, 24; 2616-2617.

Nadler S.A., (1990) Molecular approaches to studying helminth population genetics and phylogeny. *Int J Parasitol*, 20; 11-29.

Otsen M., Hoekstra R., Plas M.E., Buntjer J.B., Lenstra J.A. and Roos M.H., (2001) Amplified fragment length polymorphism analysis of genetic diversity of *Haemonchus contortus* during selection for drug resistance. *Int J Parasitol*, 31; 1138-1143.

Roos M.H., (1990) The molecular nature of benzimidazole resistance in helminths. *Parasitol Today*, 6; 125-127.

The *C. elegans* Sequencing Consortium, (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science*, 282; 2012-2018.

Thompson R.C.A. and Lymbery A.J., (1990) Intraspecific variation in parasites-what is a strain? *Parasitol Today*, 6; 345-348.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J. and Kuiper M., (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res*, 23; 4407-4414.

CHAPTER THREE

CYTOCHROME OXIDASE C SUBUNIT I POLYMORPHISMS SHOW SIGNIFICANT DIFFERENCES IN DISTRIBUTION BETWEEN A LABORATORY MAINTAINED POPULATION AND A FIELD ISOLATE OF *COOPERIA ONCOPHORA*

Reprinted from Veterinary Parasitology, Vol 116(3), Van der Veer, Kanobana, Ploeger and de Vries, pp 231-238, Copyright 2003, with permission from Elsevier

ABSTRACT

A 474 bp fragment of the mitochondrial cytochrome oxidase c subunit 1 (*cox*1) of *Cooperia oncophora* was cloned and sequenced. The overall nucleotide diversity of the *cox*1 fragment varied from 0.5-2.0 % between individuals. Two nucleotide substitutions were found within two *Rsa*I endonuclease restriction sites and were used in a PCR based restriction fragment length polymorphism (PCR-RFLP) assay to asses the intra population variation of *C. oncophora*. Testing 816 individuals revealed the existence of three different haplotypes, having either both (type I) or only one (type II and III) *Rsa*I site. Laboratory maintained individuals obtained at different time points after infection showed no significant difference in the distribution of the three haplotypes. Neither was there a difference in the distribution between male and female worms, confirming that the mitochondrial genome of *C. oncophora* is also maternally inherited. Nevertheless, there was a significant difference in the prevalence of the *Rsa*I point mutation in the *cox*1 gene between the laboratory maintained population of *C. oncophora* and a Dutch field isolate, indicating that these RFLPs can be used to study genetic variation within or among *C. oncophora* populations.

INTRODUCTION

Cooperia oncophora is a common intestinal parasitic nematode of cattle in temperate climates. Control of *C. oncophora* infections depends mainly on grazing management and the use of anthelmintics. The infection contributes to production losses in the dairy and beef industries.

C. oncophora belongs to the superfamily of the Trichostrongyloidea. Population genetic studies of other species of this family revealed a large amount of genetic variation (Dame *et al.* 1993; Le Jambre 1993; Grant and Whitington 1994). For mitochondrial (mt) DNA very high levels, up to 6.0 %, sequence difference within populations have been demonstrated (Blouin *et al.* 1995; Blouin 1998). Although there are a few published nuclear and mitochondrial sequence data for *C. oncophora* (Poot *et al.* 1997; Newton *et al.* 1998), the degree of genetic variation within or between *C. oncophora* populations has not been examined. Insight in the population genetics of the parasite population can contribute to understand adaptation of the parasite to the use of anthelmintics, different management conditions and to the host immune response (Grant 1994; Paterson *et al.* 2000).

In the present study a portion of the mt cytochrome c oxidase subunit 1 (*cox*1) gene of *C. oncophora* was sequenced in an attempt to identify genetic markers for studying the population genetic variation of *C. oncophora.* The protein coding *cox*1 gene was chosen since it is the most conserved gene between different nematode mt genomes (Okimoto *et al.* 1992; Hu *et al.* 2002a). Furthermore, the assumed maternal inheritance and the higher evolution rate of mt genomes make it useful for resolving population structure (Anderson *et al.* 1995). Two markers were identified and subsequently used in a PCR based restriction fragment length polymorphism (PCR-RFLP) assay, to examine the inter- and intra- genetic variation of a laboratory maintained population and a field population of *C. oncophora*.

MATERIALS AND METHODS

Cooperia oncophora isolates

The laboratory isolate originated from a field isolate in 1967 and was maintained in the laboratory by repeated passage through donor calves. Individual adult parasites were obtained from the intestine of female calves, which were three months old at day of infection with 100,000 infective larvae (L3), at respectively 21, 28 and 42 days after

infection (d.p.i.). Adult parasites from calves naturally infected on pasture, representing the field isolate, were kindly provided by dr. M. Eysker.

COXI PCR

To individual worms 5 µl lysis buffer was added (0.45% Nonidet P-40, 0.45% Tween 20, 10 mm Tris pH 8.0, 2.5 mM MgCl2, 50 mM KCl, 0.01% gelatine and 60 µg/ml Proteinase K) overlaid with paraffin oil and frozen for 15 min at –80°C. Subsequently, the samples were kept for 1 h at 60°C followed by 15 min incubation at 95°C. The lysis sample was used as template in the PCR with the following composition; 5 µl 10x Taq buffer (Promega), 3 µl 25 mM Mg^{2+}, 2 µl 2.5 mM dNTP's, 2.5 µl 20 pmol/µl forward primer, 2.5 µl 20 pmol/µl reverse primer, 0.25 µl 5 U/µl Taq Polymerase (Promega) and ddH_2O was added to a total volume of 50 µl. The conditions used during cycling were 5 min initial denaturation at 94°C followed by 40 cycli of 30 s denaturation at 94°C, 30 s annealing at 52°C and 30 s extension at 72°C, with a final cycle of 5 min at 72°C.

The sequence of the primers was based on conserved regions between the COX1 sequence of *Caenorhabditis elegans* and *Ascaris suum* (Okimoto *et al.* 1992) with forward primer (*COX1*F) 5'ATTCAGTTATTACAGCTCATGCTATTTT and reverse primer (*COX1*R) 5'TTACGATCAGTTAACAACATAGTAATAGCCC.

Cloning and sequencing

Pooled COXI amplicons were purified by phenol/chloroform extraction, ethanol precipitated and ligated into pUC-PCR plasmid, suited for direct TA-cloning of PCR products (de Vries 1998). Recombinant plasmids were electroporated (2.48 V, 25 µF, 200 Ω) into *Escherichia coli* JM105 electrocompetent cells. Transformed cultures were grown overnight on LB Amp+ plates at 37°C, plasmids were isolated from positive colonies using a miniprep kit (Amersham). Sequencing was performed on both strands with universal M13 primers.

The sequences were assembled using DNAStar software, the sequences of the partial mitochondrial *cox*1 gene of *C. oncophora* were submitted under Genbank accession numbers AY229868-AY229873.

PCR-RFLP assay

Based on the cloned *C. oncophora cox*1 sequence more stringent annealing primers were designed for the PCR-RFLP assay, designated COX1FN 5`TAATGCCTAGTATAAT(C/T)GGTGGTTT`3 and COX1RN

5`CCCAGCTAAAACAGGTAAAGATAAT`3. During each PCR, control samples were used containing no DNA as well as host DNA. The conditions used during cycling were, 5 min initial denaturation at 94°C followed by 40 cycli of 30 s denaturation at 94°C, 30 s annealing at 62°C and 30 s extension at 72°C, with a final cycle of 5 min at 72°C. 20 µl of the amplicon was used in the restriction reaction. The digestions with *Bgl*II, *Dra*I, *Hin*fI, *Msp*I, *Nci*I, *Rsa*I (Promega) and *Mbo*I (GibcoBRL) were all performed according to the manufacturer's instructions. Restriction fragments were separated on a 2% TBE-agarose gel and stained with ethidium bromide. Bands were detected upon ultraviolet transillumination and photographed.

Within the laboratory isolate distinction was made between individuals obtained at different time points after infection. Worms were collected from one calf at 21 d.p.i., from three calves (A-C) at 28 d.p.i. and from two calves (A and B) at 42 d.p.i. Collected worms were differentiated into males and females.

Statistical analysis

Statistical analysis was done with SPSS 10 package. Differences in frequencies were analysed with the non-parametric Chi-square test. Comparisons were made between haplotypes within a population and within haplotypes between male and female individuals. Statistical significance was defined at $P \leq 0.05$.

RESULTS

In order to examine the genetic diversity within *C. oncophora* populations, we cloned and sequenced a 474 bp fragment of the *cox*1 gene by amplification with the primers COXF and COXR derived from the *cox*1 sequence of *A. suum* and *C. elegans*.

The amplicon was digested with different restriction enzymes. Out of seven enzymes tested only *Rsa*I showed a polymorphism. Sequence analysis identified two *Rsa*I recognition sequences within the *cox*1 fragment. A 401 bp *cox*1 fragment, covering the two *Rsa*I sites, was used in the PCR/RFLP assay. Testing 816 individuals revealed the existence of three *cox*1 haplotypes. Fig. 1 shows the digestion of different amplicons with *Rsa*I. Designated type I had two *Rsa*I recognition sites at position 161 and 287 base pairs. Type II and type III had one recognition site at position 287 or 161, respectively. Fig. 2 shows two sequences of the three different types indicating the loss or gain of two *Rsa*I sites by C/T transition. Several nucleotide transitions were found and none of the 6 sequenced individuals had an

identical sequence. The highest sequence variation (2.0%) was observed between type IIB and IIIB of Fig. 2.

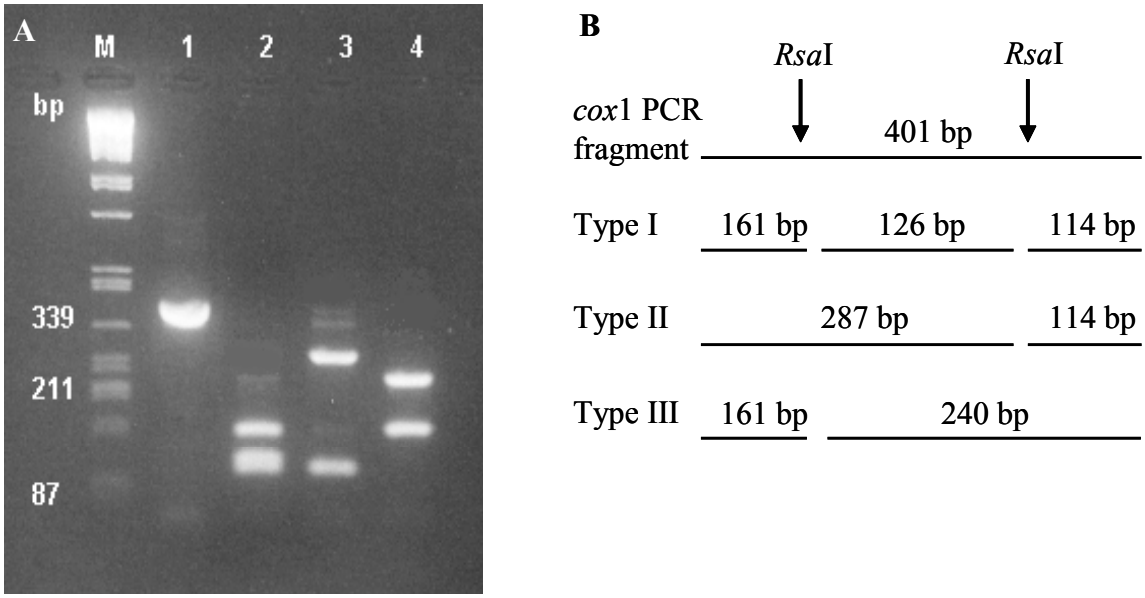

Figure 1. A; RFLP pattern of the three different *cox*1 types. Lane 1: Undigested *cox*1 PCR fragment, lane 2: type I, lane 3: type II, lane 4: type III. B; Schematic representation of the RFLP size of each fragment is given in base pairs (bp) for each type.

The goal of this research was to identify a molecular marker to study genetic variation within and between different *C. oncophora* isolates. The PCR/RFLP was used to establish if the laboratory maintained isolate compared to the field isolate had genetic differences in the distribution of the three haplotypes. *C. oncophora* is initially located in the proximal part of the duodenum whereas towards the end of the patent period worms move more towards distal parts of the intestine and individual host specific differences are observed in timing, duration and efficiency of worm expulsion (Kanobana *et al.* 2001). This raised the question whether there are genetically different subpopulations of worms that can sustain longer in their favorable habitat at the proximal part of the gut in specific individual hosts. Therefore, an initial experiment was carried out to evaluate the influences of the host and time point after infection on the distribution of the *cox*1 haplotypes within the laboratory maintained individuals. Fig. 3 shows the observed frequencies of the three types for the analysed time points after infection of the laboratory isolate. Statistical analyses showed that no significant

differences could be found in the distribution of the haplotypes, derived at different time points after infection from different hosts (A, B, C).

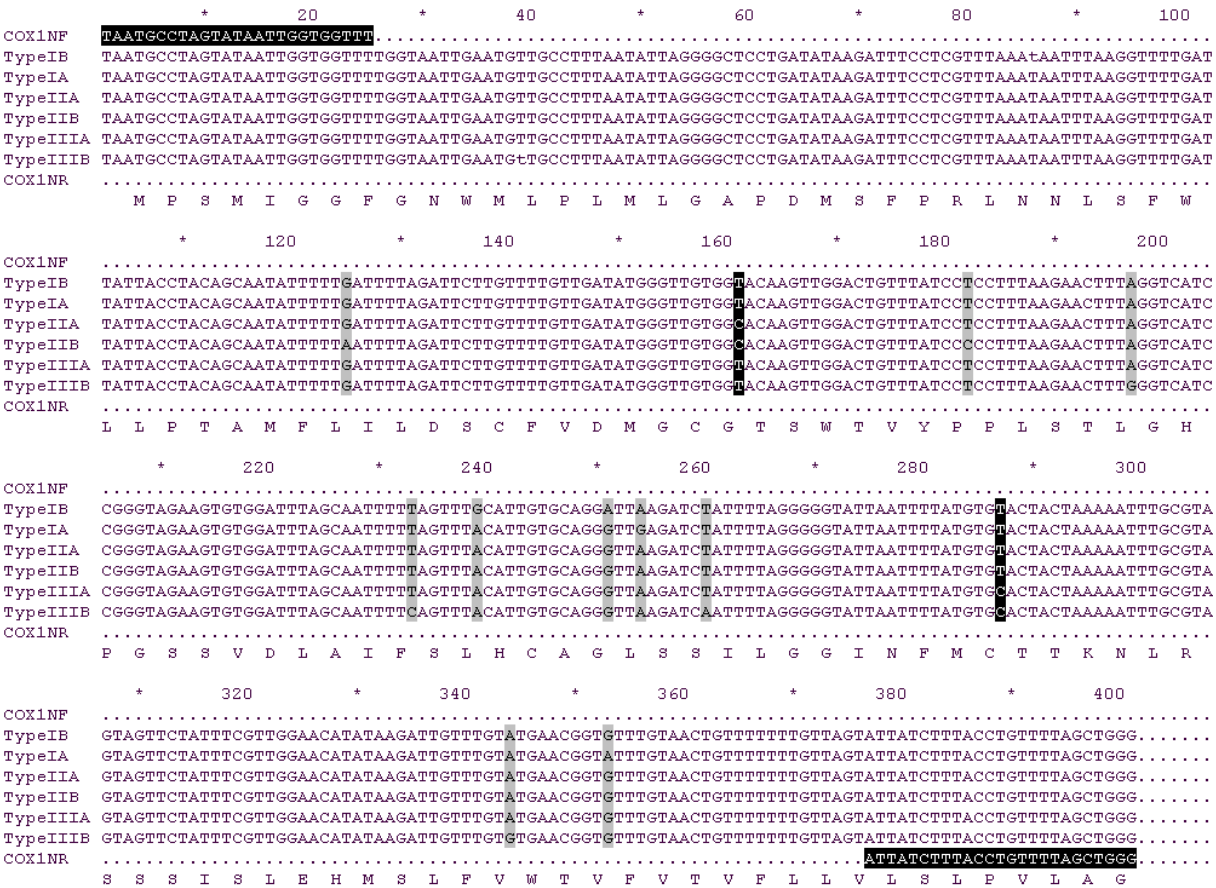

Figure 2. Alignment of the three *Rsa*I types from partial mitochondrial *cox*1 gene sequences. The sequence names indicate the different *cox*1 RFLP haplotypes (type I, II and III) as sequenced from different individuals (A and B for each type) showing other mutations (shaded in grey) in addition to the mutations causing the *Rsa*I restriction fragment polymorphism (shaded in black). The primers are indicated in black. The predicted amino acid sequence is given below the nucleotide sequences.

Mitochondrial genomes are maternally inherited (Avise *et al.* 1987) and differences between the distribution of the haplotypes between male and female individuals are not expected. This was verified here, and no significant differences in the distribution of haplotypes between males and females were observed for the field isolate and the laboratory isolate. Laboratory and field isolate were subsequently compared taking males and females together (Fig. 3). This showed a significant higher frequency of type II in the laboratory maintained isolate and of type I in the field isolate ($p<0.001$).

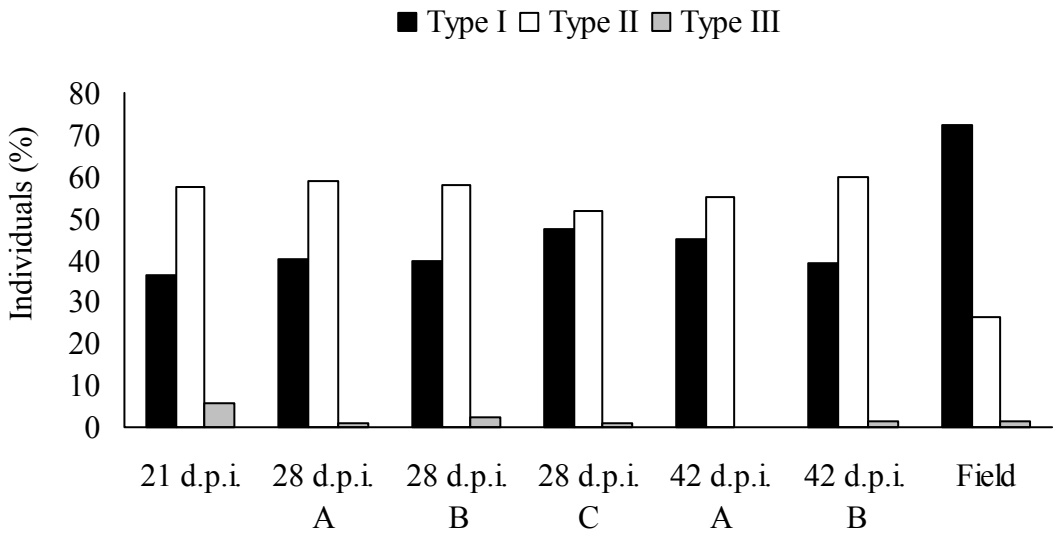

Figure 3. Frequencies (%) of the *Rsa*I types found for the different isolates and time points after infection (d.p.i.). The letters A, B and C indicated that the individuals were obtained from different hosts. On average 58 worms per host animal were analysed (minimally 45 and maximally 76).

DISCUSSION

This study revealed the presence of genetic variability within and between a laboratory- and field isolate of *C. oncophora*. Although Taq polymerase used in the PCR reactions has an error rate of 8.10^{-6} (Cline *et al.* 1996), the observed variation in the *cox*1 gene (up to 2.0%) is too high to be caused by Taq errors and can therefore be considered as genuine polymorphisms.

Considering the bias for a high AT content in nematode mitochondrial genomes (Hyman and Azevedo 1996; Hugall *et al.* 1997) the most likely events to have resulted in the described *Rsa*I RFLP are C to T conversions. In this view the preference for type I (in the field isolate), having a T at both positions, may therefore indicate a higher evolutionary rate.

Type III is characterised by the lack of a *Rsa*I recognition site at position 287, caused by the use of a TGC codon for cysteine, instead of TGT used in type I and II. The rare occurrence of type III in both isolates coincides with the codon usage found in nematode mitochondrial genomes. The codons available for cysteine are TGC and TGT. In *Ancylostoma duodenale*, *Necator americanus* and *A. suum* the TGC codon is never used. In *C. elegans* and *Oncocerca volvulus* the TGC codon is used but very rarely, 0.1% and 0.06% (Hu *et al.* 2002a).

The results showed a highly different distribution of a genetic marker between the worm population of a laboratory and a field isolate of *C. oncophora*. What the implications of this difference are can only be speculated on. The conditions under which the laboratory and field isolate have to survive differ, and is expected to be harsher for the field isolate. The laboratory isolate was collected in a different geographical region of the Netherlands than the field isolate and has been genetically isolated for almost 40 years. Nevertheless laboratory and field isolate seem equally polymorphic with respect to the mtDNA marker studied here. Grant and Whitington (1994) showed that a laboratory population of *Trichostrongylus colubriformis*, maintained over 40 years in the laboratory, was not less polymorphic than a field population isolated 30 years later. In contrast, others did demonstrate loss of genetic variability in laboratory maintained populations (Sorensen *et al.* 1999; Abu-Madi *et al.* 2000). The data presented here do also not indicate a loss of variability within the studied *Rsa*I restriction sites, given that the minor haplotypes III was present in both field and laboratory isolates.

Recent studies on partial *cox*1 sequences of hook and lungworms have been proven useful in studying the genetic population structure of these nematode species (Hu *et al.* 2002b; Hu *et al.* 2002c). Here, the described PCR/RFLP was useful as a marker clearly distinguishing different isolates. It can be used as a start for further delineation of the genetic structure of additional *C. oncophora* populations, and might in future, hopefully, be linked to functional differences between populations.

ACKNOWLEDGEMENTS

This research was supported by the Technology Foundation STW, applied science division of NWO and the technology programme of Ministry of Economic Affairs (UDG 4889). We like to thank Prof. Dr. A.W.C.A. Cornelissen and Dr. M. Eysker for the critical reading of the manuscript.

REFERENCES

Abu-Madi M.A., Mohd-Zain S.N., Lewis J.W. and Reid A.P., (2000) Genomic variability within laboratory and wild isolates of the trichostrongyle mouse nematode *Heligmosomoides polygyrus*. *J Helminthol*, 74; 195-201.

Anderson T.J., Komuniecki R., Komuniecki P.R. and Jaenike J., (1995) Are mitochondria inherited paternally in *Ascaris*? *Int J Parasitol*, 25; 1001-1004.

Avise J., Arnold J., Ball R., Bermingham E., Lamb T., Neigel J., Reeb C. and Saunders N., (1987) Intraspecific phylogeography: The mitochondrial-DNA bridge between population genetics and systematics. *Annu Rev Ecol Syst*, 18; 489-522.

Blouin M.S., (1998) Mitochondrial DNA diversity in nematodes. *J Helminthol*, 72; 285-289.

Blouin M.S., Yowell C.A., Courtney C.H. and Dame J.B., (1995) Host movement and the genetic structure of populations of parasitic nematodes. *Genetics*, 141; 1007-1014.

Cline J., Braman J.C. and Hogrefe H.H., (1996) PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res*, 24; 3546-3551.

Dame J.B., Blouin M.S. and Courtney C.H., (1993) Genetic structure of populations of *Ostertagia ostertagi*. *Vet Parasitol*, 46; 55-62.

de Vries E., (1998) pUCPCR1. A vector for direct cloning of PCR products in a double Xcm1 restriction site offering compatible single 3'-overhanging T residues. *Mol Biotechnol*, 10; 273-274.

Grant W.N., (1994) Genetic variation in parasitic nematodes and its implications. *Int J Parasitol*, 24; 821-830.

Grant W.N. and Whitington G.E., (1994) Extensive DNA polymorphism within and between two strains of *Trichostrongylus colubriformis*. *Int J Parasitol*, 24; 719-725.

Hu M., Chilton N.B. and Gasser R.B., (2002a) The mitochondrial genomes of the human hookworms, *Ancylostoma duodenale* and *Necator americanus* (Nematoda: Secernentea). *Int J Parasitol*, 32; 145-158.

Hu M., Chilton N.B., Zhu X. and Gasser R.B., (2002b) Single-strand conformation polymorphism-based analysis of mitochondrial cytochrome c oxidase subunit 1 reveals significant substructuring in hookworm populations. *Electrophoresis*, 23; 27-34.

Hu M., Hoglund J., Chilton N.B., Zhu X. and Gasser R.B., (2002c) Mutation scanning analysis of mitochondrial cytochrome c oxidase subunit 1 reveals limited gene flow among bovine lungworm subpopulations in Sweden. *Electrophoresis*, 23; 3357-3363.

Hugall A., Stanton J. and Moritz C., (1997) Evolution of the AT-rich mitochondrial DNA of the root knot nematode, *Meloidogyne hapla*. *Mol Biol Evol*, 14; 40-48.

Hyman B.C. and Azevedo J.L., (1996) Similar evolutionary patterning among repeated and single copy nematode mitochondrial genes. *Mol Biol Evol*, 13; 221-232.

Kanobana K., Vervelde L., Van Der Veer M., Eysker M. and Ploeger H.W., (2001) Characterization of host responder types after a single *Cooperia oncophora* infection: kinetics of the systemic immune response. *Parasite Immunol*, 23; 641-653.

Le Jambre L.F., (1993) Molecular variation in trichostrongylid nematodes from sheep and cattle. *Acta Trop*, 53; 331-343.

Newton L.A., Chilton N.B., Beveridge I. and Gasser R.B., (1998) Genetic evidence indicating that *Cooperia surnabada* and *Cooperia oncophora* are one species. *Int J Parasitol*, 28; 331-336.

Okimoto R., Macfarlane J.L., Clary D.O. and Wolstenholme D.R., (1992) The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. *Genetics*, 130; 471-498.

Paterson S., Fisher M.C. and Viney M.E., (2000) Inferring infection processes of a parasitic nematode using population genetics. *Parasitology*, 120 (Pt 2); 185-194.

Poot J., Kooyman F.N., Dop P.Y., Schallig H.D., Eysker M. and Cornelissen A.W., (1997) Use of cloned excretory/secretory low-molecular-weight proteins of *Cooperia oncophora* in a serological assay. *J Clin Microbiol*, 35; 1728-1733.

Sorensen E., Bogh H.O., Johansen M.V. and McManus D.P., (1999) PCR-based identification of individuals of *Schistosoma japonicum* representing different subpopulations using a genetic marker in mitochondrial DNA. *Int J Parasitol*, 29; 1121-1128.

CHAPTER FOUR

A SINGLE NUCLEOTIDE POLYMORPHISM MAP OF THE MITOCHONDRIAL GENOME OF THE PARASITIC NEMATODE *COOPERIA ONCOPHORA*

Margreet van der Veer and Erik de Vries

Parasitology (2003), in press

ABSTRACT

The 13,636 bp mitochondrial (mt) genome sequence of the trichostrongylid nematode *Cooperia oncophora* was determined. Like the mt genomes of other nematodes it is AT rich (76.75%) and cytidine is the least favourable nucleotide in the coding strand. There are two ribosomal RNA (*rrn*) genes, 22 transfer RNA (*trn*) genes and 12 protein coding genes. The relatively short AT-rich region (304 bp) and the lack of a non-coding region between two of the NADH dehydrogenase genes, *nad*3 and *nad*5, makes the mt genome of *C. oncophora* one of the smallest known to date, having only 525 bp of non-coding regions in total. The majority of the *C. oncophora* protein encoded genes are predicted to end in an abbreviated stop codon like T or TA. In total 426 single nucleotide polymorphisms (SNP) were mapped on the mt genome of *C. oncophora,* which is an average of one polymorphism per 32 bp. The most common SNPs in the mt genome of *C. oncophora* were G/A (59.2%) and C/T (28.4%) transitions. Synonymous substitutions (86.4%) were favoured over non-synonymous substitutions. However, the degree of sequence conservation between individual protein genes of different parasitic nematode species did not always correspond to the relative number of non-synonymous SNPs. The mt genome sequence of *C. oncophora* presents the first mt genome of a member of the Trichostrongyloidea and will be of importance in refining phylogenetic relationships between nematodes. The, still limited, SNP map presented here provides a basis for obtaining insight in the genetic diversity present in the different protein coding genes, *trn*, *rrn* and non coding regions. A more detailed study of the more variable regions will be of use in determining the population genetic structure of *C. oncophora*. Ultimately this knowledge will add to the understanding of the host-parasite relationship.

INTRODUCTION

Cooperia oncophora, a common parasitic nematode of cattle, belongs to the superfamily of Trichostrongyloidea. Studies of population structures at the genetic level of several strongylid species haven given insight in host-parasite relations (Blouin *et al.* 1995). Mitochondrial (mt) DNA in particular is very informative in studying aspects like population history, population size and population subdivision (Blouin *et al.* 1992; Tarrant *et al.* 1992; Dame *et al.* 1993) and the strict maternal inheritance and near absence of recombination provide a view of the straight evolutionary line (Gyllensten *et al.* 1985; Anderson *et al.* 1995).

The metazoan mt genome is circular and varies in size between 14 and 18 kb (Wolstenholme 1992; Boore 1999). For parasitic nematodes the mt genome usually encodes 12 proteins (*atp*6, *cob*, *cox*1-3 and *nad*1-4, 4L, 5-6) which are the components of the respiratory chain enzyme complexes (Okimoto *et al.* 1992; Keddie *et al.* 1998; Hu *et al.* 2002), with the exception of *Trichinella spiralis* which in addition encodes a putative *atp*8 gene (Lavrov and Brown 2001). Additionally the mt genome codes for 22 transfer RNAs (*trn*) and two ribosomal subunit RNAs (*rrn*). It generally contains at least one non-coding region which is assumed to have a function in the regulation of transcription and control of DNA replication of the mt genome (Clayton 1982). Mt genomes have been identified from *Caenorhabditis elegans*, *Ascaris suum* (Okimoto *et al.* 1992), *Onchocerca volvulus* (Keddie *et al.* 1998), *Trichinella spiralis* (Lavrov and Brown 2001), *Ancylostoma duodenale* and *Necator americanus* (Hu *et al.* 2002). The mt genome of *C. oncophora* presents the first of a member of the Trichostrongyloidea and its study can contribute to a further understanding of nematode evolution.

One of the facts making mtDNA a good marker for studying population structures is the higher rate of evolution in mt genomes compared to the nuclear genome, probably caused by frequent exposure of mtDNA to reactive oxygen metabolites (Wolstenholme 1992; Boore 1999; Wallace 1999). Therefore, the mt genome of *C. oncophora* was chosen as a target for identifying molecular markers since the higher rate of base substitution facilitates the characterization of single nucleotide polymorphisms (SNPs). To identify regions apparently enriched in variable positions an approach was taken whereby each position on the mt genome was determined from at least three different individuals. The generated data will provide a better view of the genetic diversity present in *C. oncophora* populations and ultimately contribute to dissecting the genetic population structure. The latter is an essential part for studying the effects of host immunity on the parasite population. It has previously

been demonstrated that calves experimentally infected with *C. oncophora* respond differently to infection (Kanobana *et al.* 2001) which suggests different host immune repertoires. Determining the genetic population structure can provide insight how the parasite population deals with the different pressures from the host immune response. This knowledge will be helpful in predicting the outcome of different control strategies of parasitic nematodes, like the use of anthelmintics and vaccination.

MATERIALS AND METHODS

Parasites and DNA isolation

Adult parasites were obtained from the intestine of female Holstein Friesian calves 28 days after infection (d.p.i.) with 100,000 infective *C. oncophora* larvae (L3). Before infection random samples of larvae were microscopically verified on being *C. oncophora*. Contamination with heterologous species of trichostrongylids was ruled out because samples of larvae were differentiated to the generic level by microscopical examination (MAFF 1986). DNA used as template in the PCR was obtained from a pool of worms and individual *C. oncophora* adults that were also carefully checked on bases of generally accepted morphological criteria (MAFF 1986). Total DNA was isolated by proteinase K digestion and phenol extraction as described previously (Roos *et al.* 1990). To confirm the isolated DNA was from *C. oncophora* a *Cooperia*-specific COX1 PCR was performed using the primers COX1FN 5`TAATGCCTAGTATAAT(C/T)GGTGGTTT`3 and COX1RN 5`CCCAGCTAAAACAGGTAAAGATAAT`3.

Long template PCR amplification

The complete mt DNA was amplified from 5 ng of total genomic DNA isolated from pooled (20 mg) adult worms by means of two overlapping long PCR reactions. COX1FN in combination with NAD6R 5`TTTAAATACAACTTTACTCCTGCTCTT`3 covered a ~6kb region. The second combination of COX1RN and NAD6F 5`CATATTTGGTTTTCTTACTTTATTTG`3 yielded a ~8kb product. The primers COX1FN and COX1RN are based on the sequence of the partial *cox*1 gene from *C. oncophora* (Accession numbers: AY229868-AY229873). The NAD6F & R primers are based on the *nad*6 gene sequence of *Ascaris suum* and *Caenorhabditis elegans* (Okimoto *et al.* 1992). The Expand Long Template PCR system (Roche, Mannheim, Germany) was used for amplification of the two fragments. Cycling conditions were according to the

manufacturer's protocol with modifications to annealing temperature. In short, 2 min initial denaturation at 92°C followed by 10 cycli of 30s denaturation at 92°C, 30s annealing at 55-50°C lowering each cycle with 0.5°C and 12 min extension at 68°C, followed by 30 cycles of 30s denaturation at 92°C, 30s annealing at 50-45°C and 12 min at 68°C with an additional cycle elongation of 20s for each cycle and a final cycle of 7 min at 68°C. The amplicons were digested with *Mbo*I and *Taq*I to produce overlapping clones. The restriction fragments were directly cloned into a compatible digested (*Bgl*II or *Nar*I) pUC-PCR vector (de Vries 1998). A total of 38 clones, ranging in size from 100-2000 bp, were sequenced on both strands with universal M13 sequencing primers. An additional 35 fragments, ranging in size from 309-846 bp, were obtained by long PCR reactions performed on single worms to fill gaps. For each gap a minimum of 3 individuals were sequenced. Primers for the gap filling reactions were designed on the sequences derived from the *C. oncophora* mitochondrial clones. Long PCR was performed according to the manufacturer's instructions (see above). Depending on the predicted length of the sequence the elongation time and annealing temperature were adjusted. The complete mt genome was sequenced for at least three times on both strands.

Sequence analysis

Clones and amplicons were purified using the GFX^{TM} purification kit (Amserham Biosciences). Samples were shipped to BaseClear B.V., Leiden, The Netherlands for sequence analysis using the BigDye chemistry reactions (PerkinElmer) and an Applied Biosystems 3100 genetic analyser.

The LaserGene 5.03 package (DNAStar Inc., Madison, WI, USA) was used for all sequence analyses. The SeqMan program was used for sequence assembly and identification of the SNPs. All SNPs were sequenced on both strands and manually checked by inspection of the trace files. Alignment with the *C. elegans* mt DNA sequence and BLAST analysis were used for identification of the genes. EditSeq was used to translate the protein coding genes using standard genetic codes with the modifications ATA (Met), AGA and AGG (Ser) and TGA (Trp) specific for the nematode mt genetic code (Okimoto *et al.* 1992; Jukes and Osawa 1993). Determination of the AT content and codon usage was also done with the EditSeq program. MegAlign was used for alignment of the *C. oncophora* amino acid sequences with those of *Ancylostoma duodenale*, *A. suum*, *C. elegans*, *Necator americanus*, *Onchocerca volvulus* and *Trichinella spiralis*. Pairwise alignments were performed by the ClustalW slow/accurate method (Thompson *et al.* 1997), calculation of percent identity between pairs was based on the shortest sequence, with default parameters. GeneQuest was

used to scan non-coding regions for repeats. The mt sequences from the other nematodes were downloaded from Genbank, accession numbers; *A. duodenale*: AJ417718 and *N. americanus*: AJ417719 (Hu *et al.* 2002) *A. suum*: X54253 and *C. elegans*: X54252 (Okimoto *et al.* 1992), *O. volvulus*: AF015193 (Keddie *et al.* 1998) and *T. spiralis* AF293969 (Lavrov and Brown 2001). The partial *nad*4 sequences from *Haemonchus placei* AF070825-AF070786, *Haemonchus contortus* AF070785-AF070746, *Teladorsagia circumcincta* AF070916-AF070877 and *Mazamastrongylus odocoilei* AF070876-AF070837 (Blouin *et al.* 1995).

The mitochondrial sequence data has been deposited in the EMBL/GenBank Data libraries under accession number AY265417. The single nucleotide polymorphisms have been deposited in the dbSNP Database (http://www.ncbi.nlm.nih.gov/SNP) under ID numbers ss8485731-ss8486156.

RESULTS AND DISCUSSION

General characteristics

73 fragments (47161 bp total length, 3.5-fold coverage) were sequenced on both strands and assembled in a single contig. The 13,636 bp mtDNA sequence of *C. oncophora* fits in size between those of *N. americanus* (13,604 bp) and *A. duodenale* (13,721 bp), the two smallest known mt genomes of nematodes (Hu *et al.* 2002). The size reduction of the mt genome of *C. oncophora* in comparison to *C. elegans* and *A. suum* is due to a short AT-rich region (304 bp), the lack of a non-coding region between the *nad*3 and *nad*5 genes and the compact genome organization, with only 4% non-coding base pairs.

Similar to other nematode mt genomes, the mt genome of *C. oncophora* is AT rich (76.25%) and has an unequal nucleotide composition in the coding strand (29.8% A, 46.94% T, 15.94% G and 6.43% C). The mt genome contains the genes for 12 proteins (three subunits of cytochrome c oxidase, *cox*1 *cox*2 and *cox*3; one subunit of cytochrome c-ubiquinol oxidoreductase, *cob*; seven subunits of NADH dehydrogenase, *nad*1, *nad*2, *nad*3, *nad*4, *nad*4L, *nad*5 and *nad*6; and one subunit of the ATP synthase, *atp*6; 22 tRNAs (*trns*) and the small and large subunit ribosomal RNAs, *rrn*S and *rrn*L. As in other nematodes, possibly with the exception of *T. spiralis* (Lavrov and Brown 2001), an *atp*8 gene is absent in *C. oncophora*. The relative order of the genes in the mt genome of *C. oncophora* (Fig. 1) is identical to that in *A. duodenale*, *C. elegans* and *N. americanus* (Okimoto *et al.* 1992; Hu *et al.* 2002). These are the three most closely related nematodes of which a mt genome

sequence is available indicating that within this clade no further gene rearrangements have taken place. In *A. suum* the AT rich region is located between the *trn*S (UCN) and *trn*N genes whereas in *O. volvulus* and *T. spiralis* also a number of *trn*s and protein coding genes are located differently (Okimoto *et al.* 1992; Keddie *et al.* 1998; Lavrov and Brown 2001). The larger evolutionary distance to these species is also reflected in the sequence pair distances of the complete nucleotide sequences (corrected for the different location of the genes and AT rich region of *O. volvulus* and *A. suum*) of the genomes of the six most closely related nematodes. The percent identity is the highest with *A. duodenale* (79.8 %), followed by *N. americanus* (78.4%), *C. elegans* (75.6%), *A. suum* (72.0 %) and *O. volvulus* (56.7%) (Table 1).

Table 1. Sequence pair distances in percent identity from the amino acid sequences of the 12 protein coding genes and the nucleotide sequences of the two *rrn* genes, AT-rich non-coding region and the complete mt genome. Compared for *C. oncophora* (Co) with *A. duodenale* (Ad), *A. suum* (As), *C. elegans* (Ce), *N. americanus* (Na), *O. volvulus* (Ov) and *T. spiralis* (Ts) The protein coding genes are listed from most conserved between *C. oncophora* and the other nematodes (except *O. volvulus* and *T. spiralis*) to least conserved. The ratio synonymous/non-synonymous SNPs for the protein coding genes of *C. oncophora* is given.

Gene or region	Co/Ad	Co/As	Co/Ce	Co/Na	Co/Ov	Co/Ts	ratio
Cox1	94.5	89.3	88.8	95.4	52.5	56.4	21.0
Cox2	87.9	80.6	85.7	88.7	40.1	38.2	5.5
Cox3	91.8	79.2	85.1	90.6	31.4	26.8	9.0
Atp6	86.4	73.4	77.9	86.4	21.6	13.6	1.5
Cob	83.8	74.5	76.8	80.3	50.6	34.9	3.3
Nad1	79.3	67.6	73.4	77.6	50.3	39.7	11.0
Nad5	73.6	62.6	67.9	74.0	38.9	25.5	4.67
Nad4	72.4	61.9	67.0	71.1	44.3	26.2	29.0
Nad4L	70.1	59.7	67.5	71.4	37.7	21.3	4.0
Nad3	66.7	62.2	60.4	64.0	36.0	27.0	4.0
Nad2	59.0	45.0	51.8	60.1	34.9	17.8	3.5
Nad6	56.9	54.2	52.1	58.3	24.8	14.5	0.67
*rrn*S	84.9	74.7	78.2	81.6	66.2	11.0	-
*rrn*L	80.0	71.2	76.5	77.3	66.6	32.8	-
AT-rich	53.0	60.9	65.1	58.4	50.4	-	-
Genome	79.8	72.0	75.6	78.4	56.7	-	-

Protein genes and codon usage

The genes of the mt genome of *C. oncophora* are arranged in an extremely economic fashion with a complete absence of intergenic sequences between many gene pairs (Fig. 1). Only eleven intergenic regions were found, of which the two longest, between *trn*A and *trn*P and between *nad*4 and *cox*1, are believed to be involved in regulation of transcription and control of DNA replication (Clayton 1991; Okimoto *et al.* 1992). This is the lowest number of intergenic regions found in a nematode mt genome to date. Eight out of the 12 protein genes terminate with abbreviated translation stop codons, illustrating the optimal use

of sequence (6 times T and 2 times TA, see Table 2). Most likely a TAA stop codon is created post-transcriptional after polyadenylation, as is the case in other organisms (Anderson *et al.* 1981; Ojala *et al.* 1981). For *A. duodenale*, *N. americanus*, *A. suum* and *O. volvulus* abbreviated stop codons were found in genes preceding a *trn* in contrast with *C. elegans* where the genes terminating in T or TA were followed by a protein coding gene (Okimoto *et al.* 1992; Keddie *et al.* 1998; Hu *et al.* 2002). In *C. oncophora* T and TA stop codons are directly followed by either a tRNA or protein coding gene. No distinction could be made between the genes following a T or TA stop codon.

Alternative initiation codons such as ATT, ATA and TTG have been described for other nematode mt genomes (Okimoto *et al.* 1992; Keddie *et al.* 1998; Hu *et al.* 2002). In the *C. oncophora* mt genome the ATT initiation codon, coding for Isoleucine, is used seven times as a start codon, ATA is used as the start codon for the *cob* gene and the remaining four genes, *cox*3, *nad*1, *nad*2 and *nad*4, employ a TTG codon (Table 2).

Table 1 shows the percent identities for the inferred amino acid sequences of the protein genes from *C. oncophora* compared with *A. duodenale*, *A. suum*, *C. elegans*, *N. americanus*, *O. volvulus* and *T. spiralis*. Except for *cox*1, *T. spiralis* has the lowest similarity scores when compared with *C. oncophora*. The highest identity scores were found for the three cytochrome c oxidase subunits and the lowest identities were found for the Nad2 and Nad6 proteins. For the more distantly related species except for *O. volvulus* and *T. spiralis* this pattern is slightly different with for instance relatively well conserved Nad1 and Cob genes and a poorly conserved Atp6 gene. The very low level of sequence conservation in the coding genes and also the different gene order suggests a more distant relation of *O. volvulus* to the other, more closely related, nematodes (Keddie *et al.* 1998). This distant relation is supported by strong bootstraps in a Neighbour Joining (NJ) tree based on the amino acid sequences of the protein coding genes (Fig. 2).

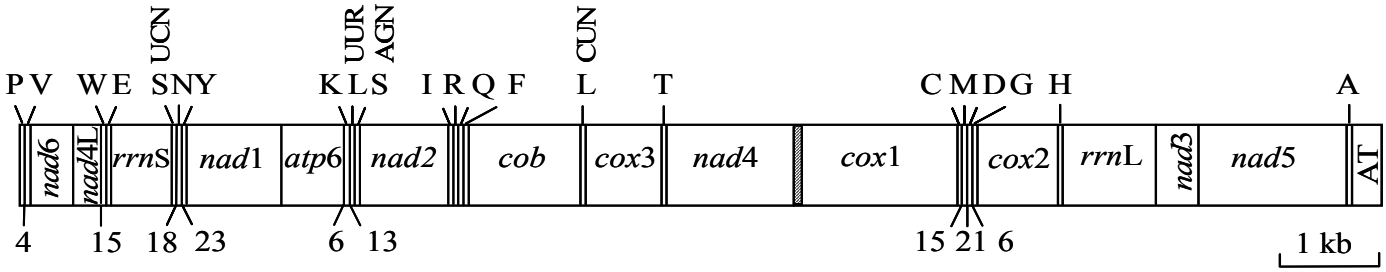

Figure 1. Schematic representation of the mitochondrial genome of *C. oncophora*. Transcription of the genes is predicted to be all in the same direction (from left to right). AT represents the AT-rich non-coding region. The long non-coding (LNC) region is shaded, the length of the other nine non-coding regions is given below. The *trn* genes are represented by their one letter amino acid code.

Table 2. Nucleotide position, AT content (%), for the genes and two non coding regions (AT-rich region and Long Non Coding (LNC) region between *nad*4 and *cox*1) of the *C. oncophora* mt genome, The translation initiation and termination codons and the number of amino acids (aa) of the inferred protein coding genes are given. To assist localization of the nine short non-coding regions positions preceded by a non-coding region are shown in bold.

Gene or region	Nucleotide Position		No. of aa	AT content	Codon	
	First	Last			Initiation	Termination
*trn*P	1	55		83.64		
*trn*V	**60**	113		77.87		
*nad*6	114	551		79.00	ATT	TA
*nad*4L	552	784	77	80.77	ATT	TAG
*trn*W	**800**	854		89.09		
*trn*E	855	908		79.63		
*rrn*S	909	1,604	527	75.57		
*trn*S (UCN)	1,605	1,657		81.13		
*trn*N	**1,676**	1,729		75.93		
*trn*Y	**1,753**	1,807		87.27		
*nad*1	1,808	2,678	370	72.07	TTG	T
*atp*6	2,679	2,278	145	77.00	ATT	TAA
*trn*K	**3,285**	3,345		73.77		
*trn*L (UUR)	**3,359**	3,413		74.55		
*trn*S (AGN)	3,414	3,465		69.32		
*nad*2	3,466	4,300	290	80.82	TTG	T
*trn*I	4,301	4,360		83.33		
*trn*R	4,361	4,414		77.78		
*trn*Q	4,415	4,468		79.63		
*trn*F	4,469	4,523		80.00		
cob	4,524	5,634	255	73.77	ATA	T
*trn*L (CUN)	5,635	5,690		85.71		
*cox*3	5,691	6,456	232	72.55	TTG	T
*trn*T	6,457	6,511		80.00		
*nad*4	6,512	7,741	234	79.84	TTG	TAA
LNC region	7,742	7,810		78.26		
*cox*1	7,811	9,390	199	70.79	ATT	TA
*trn*C	9,391	9,449		79.66		
*trn*M	**9,465**	9,522		67.24		
*trn*D	**9,544**	9,598		81.82		
*trn*G	**9,605**	9,659		74.55		
*cox*2	9,660	10,358	526	72.53	ATT	TAA
*trn*H	10,359	10,411		77.36		
*rrn*L	10,412	11,360		81.88		
*nad*3	11,361	11,694	278	78.98	ATT	T
*nad*5	11,695	13,276	77	79.06	ATT	T
*trn*A	13,277	13,332		78.57		
AT region	13,333	13,636		85.53		

The AT bias and the preference for a G over C in the coding strand has been established for other nematode mt genomes (Okimoto *et al.* 1992; Hyman and Azevedo 1996; Hugall *et al.* 1997; Keddie *et al.* 1998; Hu *et al.* 2002) and is, of course, strongly reflected in the codon usage of *C. oncophora* mt protein coding genes (Table 3). The ten most used codons (Phe (TTT), Leu (TTA), Ile (ATT), Tyr (TAT), Leu (TTG), Asn (ATT), Met (ATA), Val (GTT), Ser (AGT), Gly (GGT)) are all AT rich with 7 containing a thymidine in the 3rd position. None of the most frequently used codons contained a cytidine. In contrast, the least used codons (Pro (CCC), Ala (GCC), Arg (CGA and CGG), Asp (GAC), Ile (ATC), Leu (CTA and CTC), Cys (TGC), Gly (GGC), Thr (ACC)) mostly contain a cytidine at the 1st or 3rd position. Only two codons were never used (Ser (TCC) and Arg (CGC)).

Table 3. Frequencies (%) of amino acid (aa) codon usage in *C. oncophora* mt genes. Codons that contained a polymorphic site (2.39%) are not included in the table.

aa	Codon	Freq	aa	Codon	Freq	aa	Codon	Freq	aa	Codon	Freq
Phe	TTC	0.12	Ser	TCA	1.40	Arg	CGA	0.06	Ala	GCA	0.91
	TTT	12.70		TCC	0.0		CGC	0.0		GCC	0.06
				TCG	0.53		CGG	0.09		GCG	0.23
				TCT	2.16		CGT	0.79		GCT	1.72
Leu	TTA	10.43	Ser	AGA	2.10	Thr	ACA	0.82	Cys	TGC	0.03
	TTG	4.70		AGC	0.12		ACC	0.06		TGT	1.17
				AGG	0.47		ACG	0.29			
				AGT	3.77		ACT	1.69			
Leu	CTA	0.06	Pro	CCA	0.55	Val	GTA	3.13	Gly	GGA	0.73
	CTC	0.03		CCC	0.09		GTC	0.15		GGC	0.03
	CTG	0.20		CCG	0.18		GTG	1.26		GGG	0.73
	CTT	0.12		CCT	1.37		GTT	4.03		GGT	3.56
Ile	ATC	0.06	His	CAC	0.15	Asn	AAC	0.12	Trp	TGA	1.72
	ATT	6.75		CAT	1.34		AAT	4.47		TGG	0.35
Met	ATA	4.21	Gln	CAA	0.79	Lys	AAA	2.39	Stop	TAA	0.12
	ATG	1.90		CAG	0.38		AAG	0.79		TAG	0.03
Glu	GAA	1.43	Tyr	TAC	0.26	Asp	GAC	0.06			
	GAG	0.82		TAT	5.14		GAT	1.72			

tRNA-rRNA genes

The overall nucleotide sequences of the *trn*s, which were between 52 and 61 bp, and the predicted secondary structure of the *C. oncophora* tRNAs were similar to those of other nematodes (Okimoto *et al.* 1992; Keddie *et al.* 1998; Hu *et al.* 2002). General features, like a missing variable loop and TΨC arm, which are replaced by the TV replacement loop, were found for 20 of the *trn*s. The overall secondary structure of 7 bp in the stem structure of the amino-acyl arm is also seen for *C. oncophora*. Mismatches in the amino-acyl arm stem

structure were seen for 16 of the 22 *trn*s and most are situated at the base of the stem concerning mostly T/G pairs. All 22 *trn*s have a 5 bp anticodon arm stem combined with a 7 nt loop. Like described for other nematodes the anticodon is preceded by a pyrimidine followed by a thymidine and the anticodon is followed by a purine. In only eight cases a single mismatch in the anticodon stem was found. The consensus structure for the 20 *trn*s having a dihydrouridine (DHU) arm consists of a 4 bp stem structure and a loop varying from 4-10 nt. In 16 out of 20 *trn*s mismatches in the DHU stem were found, overall these are in the first or last bp and all but one were T/G pairs. The two *trn*s that differed in basic structure, *trn*S (AGN) and *trn*S (UCN), lack the DHU arm and instead have a variable loop of 4 (UCN) or 6 (AGN) nt. The two *trn*Ss also contain a TΨC arm which has a stem structure of 3 bp and a variable loop of 4 nt (AGN) and 5 nt (UCN).

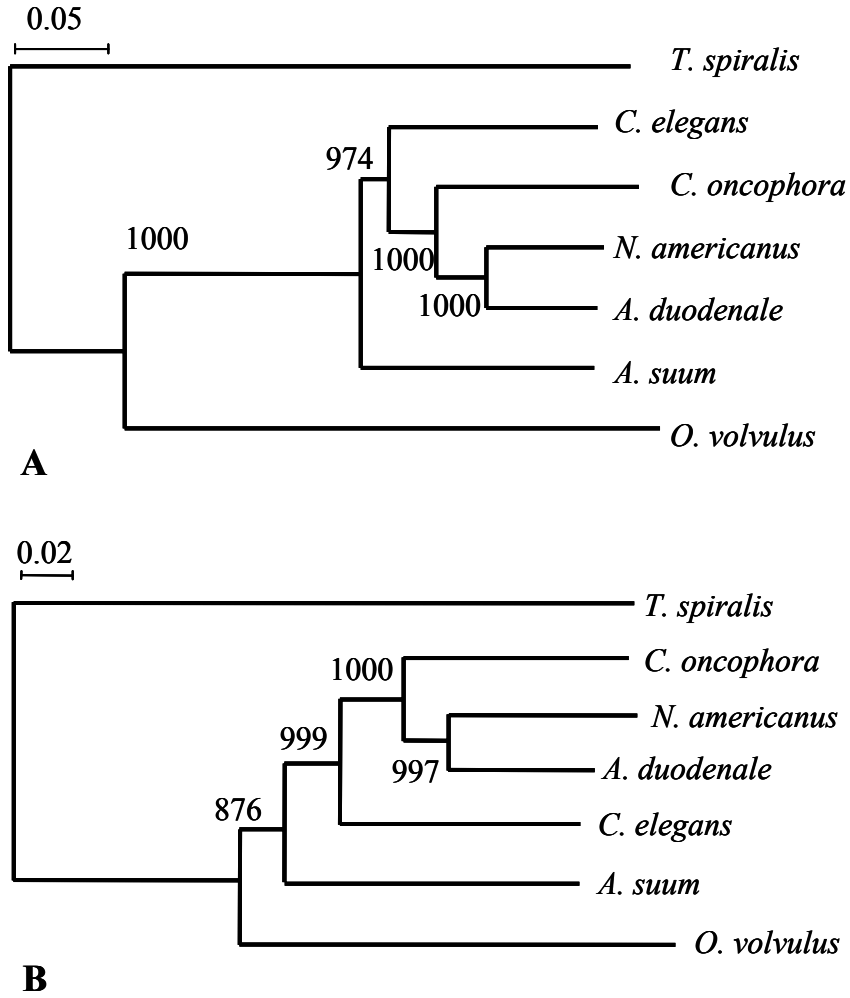

Figure 2. Neighbour Joining (NJ) Trees derived from mitochondrial data for seven nematode species. A; data derived from the amino acid sequence of the twelve protein coding genes. B; data derived from the nucleotide sequences of the *rrn* genes. Bootstrap values are given at the branches.

A pairwise comparison of the *C. oncophora rrn* genes with those of other nematodes result in a same order of conservation as observed for the protein coding genes. Nuclear *rrn* genes have been used for inferring nematode phylogenetic relationships (Blaxter *et al.* 1998; Dorris *et al.* 1999). As shown in Fig. 2 a NJ tree analysis of either concatenated mitochondrial protein encoding genes or *rrn* genes result in cladograms that support these previously described relationships with strong bootstrap values. *C. oncophora*, *A. duodenale* and *N. americanus* belonging to the Strongylida, are more closely related to each other than to *A. suum*, *C. elegans, O. volvulus* and *T. spiralis*. Additionally, the Strongylida are more closely related to the Rhabditida (*C. elegans*) than to Ascaridida (*A. suum*), Spirurida (*O. volvulus*) and Enoplida (*T. spiralis*).

Non-coding regions

The location of the 304 bp non-coding AT-rich region (85.53% AT) between the *trn*A and *trn*P genes corresponds to its location in *C. elegans*, *N. americanus* and *A. duodenale* (Okimoto *et al.* 1992; Hu *et al.* 2002). The six 43 bp direct repeats present in the AT-rich region of *C. elegans* or any other direct repeat could not be detected in *C. oncophora*. Instead, two stretches of 8 consecutive AT dinucleotides are present. The second AT stretch has an overlap with an upstream inverted repeat of 18 bp separated by one G. Like in other metazoa, the AT-rich region, or D-loop, may have a control function in transcription regulation (Clayton 1982) and contains hypervariable regions and extensive length variation (Zhang and Hewitt 1997; Meyer and Von Haeseler 2003). The hypervariablity of the AT-rich region is clearly reflected by percentage nucleotide identity between the other nematodes and *C. oncophora* (Table 1). Considering the AT content (85.53%) a percentage identity between 50 and 56% is insignificant.

The 69 bp non-coding region between the *cox*1 and *nad*4 genes (not present in *O. volvulus*) is shorter as in the 4 other nematodes (Okimoto *et al.* 1992; Keddie *et al.* 1998; Hu *et al.* 2002) and the AT content is lower (78.26%). The other nine intergenic regions of the *C. oncophora* mt genome vary in size from 4 to 23 bp. Secondary structures or repeated sequences could not be identified. None of the non coding regions were identified to harbour insertions or deletions.

Single nucleotide polymorphisms

The mt genome of *C. elegans* appears to have a two orders of magnitude higher substitution rate, 9.7×10^{-8} ($\pm 2.4 \times 10^{-8}$) per site per generation than previously estimated (Denver *et al.* 2000). Furthermore the mt genome has a lack of recombination and is strictly

maternally inherited. Consequently sequences derived from the mt genome are an excellent target for identifying molecular markers which can be used for defining the population structure. For this reason variable positions were directly determined by sequencing fragments derived from multiple individuals rather than sequencing the complete mt genome from a single worm. For each position in the mt genome a coverage of minimum three and maximum ten sequences, derived from different individuals, were used to compare for SNPs. Table 4 shows the 426 SNPs (one polymorphism per 32 bp) detected in the 13,636 bp mt genome of *C. oncophora*. No deletions or insertions were identified. 58 SNPs (13.6%) give rise to amino acid substitutions. The *nad*4L gene covers the most SNPs, one polymorphism per 23.4 bp and *nad*6 gene the least, one per 43.8 bp. The degree of conservation of the individual genes between the different nematode species (Table 1) is not very well reflected in the distribution of non-synonymous SNPs. For instance, one of the most conserved protein sequences like *atp*6, has 10 non-synonymous SNPs out of a total of 25 whereas the less well conserved *nad*4 has only 1 out of 30.

A bias for nucleotide transition (G/A or T/C) over transversions in mtDNA (Thomas and Wilson 1991; Xia *et al.* 1996; Blouin *et al.* 1998) is also very distinct in the mt genome of *C. oncophora*. From all SNP positions, 59.2% were harbouring G/A transitions, 28.4% T/C transitions, 7.5% A/T transversions and 4.9% consisted of G/T, C/G and A/C transversions. There is only one case in which three different nucleotides were seen (A, C or T) in the same position.

As expected most of the SNPs are synonymous third codon position changes (80.7%). Of the 267 SNPs found in the third position only 4 are non-synonymous. First and second codon positions are mainly non-synonymous (54 out of 67). The 12 synonymous first position changes are in Leu or Ser codons.

The number of SNPs found within the *trn* sequences is lower than average (one polymorphism per 60 bp was identified instead of the 1/32 bp for the complete mt genome). This fits with the observation that *trn* sequences are better conserved than the other sequences. In general, within non-coding regions more variation is seen which is also the case in most non-coding regions of the *C. oncophora* mt genome. For instance, in the region between *nad*4L-*trn*W, which is 15 bp in length, 5 SNPs were discovered. The 13 bp region between *trn*K- *trn*L covered 3 SNPs. The one exception is the region between the *nad*4 and *cox*1 genes (69 bp) which is a relatively long non-coding region without a single SNP. Like the AT-rich region, a control function has been attributed to this second longest non-coding region (Okimoto *et al.* 1992) and as in *C. elegans* and *A. suum*, a hairpin can be formed in this region (Fig. 3). In contrast to the hairpin structure in *C. elegans* and *A. suum*, the loop in

C. oncophora did not contain a stretch of Ts which is assumed to be involved in initiating second (L) strand synthesis (Okimoto *et al.* 1992). The absence of SNPs suggests that this region is indeed of importance for the integrity of the mt genome.

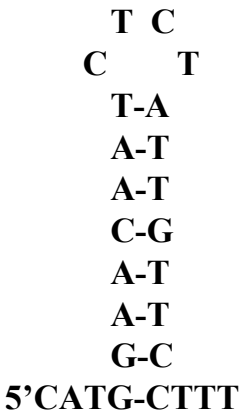

Figure 3. Potential hairpin structure found within the long non-coding region between the *cox*1 and *nad*4 genes of *C. oncophora*.

Genetic variation within *nad*4 sequences from different trichostrongylids (*Haemonchus placei*, *Haemonchus contortus*, *Teladorsagia circumcincta*, *Mazamastrongylus odocoilei*) has proven to be extremely useful in determining the genetic population structure. The data derived from the mt sequences illustrated that host mobility has a large effect on the genetic structure of the different nematodes species (Blouin *et al.* 1995). Comparing the partial *nad*4 sequences used for delineation of the population genetic structure with the corresponding sequence in *C. oncophora* revealed that of the 13 SNPs within *C. oncophora* nine were found at the same position in *T. circumcincta*, six in *M. odocoilei*, five in *H. contortus*, and four in *H. placei*.

Table 4. Single nucleotide polymorphisms (426) identified and mapped in the mitochondrial genome of *C. oncophora*. For protein coding genes positions are given in bp starting at the first position of their translation initiation codon. For the *trns*, *rrns* and non-coding regions the nucleotides were numbered in the same direction as the protein coding genes. The frequency of nucleotides at each variable position is given in parenthesis after the nucleotide position where they occur and bold positions indicate that the substitution is non-synonymous. The initiation (ini) and termination (ter) coordinates refer to the nucleotide positions in the complete mt genome sequence. The frequency given in parenthesis after the gene or region indicates the average occurrence of SNPs for that specific part of the mt genome.

Gene or region ini..ter	Substitution						
	C/T	A/G	A/T	A/C/T	G/T	A/C	G/C
*atp*6 (1/24) 2679..3278	75 (1/3), **107** (1/3), 108 (1/3), 117 (2/4), 192 (1/5), 525 (1/7)	**73** (1/3), **106** (1/3), **118** (1/5), **139** (5/1), **178** (3/3), 240 (3/3), **265** (1/5), 279 (5/1), 282 (1/5), 303 (2/6), 351 (1/7), 372 (1/7), 429 (7/1), 480 (7/1), **514** (7/1), 570 (7/1)			129 (4/2), **370** (1/7)		**227** (4/2)
*cox*1 (1/35.9) 7811..9390	30 (1/3), 63 (1/3), 210 (3/1), 231 (1/3), 396 (4/4), 417 (1/7), 468 (1/7), 522 (1/6), 684 (2/2), 777 (1/4), 849 (2/3), 1392 (1/3), 1491 (1/3), 1533 (1/3)	66 (3/1), 156 (3/1), 171 (3/1), 327 (7/1), 339 (6/2), 405 (1/7), 432 (7/1), 483 (6/2), 489 (7/1), 579 (6/1), 588 (1/5), 852 (4/1), 888 (3/2), 912 (1/4), 978 (4/1), 1014 (3/2), 1020 (4/1), 1098 (3/1), 1125 (3/1), 1335 (2/1), 1446 (1/3), 1467 (1/3), 1473 (1/3), 1476 (1/3), **1576** (2/2)	**497** (1/7), 546 (1/6), 747 (3/2), 1227 (1/2), 1536 (1/3)				
*cox*2 (1/26.9) 9660..10358	**149** (1/4), 348 (1/4), 558 (4/1), 633 (2/1)	168 (2/3), 273 (7/1), 276 (6/2), 306 (5/1), 309 (1/5), 318 (2/4), 417 (1/4), 423 (4/1), 441 (3/2), 462 (2/3), 486 (3/2), 519 (4/1), **557** (1/4), 573 (1/4), 600 (1/3), 648 (2/1), **695** (2/1)	141 (1/4), 540 (1/4)		**499** (4/1), 612 (3/1)	270 (5/2)	
*cox*3 (1/25.5) 5691..6456	342 (1/5), 349 (1/5), 369 (1/5), 417 (1/5), 435 (5/1), 540 (1/6), 552 (1/5), 612 (2/3), 717 (3/2)	81 (6/1), 138 (3/4), 177 (5/2), 183 (2/5), 195 (5/2), 276 (5/2), 306 (2/5), 339 (3/3), **386** (5/1), 444 (5/1), 492 (4/3), 513 (1/6), 522 (1/6), 543 (6/1), **568** (2/4), 648 (4/1), 759 (2/1)	192 (2/5), 534 (1/6)	33 (4/1/2)	**216** (5/2)		

Gene or region ini..ter	Substitution						
	C/T	A/G	A/T	A/C/T	G/T	A/C	G/C
cob (1/25.8) 4524..5634	100 (1/4), **170** (4/1), **220** (1/5), **278** (1/3), 501 (5/2), 559 (1/6), 576 (1/6), 759 (1/6), 768 (1/4), 786 (3/2), 868 (4/1), **926** (1/3), 963 (1/2), 991 (1/4), 1020 (8/1), 1074 (5/2)	**14** (1/3), 21 (3/1), 60 (4/1), 69 (1/4), 72 (2/3), 81 (4/1), 186 (4/1), 201 (1/4), 249 (1/3), **292** (1/3), 342 (2/5), 360 (6/1), 435 (6/1), 552 (6/1), 561 (2/5), 591 (5/2), 795 (4/1), 906 (4/1), **907** (4/1), 933 (1/2), 1017 (4/5)	**268** (1/3), **269** (1/3), 465 (6/1), 1032 (5/4)				306 (1/3), **925** (3/1)
*nad*1 (1/24.2) 1808..2678	16 (3/1), 237 (1/3), 249 (1/3), 285 (2/2), 322 (3/1), 474 (1/3), 528 (1/3), 666 (3/1), 699 (3/1), 744 (1/3)	21 (1/3), 33 (1/3), 69 (1/3), 90 (2/2), 117 (3/1), 126 (3/1), 258 (1/3), 291 (3/1), 294 (3/1), 303 (1/3), 357 (3/1), 372 (2/2), 405 (1/3), 429 (3/1), 459 (3/1), **469** (3/1), 546 (1/3), 591 (3/1), 630 (3/1), 636 (1/3), **637** (1/3), 690 (2/2), 705 (3/1), 729 (3/1)	165 (1/3)		**164** (1/3)		
*nad*2 (1/30.9) 3466..4300	18 (1/3), 129 (3/1), 174 (1/3), 267 (2/2), 549 (3/1), 564 (1/3), 619 (2/2), 675 (1/3), 807 (1/3)	45 (3/1), 54 (1/3), **55** (1/3), 78 (1/3), 90 (1/3), 111 (3/1), **167** (3/1), 198 (3/1), 420 (2/1), 483 (3/1), **544** (1/3), 672 (3/1), 702 (3/1), **752** (1/3), **763** (3/1), 765 (3/1), 816 (3/1)	**757** (3/1)				
*nad*3 (1/33.4) 11361..11694	30 (1/2), **59** (1/2)	21 (1/2), 72 (2/1), 99 (2/1), 174 (1/2), 183 (2/1), 234 (1/2), 273 (2/1)	**81** (2/1)				
*nad*4 (1/41.0) 6512..7741	252 (2/1), 453 (1/3), 561 (1/2), 564 (1/2), 604 (1/2), 708 (1/2), 756 (2/1), 840 (1/2), 990 (1/3), 993 (1/3)	21 (2/1), 78 (2/1), 111 (2/1), 147 (1/2), 231 (2/1), 273 (1/2), 441 (1/2), 474 1/2), 504 (2/1), 585 (2/1), 792 (1/2), 795 (1/2), 831 (2/1), 942 (4/1), 951 (4/1), 996 (3/1), 1059 (3/1), 1143 (2/2)	984 (1/3), **1040** (3/1)				
*nad*4L (1/23.4) 551..784	69 (7/1), 173 (2/6), 210 (2/6), 222 (2/6)	126 (7/1), 156 (1/7), 159 (3/5), **192** (7/1), 201 (3/5), **230** (6/2)					

Gene or region ini..ter	Substitution						
	C/T	A/G	A/T	A/C/T	G/T	A/C	G/C
*nad*5 (1/31.0) 11695..13276	297 (3/1), 369 (1/3), 435 (1/3), 489 (1/3), 534 (3/1), 585 (3/1), 588 (1/3), 658 (1/3), 729 (1/4), 768 (3/1), 769 (1/3), 849 (3/1), **1061** (5/2), 1098 (1/6), 1111 (1/6), 1119 (5/2), 1155 (1/6), 1207 (1/5), 1236 (5/1), 1332 (3/3), **1339** (1/5)	276 (3/1), 306 (1/3), 315 (3/1), 324 (3/1), 372 (2/2), 492 (3/1), 528 (3/1), 561 (1/3), 609 (2/2), 681 (3/1), 786 (1/3), **911** (2/2), 930 (1/3), **937** (3/1), 975 (1/3), 984 (3/1), 1026 (5/2), 1029 (1/6), 1083 (4/3), 1170 (1/6), **1171** (5/2), **1195** (1/5), 1197 (5/1), **1234** (3/3), 1260 (1/5), **1316** (5/1), 1458 (5/1), 1557 (5/1)	**1497** (4/2)			1416 (5/1)	
*nad*6 (1/43.8) 114..550	174 (1/2), **300** (1/4)	93 (1/2), **118** (2/1), 234 (2/2), 255 (3/2)			**17** (2/1), **92** (2/1), **161** (2/1)		**111** (1/2)
*rrn*S (1/46.4) 909..1604	394 (7/1)	5 (5/2), 95 (7/1), 194 (6/7), 200 (3/9), 201 (11/1), 237 (11/1), 239 (2/10), 284 (10/1), 390 (7/1), 403 (1/7), 445 (1/6), 545 (6/1)	18 (6/2), 243 (10/2)				
*rrn*L (1/43.1) 10412..11360	26 (1/2), 159 (1/7), 269 (6/2), 499 (10/1)	13 (2/1), 84 (2/1), 164 (7/1), 228 (5/3), 257 (4/4), 276 (7/1), 297 (7/1), 316 (7/1), 339 (7/1), 341 (6/2), 574 (1/10), 619 (10/1), 773 (2/1), 856 (1/2), 890 (1/2)	194 (7/1), 201 (7/1), 818 (1/2)				
*trn*H 10359..10511			17 (2/2)				
*trn*V 60..113			54 (2/1)			32 (1/2)	
*trn*W 800..854		24 (7/1)					
trnE 855..908	45 (1/7)	16 (2/6), 46 (7/1)					

Gene or region ini..ter	Substitution						
	C/T	A/G	A/T	A/C/T	G/T	A/C	G/C
*trn*N 1676..1729		16 (1/6)					
*trn*K 3285..3345		16 (1/7)				15 (7/1)	
*trn*L UUR 3359..3413	54 (3/1)	43 (3/1), 46 (1/3)					
*trn*R 4361..4414		47 (2/2)					
*trn*Q 4415..4468		15 (1/3)					
*trn*F 4469..4523		18 (2/2), 49 (3/1)	48 (2/2)				
*trn*M 9465..9522		39 (3/1)					
*trn*G 9605..9659		48 (4/1)	17 (2/3)				
AT rich 13333..13636	98 (2/4), 109 (1/5), 291 (2/2)	46 (2/4), 172 (3/1)	65 (2/4)		132 (3/1), 253 (2/1)	124 (3/1)	
*nad*4L-*trn*W 785..799	8 (1/7)	2 (7/1), 5 (4/4), 6 (1/7)	15 (1/7)				
*trn*S- *trn*N 1658…1675			16 (6/1)				
*atp*6- *trn*K 3279..3284		1 (3/5)					
*trn*K- *trn*L 3346..3358	8 (3/1)	3 (1/3), 7 (3/1)					
*trn*M- *trn*D 9523..9543	18 (2/2)	20 (2/2)	2 (2/2)				
*trn*N-*trn*Y 1728..1752	12 (1/3)	10 (1/3), 19 (3/1), 23 (3/1)					

CONCLUSION

The SNP map of the complete mt genome of *Cooperia oncophora* presented herein is the first of any parasitic nematode and also represents the first full length sequence of a trichostrongylid mt genome. The latter is of importance as it can be included in studies aiming at the refinement of nematode systematics and mt evolution in general. A SNP map will be of use for studies on *C. oncophora* population genetics. The current SNP map is based on an average 3.5 fold coverage and thus many SNPs that have been established in the population at a low frequency will not have been detected yet. Also, many of the singleton polymorphic sites reported here will have been selected by chance and do not necessarily represent polymorphisms with high frequencies. On the other hand, the SNP map does already provide a considerable set of parsimony informative positions as well as non synonymous substitutions that may be subject of selection. With the help of this map hypervariable regions may be selected which can be searched more intensively for additional informative positions that can be used in determining population structures. As new technologies to score SNPs in a semi-automated way are being developed, it is the aim to implement a high throughput method for marker-assisted screening of potential selection in populations of *C. oncophora* during infection of the host.

Regarding the high similarity of several genes from *C. oncophora* with the mt genes of other nematodes and the occurrence of SNPs within these nematodes at the same position as compared to *C. oncophora*, the presented SNP map will be helpful in determining SNPs in the mt genomes of related nematodes.

ACKNOWLEDGEMENTS

Harm Ploeger is gratefully acknowledged for supporting this study and critically reading the manuscript. The authors whish to thank Prof. Dr. A.W.C.A. Cornelissen for his helpful suggestions. This research was supported by the Technology Foundation STW, applied science division of NWO and the technology programme of Ministry of Economic Affairs (UDG 4889).

REFERENCES

Anderson S., Bankier A.T., Barrell B.G., de Bruijn M.H., Coulson A.R., Drouin J., Eperon I.C., Nierlich D.P., Roe B.A., Sanger F., Schreier P.H., Smith A.J., Staden R. and Young I.G., (1981) Sequence and organization of the human mitochondrial genome. *Nature*, 290; 457-465.

Anderson T.J., Komuniecki R., Komuniecki P.R. and Jaenike J., (1995) Are mitochondria inherited paternally in *Ascaris*? *Int J Parasitol*, 25; 1001-1004.

Blaxter M.L., De Ley P., Garey J.R., Liu L.X., Scheldeman P., Vierstraete A., Vanfleteren J.R., Mackey L.Y., Dorris M., Frisse L.M., Vida J.T. and Thomas W.K., (1998) A molecular evolutionary framework for the phylum Nematoda. *Nature*, 392; 71-75.

Blouin M.S., Dame J.B., Tarrant C.A. and Courtney C.H., (1992) Unusual population genetics of a parasitic nematode; mtDNA variation within and among populations. *Evolution*, 46; 470-476.

Blouin M.S., Yowell C.A., Courtney C.H. and Dame J.B., (1995) Host movement and the genetic structure of populations of parasitic nematodes. *Genetics*, 141; 1007-1014.

Blouin M.S., Yowell C.A., Courtney C.H. and Dame J.B., (1998) Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Mol Biol Evol*, 15; 1719-1727.

Boore J.L., (1999) Animal mitochondrial genomes. *Nucleic Acids Res*, 27; 1767-1780.

Clayton D.A., (1982) Replication of animal mitochondrial DNA. *Cell*, 28; 693-705.

Clayton D.A., (1991) Replication and transcription of vertebrate mitochondrial DNA. *Annu. Rev. Cell. Biol.*, 7; 453-478.

Dame J.B., Blouin M.S. and Courtney C.H., (1993) Genetic structure of populations of *Ostertagia ostertagi*. *Vet Parasitol*, 46; 55-62.

de Vries E., (1998) pUCPCR1. A vector for direct cloning of PCR products in a double Xcm1 restriction site offering compatible single 3'-overhanging T residues. *Mol Biotechnol*, 10; 273-274.

Denver D.R., Morris K., Lynch M., Vassilieva L.L. and Thomas W.K., (2000) High direct estimate of the mutation rate in the mitochondrial genome of *Caenorhabditis elegans*. *Science*, 289; 2342-2344.

Dorris M., De Ley P. and Blaxter M.L., (1999) Molecular analysis of nematode diversity and the evolution of parasitism. *Parasitol Today*, 15; 188-193.

Gyllensten U., Wharton D. and Wilson A.C., (1985) Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. *J. Hered.*, 76; 321-324.

Hu M., Chilton N.B. and Gasser R.B., (2002) The mitochondrial genomes of the human hookworms, *Ancylostoma duodenale* and *Necator americanus* (Nematoda: Secernentea). *Int J Parasitol*, 32; 145-158.

Hugall A., Stanton J. and Moritz C., (1997) Evolution of the AT-rich mitochondrial DNA of the root knot nematode, *Meloidogyne hapla*. *Mol Biol Evol*, 14; 40-48.

Hyman B.C. and Azevedo J.L., (1996) Similar evolutionary patterning among repeated and single copy nematode mitochondrial genes. *Mol Biol Evol*, 13; 221-232.

Jukes T.H. and Osawa S., (1993) Evolutionary changes in the genetic code. *Comp. Biochem. Physiol. B*, 106; 489-494.

Kanobana K., Vervelde L., Van Der Veer M., Eysker M. and Ploeger H.W., (2001) Characterization of host responder types after a single *Cooperia oncophora* infection: kinetics of the systemic immune response. *Parasite Immunol*, 23; 641-653.

Keddie E.M., Higazi T. and Unnasch T.R., (1998) The mitochondrial genome of *Onchocerca volvulus*: sequence, structure and phylogenetic analysis. *Mol Biochem Parasitol*, 95; 111-127.

Lavrov D.V. and Brown W.M., (2001) *Trichinella spiralis* mtDNA: a nematode mitochondrial genome that encodes a putative ATP8 and normally structured tRNAS and has a gene arrangement relatable to those of coelomate metazoans. *Genetics*, 157; 621-637.

MAFF, (1986) Manual of Veterinary Parasitological Laboratory Techniques. *(Technical Bulletin No. 18, HMSO, London)*,

Meyer S. and Von Haeseler A., (2003) Identifying site-specific substitution rates. *Mol Biol Evol*, 20; 182-189.

Ojala D., Montoya J. and Attardi G., (1981) tRNA punctuation model of RNA processing in human mitochondria. *Nature*, 290; 470-474.

Okimoto R., Macfarlane J.L., Clary D.O. and Wolstenholme D.R., (1992) The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. *Genetics*, 130; 471-498.

Roos M.H., Boersema J.H., Borgsteede F.H.M., Cornelissen J., Taylor M. and Ruitenberg E.J., (1990) Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Haemonchus contortus*. *Mol. Biochem. Parasitol.*, 43; 77-88.

Tarrant C.A., Blouin M.S., Yowell C.A. and Dame J.B., (1992) Suitability of mitochondrial DNA for assaying interindividual genetic variation in small helminths. *J Parasitol*, 78; 374-378.

Thomas W.K. and Wilson A.C., (1991) Mode and tempo of molecular evolution in the nematode *Caenorhabditis*: cytochrome oxidase II and calmodulin sequences. *Genetics*, 128; 269-279.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D.G., (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*, 25; 4876-4882.

Wallace D.C., (1999) Mitochondrial diseases in man and mouse. *Science*, 283; 1482-1488.

Wolstenholme D.R., (1992) Animal mitochondrial DNA: structure and evolution. *Int Rev Cytol*, 141; 173-216.

Xia X., Hafner M.S. and Sudman P.D., (1996) On transition bias in mitochondrial genes of pocket gophers. *J Mol Evol*, 43; 32-40.

Zhang D.-X. and Hewitt G.M., (1997) Insect Mitochondrial Control Region: A Review of its Structure, Evolution and Usefulness in Evolutionary Studies. *Biochem. Syst. Ecol.*, 25; 99-120.

CHAPTER FIVE

GENETIC POPULATION STRUCTURE OF THE PARASITIC NEMATODE *COOPERIA ONCOPHORA* BEFORE AND AFTER SERIAL PASSAGE THROUGH IMMUNIZED HOSTS AS EVALUATED BY MITOCHONDRIAL SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS)

Margreet van der Veer, Harm W. Ploeger and Erik de Vries

To be submitted for publication

ABSTRACT

In general, parasitic nematode populations display extensive genetic variation but detailed knowledge on the relation between the extent of variation and the outcome of an infection is rather limited. Here, a serial passage of the bovine intestinal nematode *Cooperia oncophora* through primed hosts was performed in order to analyse whether application of such an immune pressure under controlled conditions leads to phenotypic and genotypic adaptations. A trend of significantly increased egg shedding in nine subsequent generations could be detected, suggesting an increased capacity of the parasite population to withstand immune pressure. To examine the effects on population complexity after nine generations mitochondrial (mt) single nucleotide polymorphisms (SNPs) of the initial population and generation nine were determined. Six regions of the mitochondrial (mt) genome, covering a total of 1061 bp comprising coding, non-coding and ribosomal sequences were selected for SNP analysis. In total 50 individuals of each population were haplotyped on the basis of identified SNPs. The genetic structures of both populations were comparable with other nematode species having high within population diversity. No differences could be detected in haplotype or nucleotide diversity after the passage experiment indicating that the experimental passage had no influence on genetic variability of the worm population.

INTRODUCTION

The last decennia have seen an enormous increase in the occurrence of anthelmintic resistance in parasitic nematodes of livestock. This particularly occurred in small ruminant nematodes, but recently there are also several reports of anthelmintic resistance developing in the cattle nematode *Cooperia oncophora* (Coles *et al.* 2001; Familton *et al.* 2001) a parasite contributing to production losses. This has prompted research for other parasite control strategies, including development of vaccines against several important and pathogenic nematode species (Emery 1996; Sen *et al.* 2000) and selective breeding of host animals for increased genetic resistance against parasitic infections (Kloosterman *et al.* 1992; Gasbarre *et al.* 2001; Bishop and Stear 2003). Both these strategies have in common that they aim for increased levels of herd immunity in the host population and hence create a more adverse environment for the parasites. While it is generally accepted that probably no parasite control strategy will be capable of eradicating parasitic infections completely, the question arises how fast and to what extent parasitic nematodes can adapt to structurally changed host immunity related environmental conditions. The answer is important for evaluating the long-term effectiveness of strategies aiming for increased levels of herd immunity, the importance of which is underlined by the often rapid development of anthelmintic resistance in these parasitic organisms.

To date several studies have been conducted to evaluate how rapid parasite species may adapt to immunologically more resistant hosts, both in ruminants as in laboratory animals (Wescott and Todd 1966; Adams 1988). Generally, these studies looked at phenotypic changes in variables such as rates of parasite establishment and fecundity over a number of generations using serial passage experiments. Overall, results were inconclusive, sometimes showing no apparent phenotypic changes at all (Albers and Burgess 1988; Woolaston *et al.* 1992) and sometimes resulting in clearly better adapted lines of parasites (Su and Dobson 1997). Nonetheless, it is known that several ruminant parasitic nematode species show unusual large genetic heterogeneity (Blouin *et al.* 1992; Blouin *et al.* 1995). Further, it is not unreasonable to assume that any structural change in the environment parasites encounter, will potentially be a strong selective pressure causing parasite adaptation. It would therefore be interesting to not only investigate phenotypic changes, but also genetically monitor any adaptation.

Here, we examine both phenotypic and genetic changes occurring in the cattle nematode *C. oncophora* during and after a series of consecutive passages through immunized calves, thereby attempting to mimic a generally increased level of herd immunity. Ideally,

monitoring genetic changes would involve studying the relevant genetic loci or using a set of well-defined genetic markers distributed over the complete nuclear genome. Both knowledge on relevant loci and an established set of nuclear genome markers are lacking. However, without the aim of identifying specific genetic loci, there are possibilities to assess changes in the population structure per se as might be caused by a series of consecutive passages through a more resistant host population.

The sequencing of the complete mitochondrial (mt) genome of *C. oncophora*, based on different individuals, allowed the identification of 426 single nucleotide polymorphisms (van der Veer and de Vries 2003). A selection of six mt regions with a total length of 1061 basepairs (bp) was made based on the presence of multiple SNPs. Here, SNP analysis has been used to examine the genetic population structure of the nematode *C. oncophora*, and to monitor whether changes occurred in this structure following a series of passages through immunized host animals.

MATERIALS AND METHODS

Laboratory Cooperia oncophora isolate

The laboratory isolate (L population) originates from a field isolate in 1967 and was maintained by repeated passage through naïve donor calves. Adult parasites were recovered from 3-month old female Holstein Friesian calves, necropsied 28 days after (d.p.i.) primary infection with 100,000 infective larvae (L3).

Adaptation of Cooperia oncophora to 'ímmunized' hosts

It was attempted to adapt the laboratory maintained *C. oncophora* isolate (L) to 'immunized' calves in order to simulate a generally increased level of herd immunity as may be achieved through vaccination or selectively breeding less susceptible hosts.

The experimental design consisted of a primary immunization dose of 30,000 L3 from the L population, followed by a treatment with oxfendazole (Systamex 2.265, Schering-Plough Animal Health) 35 dpi. Subsequently, 42 dpi, the calves were challenged with a single dose of 100,000 L3 of successive generations of *C. oncophora* starting with the L population. Each successive generation consisted of equivalent numbers of L3 from all calves excreting eggs 21-28 days following the challenge infection with the preceding generation. For each generation five female Holstein Friesian calves were used. This was continued up to generation nine resulting in a *C. oncophora* population designated 'P'.

During each passage faecal egg counts were done 3 times weekly between days 14-35 after the primary immunizing infection and daily between days 14-28 after the challenge infection using a modified McMaster technique (Gordon and Whitlock 1939). Larval cultures were done according to the Baermann technique (Borgsteede and Hendriks 1973). Adult parasites necessary for SNP analysis were recovered from five 3-month old female Holstein Friesian calves, necropsied 28 d.p.i. with 100,000 L3 obtained from the P population.

Target sequences

Six regions were selected for analysis based on the presence of a relatively large number of polymorphic sites as determined from a recently established SNP map (van der Veer and de Vries 2003). The primers used for amplification of the selected regions were designed based on the total mt sequence of *C. oncophora* (GenBank Accession Number AY265417) and are listed in Table 1.

Table 1. Oligo nucleotide sequences of the used primers and the size (without primers) of the amplified fragment (bp).

Oligo name	Sequence `5 → 3`	Size (bp)
Atp6F	GGA AGG TAT GTT AAA TAT TGT GGT	
Atp6R	TTA TTA ACG TCC TTA TTC ATG CT	177
AtpKF	ATA ATG GAG TGT TTT GTA T	
AtpKR	CAG GTT TCC CTA AAA CAA ATT TA GCT ATT	213
Cox3F	TTA TTG CTT TTG TTT GAG GTA AA	
Cox3R	TAA AGG AAC ACC AAA AGG ATT AA	189
Nad4F	TTG GCT GCT TAT TCT TCT GT(C/T) AC	
Nad4R	GAA GAA ACA TGA TAA AAT TCA CCA	112
Nad5F	GTA ACT TTG TTT TGT TTA TGT GGT T	
Nad5R	CAT CAC AAA AAA ACG ACA GAA A	221
RrnSF	AGA ATA ATC GGC TAG GCT TG	
RrnSR	TTT AAA TAC AAC TTT ACT TCT GCA CTT	149

Four fragments encode parts of the proteins ATP synthetase subunit 6 (*atp6*), cytochrome c oxidase subunit 3 (*cox3*) and NADH dehydrogenase subunits 4 and 5 (*nad4, nad5*). A fifth fragment encodes a variable region of the small subunit rRNA (*rrnS*). The last fragment consists of a partial protein coding sequence, transfer RNA (*trn*) genes and two non-coding sequences. From this sequence the nucleotide (nt) positions 1-56 (primer not included) cover the 5` end of the *atp*6 gene with position 54-56 representing the stop codon. This part of the sequence was combined with the 5` end of the other *atp*6 sequence. The tRNA

sequences were also combined, nt positions 63-123 (*trn*K), 137-191 (*trn*L) and from 192-213 (22 nt of the *trn*S) were considered as one sequence of 138 bp designated "tRNA". The remaining 19 nts covered two non-coding regions. Together the selected sequences cover 7.74% of the complete mt genome of *C. oncophora* (van der Veer and de Vries 2003). Sequences studied herein are deposited in GenBank under accession numbers AY291599-AY292198.

SNP PCR

To individual worms 25 µl lysis buffer was added (0.45% Nonident P-40, 0.45% Tween 20, 10 mm Tris pH 8.0, 2.5 mM MgCl2, 50 mM KCl, 0.01% gelatine and 60µg/ml Proteinase K) overlaid with paraffin oil and frozen for 15 min at –80°C. Subsequently the samples were kept for 1 h at 60°C followed by 15 min incubation at 95°C. From the lysis sample 2.5µl was used as template in the PCR with the following composition; 5µl 10x *Pfu* reaction buffer (Promega), 1µl 2.5mM dNTP's, 2.5µl 20pmol/µl forward primer, 2.5µl 20pmol/µl reverse primer, 0.5µl 2-3U/µl *Pfu* DNA Polymerase (Promega) and ddH_2O was added to a total volume of 50 µl. The conditions used during cycling were, 5 min initial denaturation at 94°C followed by 40 cycli of 30s denaturation at 94°C, 30s annealing at 55°C and 2.5 min extension at 73°C, with a final cycle of 5 min at 73°C.

Sequence analysis

Amplicons were purified using the GFX^{TM} PCR purification kit (Amersham biosciences) and diluted ten-fold with ddH_2O. From the diluted sample 5µl was used as a template in the sequence reaction which further contained 1µl BigDye Terminator reagents (applied biosytems) 1µl primer (3.2pmol/µl), 7µl buffer (200mM Tris, pH 9.0, 5mM $MgCl_2$) and to which ddH_2O was added to a total volume of 20 µl. Cycling conditions were; 10s 96°C, 5s 50°C, 4 min 60°C, 25 cycli. Samples were desalted and run on an applied biosystems 3700 genetic analyzer. The LaserGene 5.03 package (DNAStar Inc., Madison, WI, USA) was used for assembling and alignment of the sequences. In short, the SeqMan program was used for general assembling of all forward and reverse reactions of the same sample. After visual controls the consensus sequences were saved as a single file for further analysis. Pairwise alignments were done by the ClustalW slow/accurate method, with default parameters. DnaSP version 3.50 was used for calculation of haplotypes, nucleotide diversity and polymorphic sites (Rozas and Rozas 1999). Most parsimonious trees were sought using PAUP* (Swofford 1998) using steepest descent, and 10000 random additions of taxa, with a maximum of 25 trees per random addition. These resulting trees were fed into MacClade

(Maddison and Maddison 1992) and used to reconstruct the average number of mutations for parsimony informative sites over all trees

Statistical analysis

Where used statistical analysis is indicated. Statistical significance was assumed at P< 0.05.

RESULTS

The passage experiment

The average cumulative epg between day 14-28 following the primary infection with 30,000 L3 varied between 5763 to 19939 epg, being equal to or higher than the cumulative epg following the challenge infections with 100,000 L3 (see Fig. 1). By reference, the cumulative epg of 415 calves primary infected with 100,000 L3 of the L population since 1993 was 30,093 (median: 29,560 epg-range: 0-124,540 epg). For each generation and each individual calf the cumulative epg of the challenge infection was calculated. These were plotted against generation number (Fig. 1) and tested under the null hypothesis that no change in level of epg would occur, or in other words, that no phenotypic adaptation occurred. Regression analysis demonstrated that there is a clear increase in egg output with generation number (P<0.05).

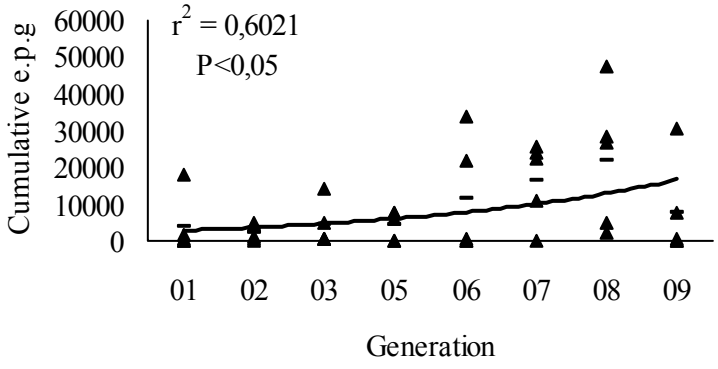

Figure 1. Individual cumulative egg output of subsequent generations during the passage experiment from the day of challenge until day 28. The regression analysis line was estimated on the mean values of the five animals of each generation. Horizontal bars indicate the mean value for that generation.

SNP analysis

From both the L and P population (9th generation) 50 individual adults were analysed separately and were compared in order to answer the question whether any change in population structure complexity, as derived from mtDNA data could be found after serial passage. To provide a single overview of the properties of all substitutions encountered, the 50 sequences of the two populations were also combined and analysed as being derived from a single population. For easy reference, Fig. 2 provides a comprehensive documentation of the sequence features. The two short non-coding regions preceding *trn*K (6bp) and *trn*L (13bp) were extremely variable and therefore not included in the analysis. Also 6 positions in the *rrn*S and *trn* genes at which an insertion occurred are left out. In total 130 positions in the L population and 154 positions in the P population were identified to harbour single base changes of which 92 were shared between both populations. Singleton polymorphic sites represented 55% of the polymorphic sites of the L population (71 sites) and 53% of the P population (81 sites). Combining both populations resulted in a reduction of the percentage of singleton polymorphic sites to 38% (73 sites) and, consequently, in an increase in the number of parsimony informative polymorphic sites (119 sites). Taken together an average of one polymorphism every 8.1 basepairs for the L population and one polymorphism every 6.9 basepairs in the P population was established.

⟶ **Figure 2**. Nucleotide sequences of the studied mitochondrial regions. The sequences shown represent the majority consensus (>50%). Third codon positions of the four protein encoding genes are lined up and indicated by an asterix. Different types of mutations can be distinguished by their shading colour which is given in the legend. For the 25 positions at which transversions occurred, the observed substitution is explained in the legend. Site frequencies and the estimated number of mutations are given below the sequence. Positions with a high site frequency explained by a single or very few mutations are indicated in red.

Synonymous transition
Non-synonymous transition
Synonymous transversion
Non-synonymous transversion
Transition + transversion
TRANSVERSIONS
Gene Pos. Type Nr. L P
ATP6 T18 T>A 0 2
T22 T>A 0 1
G39 G>T 1 1
A57 A>T 1 1
T66 T>A 0 2
G137 G>C 0 1
G178 G>T 1 0
COX3 T24 T>A 1 2
A27 A>C 0 1
T144 T>A 1 1
T174 T>A 1 0
ND4 T57 T>A 3 2
C92 C>A 0 2
ND5 A27 A>C 0 1
A57 A>T 0 4
G115 G>T 1 1
T150 T>A 0 1
G165 G>T 1 0
rmS A4 A>T 0 1
A5 A>T 0 1
A27 A>T 0 1
T28 T>A 1 0
T36 T>G 0 1
A39 A>T 0 1
G110 G>T 2 0
ATP6_L
ATP6_P
COX3_L
COX3_P
NAD4_L
NAD4_P
NAD5_L
NAD5_P
tRNA_L
tRNA_P
rmS_L
rmS_P

Table 2. Sequence polymorphism of the different fragments and all fragments combined calculated for each population separately and for both combined. The sequence analysis was performed on 50 individuals of each population; the number of bp given for each region indicates the total number of nucleotides used for the calculations. Non coding regions and sequence gaps were omitted.

	Region	Combined	Laboratory	Passaged
Number of haplotypes	*atp6* 233 bp	51	31	30
Genetic diversity (*h*)		0.948	0.941	0.958
% Polymorphic sites		21.46	15.45	14.59
Nucleotide diversity (π)		0.01294	0.01100	0.01486
Number of haplotypes	*cox3* 189 bp	47	29	32
Genetic diversity (*h*)		0.958	0.953	0.964
% Polymorphic sites		16.93	13.22	14.29
Nucleotide diversity (π)		0.01590	0.01591	0.01602
Number of haplotypes	*nad4* 112 bp	40	22	25
Genetic diversity (*h*)		0.865	0.842	0.893
% Polymorphic sites		27.68	16.96	21.43
Nucleotide diversity (π)		0.01911	0.01472	0.02334
Number of haplotypes	*nad5* 221bp	40	24	28
Genetic diversity (*h*)		0.900	0.904	0.895
% Polymorphic sites		17.65	10.86	15.84
Nucleotide diversity (π)		0.01140	0.01106	0.01129
Number of haplotypes	*rrn*S 149 bp	43	25	30
Genetic diversity (*h*)		0.929	0.920	0.944
% Polymorphic sites		20.81	12.75	17.45
Nucleotide diversity (π)		0.01555	0.01476	0.01643
Number of haplotypes	*tRNA* 138bp	11	9	9
Genetic diversity (*h*)		0.503	0.558	0.447
% Polymorphic sites		6.52	5.07	5.80
Nucleotide diversity (π)		0.00480	0.00524	0.00437
Number of haplotypes	*Combined* 1042 bp	86	47	48
Genetic diversity (*h*)		0.997	0.998	0.998
% Polymorphic sites		18.43	12.48	14.78
Nucleotide diversity (π)		0.01307	0.01208	0.01406

Table 2 summarizes the diversity as observed for the individual protein encoding gene fragments, the combined *trn* gene fragments and the *rrn*S gene fragment. The percentage of polymorphic sites within the protein regions ranged from 10.86% (*nad5,* L) to 21.43% (nad4, P) in the individual populations and reached as high as 27.68% (*nad*4) considering the combined population. Although the complete *rrn* and *trn* gene sequences constitute the more conserved parts of the mt genome (Anderson *et al.* 1982; Mears *et al.* 2002) the *rrn*S region selected here displayed variability within the same range (20.81% in the combined populations). The *trn* genes were clearly more conserved. From Fig. 2 it can be seen that the majority of polymorphic sites are located at the end of the *trn*S, causing predominantly some mispairing in the amino-acyl arm of the *trn*S. Overall, all diversity measures showed high similarity between the two populations.

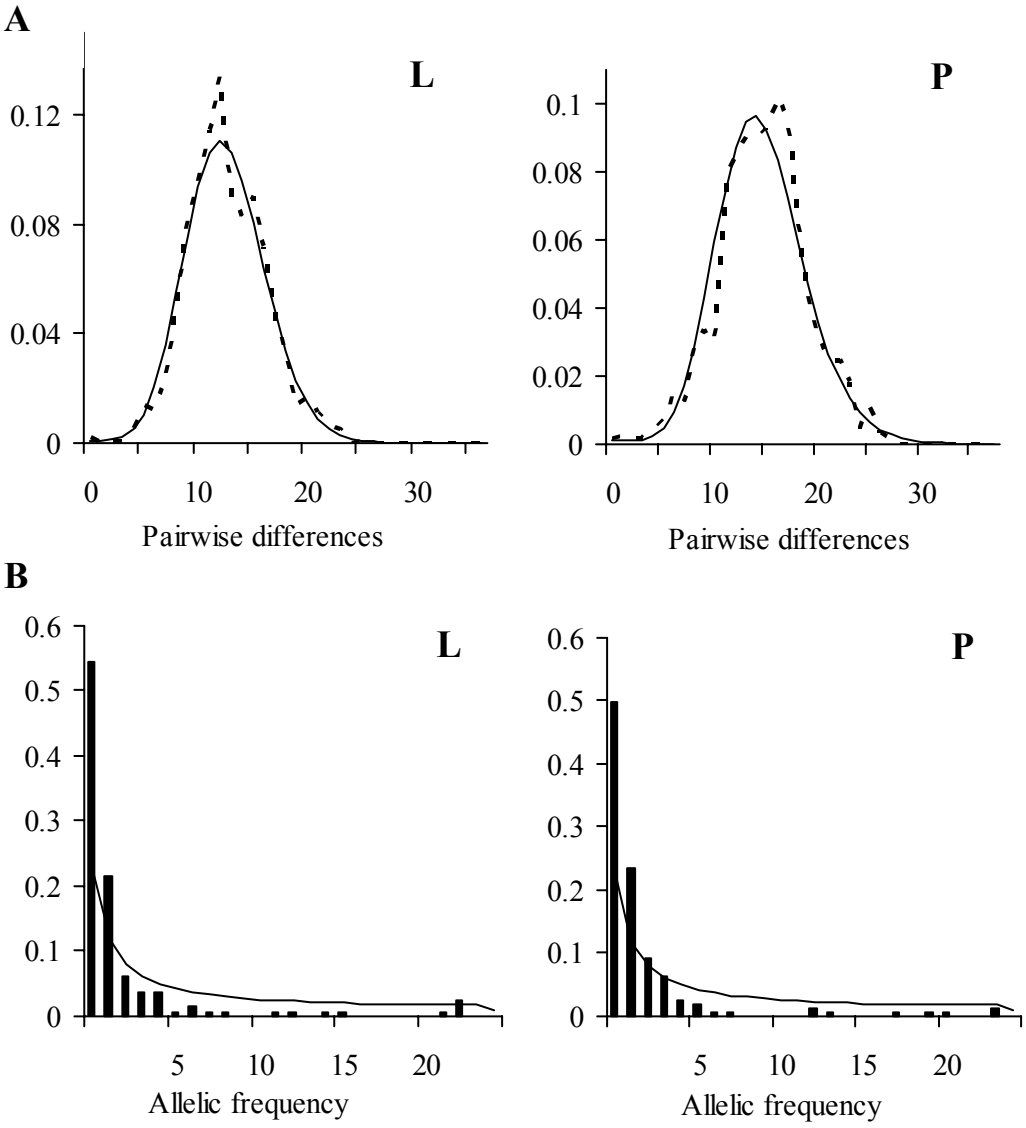

Figure 3. A; The number of pairwise differences plotted against their frequencies observed for the 50 sequences of L population and P population (dotted lines). For both populations a wave-like curve, as expected after a recent growth or decline (solid line), fitted the observed curve optimally at τ=11.921 and θ_i=0.665 for the L population and τ=12.792 and θ_i =1.855 for the P population. B; Allelic site frequencies observed for the L and P population (bars), solid line indicates distribution expected for a population in equilibrium.

Fig. 3 presents a view on the L and P population structures by plotting the pairwise differences (3A) and site-frequency spectra (3B) of the combined sequence data (excluding the two short non-coding regions). The pairwise difference plots displayed a wave-like shape that has previously been noted for human population studies using mtDNA (Rogers and Harpending 1992; Rogers *et al.* 1996) and has recently been analysed in depth for

parasitic nematodes (Mes 2003). Simulations have provided evidence that such a shape is characteristic for a population that has undergone a recent population expansion (Rogers and Harpending 1992; Rogers *et al.* 1996) and the values for the parameters of τ (a measure for the time since population expansion) and θ_i (a measure for the initial population size) by which curves (dotted lines in Fig. 3A) that fitted the observed graphs were obtained, are indicated in the legend. Most relevant to this study, no clear differences were observed between the populations. The same holds true for the observed site frequency spectra. Also in this case an over-representation of singleton polymorphic sites as compared to the distribution expected for a population in equilibrium (straight lines in Fig. 3B) is similar to what has been described before (Mes 2003) and suggestive for a recent population expansion.

As shown in Fig. 2 by indicating the frequency at which mutations are observed at all polymorphic sites, there is a small number of sites at which a high site frequency is found (e.g. at 20 positions in the combined populations, 8 or more mutations were present). This could either represent sites displaying a high mutation rate or mutations that have become established at relatively high abundance in the population by whatever expansion (or decline) mechanism. To gain some insight in this, most parsimonious trees were constructed to obtain an estimate of the number of mutations that has taken place. For the L population 5330 trees with 224 mutations were found. In the P populations 10,000 trees with a length of 270 mutations were selected from a much larger number of trees. Because these trees were derived from many independent random additions, this subset of trees was sufficiently accurate to estimate the number of mutations. At nearly all positions, values for site-frequencies and mutation rates corresponded very well between both populations (a few exceptions are described in more detail below). This analysis clearly identified a number of positions displaying a high mutation rate but also a number of positions at which a high site frequency could be explained by a single or very few mutations (frequencies indicated in red in the figure). A simple but unlikely explanation for the occurrence of such sites is a recent expansion of genotypes harbouring the mutation concerned. This explanation does not hold for the six sites most prominently displaying this feature in the current data sets as revealed by a pairwise difference analysis on subsets of sequences (results not shown). For each of the six polymorphic positions data sets were split in two subsets containing either the one or the other nucleotide type after which a pairwise difference analysis demonstrated distribution profiles similar to those of the total set. These positions therefore display variation present in the population for a longer time and are used for comparison of the two populations (Table 3). Only a limited number of sequence combinations were detected and

occurred mainly at similar frequencies in the two populations. The largest difference was noted for the combination GAGACG which occurred 13 times in the P population and 5 times in the L population. A closer inspection of this group shows that it contains all individuals having a non-synonymous A to G transition at position 88 in *atp*6_L. For both populations this transition was estimated to be caused by a single mutation event causing an Ile to Val substitution in the encoded protein.

Table 3. Frequency distribution of genotypes based on six highly variable positions having a low mutation rate. Sequences are composed of position 88 of *atp*6, positions 27, 138 and 171 of *cox*3, position 82 of *nad*4 and position 39 of *rrn*S.

	Laboratory	Passaged
GAGACG	5	13
AGGGTA	8	8
AGAACA	8	4
AAGATG	6	5
AAGGTG	8	3
AGGATA	4	3
AGAATA	2	4
AAGGTA	2	2
AGAGCA	2	2
AAAGTA	1	1
AGAGTA	1	1
AAGGCG	1	-
AGAATG	1	-
AGGATG	1	-
AAGACG	-	1
AAGATA	-	1
ACGGTG	-	1
AGAATT	-	1

Nature of the SNPs

As analysis of substitution patterns in mtDNA of other parasitic nematodes has mainly been confined to the 3'-end of the nad4 gene, a more detailed examination of the data sets now available for six *C. oncophora* gene fragments was undertaken. The distribution of polymorphic sites over first, second and third codon positions was, not unexpectedly, variable between the four protein encoding gene fragments (Table 4). The largest difference was observed between the *atp*6 and *cox*3 genes. Their total percentage of polymorphic sites was similar (21% and 17% respectively) but these were completely restricted to the third codon position for *cox*3 whereas 32% of the polymorphic positions of *atp*6 were located at first and second codon positions. Obviously, this distribution has consequences for the

number of non-synonymous substitutions which were absent from the *cox*3 gene fragment but occurred at 26% of the polymorphic sites in the *atp*6 gene fragment.

Table 4. Characteristics of the SNPs. The codon positions where the SNPs occurred are given for the protein coding regions. For each region the characteristics of SNPs identified are given by the percentage transversion, transitions and non synonymous positions. The percentage >2nt indicates the positions that contained more than two variable nucleotides at one position and therefore harbouring a transition as well as a transversion.

Laboratory	*atp*6	*cox*3	*nad*4	*nad*5	*rrn*S	*trn*	Average
1st	9	-	4	4			
2nd	3	-	-	-			
3rd	24	25	15	20			
Total SNP	36	25	19	24	19	7	
% Transitions	91.67	88.00	94.74	91.67	89.47	100.00	92.59
% Transversions	5.56	4.00	0.00	4.17	5.26	-	3.17
% >2 nt	2.78	8.00	5.26	4.17	5.26	-	4.25
% ns SNP	22.22	0.00	0.00	8.33	-	-	9.62
Passaged	*atp*6	*cox*3	*nad*4	*nad*5	*rrn*S	*trn*	Average
1st	8	-	5	6			
2nd	2	-	1	3			
3rd	23	25	18	26			
Total SNP	34	27	24	35	26	8	
% Transitions	82.35	88.88	91.67	91.43	80.77	100.00	89.18
% Transversions	2.94	3.71	4.17	5.71	15.39	-	5.32
% >2 nt	14.71	7.41	4.17	2.86	3.84	-	5.50
% ns SNP	30.30	0.00	8.33	17.14	-	-	15.39

Mt genomes of nematodes are characterized by a high AT-content and population studies using the 3'-end of *nad*4 have suggested a substitution pattern with a relatively low transition/transversion (ts/tv) ratio. The four protein encoding fragments studied here have an overall AT-content of 75.9% (74.0% at first, 69.6% at second and 84.0% at third codon position respectively). On average 90.89% of the polymorphic sites carries a transition and 4.25% a transversion. In addition 4.88% of the polymorphic sites have a transversion in addition to a transition. Sites with two different transversions did not occur. The ratios between polymorphic sites carrying a transition and those carrying a transversion are not very different for the four protein encoding genes and the *rrn*S gene (no transversions occur in the tRNA genes). A more accurate view on the transition and transversion rates underlying the observed substitution patterns is obtained by analysis of the neutral third codon position of four-fold degenerate codons that are, in theory, not subject to selection. In total 83 four-fold degenerate positions were present with an AT-content of 87.3% and no C at all. Table 5 displays the observed transversions and transitions indicating both the number

of positions as well as the estimated mutation rate. A to G transitions are clearly the most abundant but after correction for the A and G content the G to A mutation rate is relatively higher. T to A changes were the most frequent transversions, also after correction for the high T content.

Table 5. Substitution patterns observed at the third codon position of the 83 four-fold degenerate codons present in the combined protein coding regions. Colums should be read vertically, e.g. a G to A transition occurred at 7 positions in the L as well as P population. The sum of the individual mutation rates at these positions is given, e.g. 19.3 for the L population and 19.0 for the P population for G to A transitions.

		G		**A**		**T**		**C**	
		12.7%		30.7%		56.6%		0%	
		L	P	L	P	L	P	L	P
G	Positions	n.a		15	21	0	0	0	0
	Rate			31.0	42.5	0	0	0	0
A	Positions	7	7	n.a		4	6	0	0
	Rate	19.3	19.0			4	7	0	0
T	Positions	2	1	1	1	n.a		0	0
	Rate	2	1	1	1			0	0
C	Positions	0	0	0	2	12	17	n.a	
	Rate	0	0	0	2	18.4	23		

Non-synonymous SNPs

Table 6 shows the characteristics of the non-synonymous amino acid substitutions in the protein encoding regions. Within the *cox*3 sequences no nsSNPs were observed. The *atp*6 region had an increase of the total amino acid substitutions of 56.5%. This increase was mainly caused by the A→G substitution on position 88 which showed an increase from 5 to 13 individuals having the particular substitution. The *nad5* sequence of the P population carries two remarkable non-synonymous substitutions (positions 57 and 164), both occurring 4 times and characterized by a high mutation rate of 4. For the *nad*4 region, having no amino acid substitutions prior to passage, a total of 4 amino acid substitutions were present in the passaged population. Six out of 10 nsSNP positions in the L population were identified in the P population and an additional 12 positions were found that were only

present in the latter. Just one non-conservative amino acid replacement was shared between the populations, the remaining 7 were unique for either one of the populations.

Table 6. Characteristics of the amino acid substitutions. For each amino acid replacement the sequence site, codon position, substitution and frequency (between brackets) in which they occurred are given (G/A means that the nucleotide G was substituted for an A etc.). The type of amino acid (aa) replacements is given (non conservative substitutions are indicated in bold).

Region	Laboratory	aa change	Passaged	aa change
*atp*6	3; 2nd A/G (1)	**Lys → Ser**	21; 1st T/A (1)	Leu →Met
	14-16; 1st-3rd	**Val → Thr**	24; 1st G/A (2)	Val → Ile
	GTT/ACC (1)*		48; 2nd G/A (1)	Ser → Asn
	35; 1st A/G (1)	**Ser → Gly**	53; 1st C/T (1)	**Pro → Ser**
	51; 2nd T/C (1)	**Leu → Ser**	68; 1st G/A (1)	Val → Ile
	68; 1st G/A (2)	Val → Ile	86; 1st A/G (13)	Ile → Val
	86; 1st A/G (5)	Ile → Val	89; 1st G/A (1)	Val → Met
	89; 1st G/A (1)	Va l → Met	135; 2nd G/C (1)	Cys → Ser
	173; 1st G/A (1)	Val → Met	149; 1st A/G (1)	Ile → Val
			173; 1st G/A (1)	Val → Met
*cox*3	0		0	
*nad*4	0		59; 1st G/A (2)	**Gly → Ser**
			90; 2nd C/A (2)	**Thr → Lys**
*nad*5	99; 1st A/G (1)	Ile → Val	56; 3rd A/T (4)	Leu → Phe
	114; 1st G/T (1)	**Gly → Cys**	99; 1st A/G (2)	Ile → Val
			114; 1st G/T (1)	**Gly → Cys**
			163; 2nd T/C (4)	Val → Ala
			165; 1st A/G (1)	Ile → Val
			173; 1st A/G (1)	Ile → Val

* Indicates the replacement of a complete codon and is counted as 1 event.

DISCUSSION

Serial passage experiments have been employed in several organisms, especially in viruses and bacteria where such experiments benefit from much shorter generation times. However there are examples of serial passage in more complex organisms like parasitic nematodes (Ebert 1998). Selection experiments from Dobson and Tang (1991) provided evidence for the selection of parasites that were able to resist the host immune response. Their results demonstrated that the improved biological performance of selected

Heligmosomoides polygyrus was specific to the genotype and immune status of the mice through which the parasite phenotype was passaged. Similarly, the passage of *C. oncophora* through immunized hosts resulted in better performing worms in terms of significantly increased egg output. The molecular basis of this phenomenon remains unresolved however and is complicated by variable responses of the host as demonstrated by the fact that still animals remain showing virtually no egg output. Although various reports assign the observed effects of repeated passage on life history traits to genetic changes this hypothesis has hardly received experimental support (Chehresa *et al.* 1997; Reyda and Nickol 2001). Here it was demonstrated that repeated passage for nine generations, resulting in a better performing population in immune hosts, did not cause large changes in the genetic complexity of the population. Nevertheless, it is possible that nuclear genotypes have been selected during passage without the loss of genetic diversity as revealed by studies on mtDNA. In contrast to most previously described serial passage experiments that were performed with genetically uniform hosts like inbred mice, here the natural host was used. Consequently, heterogeneity of the bovine host could have been of influence in maintaining polymorphism in the passaged population. Other additional experimental factors could have contributed to the observed lack of genetic changes between the two populations. The number of generations might not have been high enough to observe an effect on population complexity, the effects of subsequent passages may be counteractive or the applied selection criteria (e.g. a single pre-immunization) may not be restrictive enough.

The use of high resolution sequencing, allowing identifying all SNPs present in the selected mt DNA regions, has greatly increased the precision to determine variation in the mt genome of *C. oncophora*. It is demonstrated that the AT and transition bias as established for other nematode mt genomes (Hyman and Azevedo 1996; Hugall *et al.* 1997) was also clearly present in *C. oncophora*. The AT content of the first, second and third codon position was, with the exception of the *cox*3 gene comparable as described by Blouin et al. (1998), with increasing AT content from second to first to third position. The differences between the polymorphisms found was mainly expressed in the number of non synonymous SNPs, which was the highest for the *atp*6 gene.

Although the laboratory maintained population did not have the opportunity to mix with other populations for over 30 years, the genetic variability is present and for the nucleotide diversity of the *nad*4 region it is comparable with the partial *nad*4 sequences of other nematodes such as *Haemonchus contortus* (0.026) *Haemonchus placei* (0.019) and *Teladorsagia circumcincta* (0.024) and *Ostertagia ostertagi* (0.027) (Blouin *et al.* 1992; Blouin *et al.* 1995). The overall nucleotide diversity was even higher in *C. oncophora* than

found in *Necator americanus* (0.0012) (Hawdon *et al.* 2001), which can be explained by population size, which is substantially larger in *C. oncophora* and resembles more the population sizes from the other parasitic nematodes from livestock (Blouin *et al.* 1995). The population size of *C. oncophora*, even cultivated under laboratory circumstances, remains large and may therefore be a reasonable explanation for the apparent maintenance of substantial genetic variation.

CONCLUSION

The results of this study provide the first extensive sampling of multiple mitochondrial sequence diversity in any parasitic trichostrongylid nematode. Although the genetic diversity in *C. oncophora* is slightly lower than observed in other nematodes, it is still high compared to other metazoans and resembles that of other parasitic nematodes of livestock. The observed phenotypic changes in *C. oncophora* during the passage experiment were not mirrored by any clear changes in the genetic population structure. Hence, we could not demonstrate whether the observed phenotypic differences were the result of genetic adaptation or that the parasites just demonstrated plasticity in response to the experimental protocol.

ACKNOWLEDGEMENTS

This research was supported by the Technology Foundation STW, applied science division of NWO and the technology programme of Ministry of Economic Affairs (UD 4889). The authors like to thank Prof. Dr. A.W. C. A. Cornelissen for critical reading of the manuscript.

REFERENCES

Adams D.B., (1988) Infection with *Haemonchus contortus* in sheep and the role of adaptive immunity in selection of the parasite. *Int J Parasitol*, 18; 1071-1075.

Albers G.A. and Burgess S.K., (1988) Serial passage of *Haemonchus contortus* in resistant and susceptible sheep. *Vet Parasitol*, 28; 303-306.

Anderson S., de Bruijn M.H., Coulson A.R., Eperon I.C., Sanger F. and Young I.G., (1982) Complete sequence of bovine mitochondrial DNA. Conserved features of the mammalian mitochondrial genome. *J Mol Biol*, 156; 683-717.

Bishop S.C. and Stear M.J., (2003) Modeling of host genetics and resistance to infectious diseases: understanding and controlling nematode infections. *Vet Parasitol*, 115; 147-166.

Blouin M.S., Dame J.B., Tarrant C.A. and Courtney C.H., (1992) Unusual population genetics of a parasitic nematode; mtDNA variation within and among populations. *Evolution*, 46; 470-476.

Blouin M.S., Yowell C.A., Courtney C.H. and Dame J.B., (1995) Host movement and the genetic structure of populations of parasitic nematodes. *Genetics*, 141; 1007-1014.

Blouin M.S., Yowell C.A., Courtney C.H. and Dame J.B., (1998) Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Mol Biol Evol*, 15; 1719-1727.

Borgsteede F.H.M. and Hendriks J., (1973) Een kwantitatieve methode voor het kweken en verzamelen van infectieuze larven van maagdarm wormen. *Tijdschr Diergeneesk*, 98; 280-286.

Chehresa A., Beech R.N. and Scott M.E., (1997) Life-history variation among lines isolated from a laboratory population of *Heligmosomoides polygyrus bakeri*. *Int J Parasitol*, 27; 541-551.

Coles G.C., Watson C.L. and Anziani O.S., (2001) Ivermectin-resistant *Cooperia* in cattle. *Vet Rec*, 148; 283-284.

Dobson C. and Tang J.M., (1991) Genetic variation and host-parasite relations: *Nematospiroides dubius* in mice. *J Parasitol*, 77; 884-889.

Ebert D., (1998) Experimental evolution of parasites. *Science*, 282; 1432-1435.

Emery D.L., (1996) Vaccination against worm parasites of animals. *Vet Parasitol*, 64; 31-45.

Familton A.S., Mason P. and Coles G.C., (2001) Anthelmintic-resistant *Cooperia* species in cattle. *Vet Rec*, 149; 719-720.

Gasbarre L.C., Leighton E.A. and Sonstegard T., (2001) Role of the bovine immune system and genome in resistance to gastrointestinal nematodes. *Vet Parasitol*, 98; 51-64.

Gordon H.M. and Whitlock A.V., (1939) A new technique for counting nematode eggs in sheep faeces. *J Coun Sci Ind Res Aust*, 12; 50-52.

Hawdon J.M., Li T., Zhan B. and Blouin M.S., (2001) Genetic structure of populations of the human hookworm, *Necator americanus*, in China. *Mol Ecol*, 10; 1433-1437.

Hugall A., Stanton J. and Moritz C., (1997) Evolution of the AT-rich mitochondrial DNA of the root knot nematode, *Meloidogyne hapla*. *Mol Biol Evol*, 14; 40-48.

Hyman B.C. and Azevedo J.L., (1996) Similar evolutionary patterning among repeated and single copy nematode mitochondrial genes. *Mol Biol Evol*, 13; 221-232.

Kloosterman A., Parmentier H.K. and Ploeger H.W., (1992) Breeding cattle and sheep for resistance to gastrointestinal nematodes. *Parasitol Today*, 8; 330-335.

Maddison W.P. and Maddison D.R., (1992) Analysis of phylogeny and character evolution. Sinauer, Sunderland

Mears J.A., Cannone J.J., Stagg S.M., Gutell R.R., Agrawal R.K. and Harvey S.C., (2002) Modeling a minimal ribosome based on comparative sequence analysis. *J Mol Biol*, 321; 215-234.

Mes T.H., (2003) Demographic expansion of parasitic nematodes of livestock based on mitochondrial DNA regions that conflict with the infinite-sites model. *Mol Ecol*, 12; 1555-1566.

Reyda F.B. and Nickol B.B., (2001) A comparison of biological performances among a laboratory-isolated population and two wild populations of *Moniliformis moniliformis*. *J Parasitol*, 87; 330-338.

Rogers A.R. and Harpending H., (1992) Population growth makes waves in the distribution of pairwise genetic differences. *Mol Biol Evol*, 9; 552-569.

Rogers A.R., Fraley A.E., Bamshad M.J., Watkins W.S. and Jorde L.B., (1996) Mitochondrial mismatch analysis is insensitive to the mutational process. *Mol Biol Evol*, 13; 895-902.

Rozas J. and Rozas R., (1999) DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, 15; 174-175.

Sen L., Ghosh K., Bin Z., Qiang S., Thompson M.G., Hawdon J.M., Koski R.A., Shuhua X. and Hotez P.J., (2000) Hookworm burden reductions in BALB/c mice vaccinated with recombinant *Ancylostoma* secreted proteins (ASPs) from *Ancylostoma duodenale*, *Ancylostoma caninum* and *Necator americanus*. *Vaccine*, 18; 1096-1102.

Su Z. and Dobson C., (1997) Genetic and immunological adaptation of *Heligmosomoides polygyrus* in mice. *Int J Parasitol*, 27; 653-663.

Swofford D.L., (1998) PAUP*. Phylogenetic analysis using Parsimony (* and other methods). Sinauer Associates, Sunderland

van der Veer M. and de Vries E., (2003) A single nucleotide polymorphism map of the mitochondrial genome of the parasitic nematode *Cooperia oncophora*. submitted for publication.

Wescott R.B. and Todd A.C., (1966) Adaptation of *Nippostrongylus brasiliensis* to the mouse. *J Parasitol*, 52; 233-236.

Woolaston R.R., Elwin R.L. and Barger I.A., (1992) No adaptation of *Haemonchus contortus* to genetically resistant sheep. *Int J Parasitol*, 22; 377-380.

CHAPTER SIX

GENERAL DISCUSSION

In the last decades much effort has been undertaken to examine the efficiency of using genetically more resistant hosts or to employ vaccination strategies to combat the negative effects of parasitic infections, among them gastro-intestinal nematodes of domesticated ruminants of which *Cooperia oncophora* is one. The research efforts were prompted by the development of anthelmintic resistance in many parasitic nematode species. This development however also made clear that parasite populations are heterogeneous and can adapt to control strategies. Clearly, due to existing genetic variation, recurrent mutation and recombination, populations of hosts and parasites are in a continues dynamic state of change. They are evolving in relation to one another, to features of their environment, and to agents as drugs and vaccination. Obviously, there is a need to address dynamics such as adaptation of parasite populations to host immunity. This can be done two-fold. First, it is possible to study genes from which is known that they are associated with resistance of the parasite to particular changes in the environment. As some of the gene polymorphisms responsible for the development of anthelmintic resistance are known the gene(s) responsible for withstanding, evading or modulating host immunity are not. The difficulty of studying a trait that might be under selection without knowledge which gene(s) is (are) involved, is that the complete genome has to be monitored in order to recognize a change after adaptation. For this purpose it was initially attempted to identify genetic markers.

THE MOLECULAR MARKERS

Amplified fragment length polymorphism

The approach taken for identification of markers which could assist monitoring the genetic composition of parasite populations was the amplified fragment length polymorphism (AFLP). This technique is used for assessing genetic polymorphism and characterization of molecular markers throughout the complete genome without the need for prior knowledge of the genome under study (Vos *et al.* 1995; Janssen *et al.* 1996). The AFLP has not been extensively used for the characterization of polymorphism in parasitic worms, which is primarily caused by the efforts required to optimise the technique and the rather labour intensive nature of the AFLP. Because basic knowledge of the genetic make-up of *C. oncophora* was lacking, the AFLP was used to validate its use to generate genetic markers and to gain insight in the genetic polymorphism in *C. oncophora* (Chapter 2). It was concluded that the genetic variation in *C. oncophora* populations is extremely high and that the interpretation of the AFLP fingerprinting patterns is not applicable without the use

of an internal size marker and computerized data processing. The AFLP was not useful for identifying comprehensive genetic markers, as it resulted in the majority of the markers not being shared between individuals. These results are in agreement with those found for the parasitic nematode of sheep *H. contortus* (Otsen *et al.* 2001) where high within population variation was discovered. Since for *C. oncophora* the building of a genetic map was not feasible the search for loci associated with reaction to host immunity was only possible by typing large numbers of individuals at many loci and different populations and subsequently search for an association with a marker or set of markers correlated with the phenotype of interest. Unfortunately, as stated before, the variation in the complete genome was overwhelming and locating markers was not feasible.

cDNA-AFLP

An alternative approach for the AFLP was studied; hereby messenger RNA (mRNA) was used as a template. This allows studying the genes that are expressed within the population at the time of sampling. The application of cDNA-AFLP resulted, as expected, in a less complex fingerprinting pattern but failed when used on individual worms. Nevertheless this approach is quite valuable when gene activity of a complete population is under study. The cDNA-AFLP can provide insight which genes are active during the parasitic phase compared to, for instance, free living phases as larvae. Moreover when different hosts are used, ie immune and susceptible, the expression patterns can assist identifying genes associated with escaping or altering host immunity. The cDNA-AFLP provides a tool for the comparison of different populations instead of concentrating on individual worms.

Restriction fragment length polymorphism

Given the difficulties encountered with the AFLP, and since the project aimed at providing an answer whether the parasites are able to adapt genetically after immune selection and not explaining the mechanism or gene(s) responsible for the adaptation, a different strategy was used. Following different populations by complexity of the genetic structure can provide an answer whether genetic changes occur in populations subjected to host immunity compared to populations that do not encounter host immunity. The alterations in complexity can indicate whether a population has undergone a selective sweep. For studies of population genetic structures the mitochondrial (mt) DNA has been used extensively. Two properties make it particular useful for such studies. First the high nucleotide substitutions compared with nuclear DNA (Blouin *et al.* 1998), and second the maternal inheritance which excludes recombination (Wolstenholme 1992). In addition the

mt genome is assumed a neutral marker (i.e. not under selection) and therefore it is ideal for determining the genetic structure of a population. The lack of target sequences or plausible candidate genes, is less of a problem using the mt genome. The complete mt genome could function as the target sequence for identifying genetic markers. Furthermore the complete mt sequence of two related nematodes, *Ascaris suum* and *Caenorhabditis elegans*, were available (Okimoto *et al.* 1992) facilitating appropriate primer design. An initial study to determine whether molecular variation was present within the mt genome of *C. oncophora* was performed on one of the most conserved regions, the cytochrome oxidase subunit I gene (*cox*1) (Chapter 3). One restriction endonuclease was found to identify a polymorphism between individuals. This PCR based restriction fragment length polymorphism (RFLP) was tested on a laboratory population of *C. oncophora* and individuals isolated from the field. A clear difference in occurrence of the different RFLP types was observed. Although all haplotypes were present in the two populations the laboratory population showed a preference for a different type than the field isolate. The polymorphisms did not cause any amino acid substitutions and are therefore difficult to subscribe to any function or alteration in function of the protein they code for. So, no conclusion can be made concerning this difference, rather it just indicated that populations can differ in allele frequencies and consequently, that the mt polymorphisms can be used as genetic markers for differentiating between populations. Although the studied locus showed polymorphism the use of only one locus is not as informative as the use of multiple loci when studying population structure (Anderson 2001). Therefore, additional polymorphic loci of the mt genome were identified.

Mitochondrial single nucleotide polymorphisms

The complete mt genome of *C. oncophora* was sequenced with a minimally 3-fold coverage. As a consequence 426 single nucleotide polymorphisms (SNPs) could be identified (Chapter 4). A selection of these SNP markers was made predominantly based on the occurrence of non-synonymous amino acid substitutions for the typing of two *C. oncophora* populations. The use of 100 individuals derived from two different populations revealed the heterogeneity of the mt genome. The number of polymorphisms found in the 1056 base pairs (bp) that were analysed for each individual was high and predominantly caused by the numerous singleton SNPs found between the individual worms. Clearly, there was a relation between the location of the sequence and the number of polymorphisms found, the conserved nature of several sequences over others was reflected in the number of SNPs. Although the studied sequences were selected for containing non-synonymous

substitutions, the majority of the identified SNPs were synonymous substitutions, located in the third codon position. Of the non-synonymous SNPs the majority resulted in conservative amino acid substitutions. SNPs clearly occurred most often in the non-coding regions. And it was in these non-coding regions, with the exception of one tRNA and rRNA sequence, where the insertion and/or deletion (indel) occurred. The SNPs were found to discriminate a total of 86 haplotypes within 100 individual worms, and although the worms were derived from two different populations this was not reflected in their haplotype variability. The mt genome sequence data reflect a straight evolutionary line and can therefore be used to identify if all the females contribute equally to the subsequent generation. If certain haplotypes would be more prominently present in subsequent generations it provides evidence that these individuals were more productive and therefore fitter. Since the fitness is a consequence of their genetic make up, these individuals would be of interest for further study.

In conclusion, since a marker must have certain sensitivity in detecting polymorphisms within a DNA sequence, the AFLP is an example that it rendered too much variation. The individual worms were too different so there was nothing to link them. Microsatellite markers, as AFLP markers, in general cover the complete genome and are therefore applicable for the identification of parts of the genome that contain the gene(s) under selection. That no microsatellite analysis was conducted is of a combined motivation. Previous studies were not conclusive in applying microsatellite analysis in parasitic nematodes (Callaghan and Beh 1994; Fisher and Viney 1996; Hoekstra *et al.* 1997). While in other organisms microsatellite markers are widespread and abundant, in the studied nematodes both characteristics were lacking. Consequently the microsatellites were not as informative as might have been expected. Moreover the identification of microsatellites is very laborious and with the low success rate of previous studies this technique was not used. The RFLP study is an example that it contained rather little information when compared to the SNP analysis. Using the former within 800 individuals sampled three haplotypes were discovered, while with the latter among 100 sampled individuals 86 haplotypes were identified. Although neither AFLP nor RFLP markers were subsequently used for further studies, as pilot experiments they were informative about the state of the specific marker(s) and useful for deciding on the direction for this study. Obviously the SNP study was the most informative not only concerning the amount of diversity within a population but additional information concerning the state of the polymorphisms were revealed using this approach. It endorsed the genetic variability and complexity identified with the AFLP. But

in contrast with the AFLP the location of the markers were all known and were applicable for haplotyping individual worms.

POPULATION DIVERSITY

Assuming a genome size for *C. oncophora* comparable to that of its free living relative *C. elegans*, it will approximately cover 100 million base pairs. This large genome provides the worms with the opportunity to develop sophisticated mechanisms to escape host immune responses. In addition it provides the worms the ability to display phenotypic variation which can be confused with genetic adaptation. Therefore it is of importance to have molecular markers that can detect genetic changes within the population. For the elucidation of the genetic structure of a population, sequence polymorphisms are needed. However, knowledge of the extent of genetic variation of *C. oncophora* populations and the role of this diversity in the interaction with the host was unknown. While most studies on the population structure of parasitic nematodes in general use just one region of a mt encoded gene combined with a limited number of individuals (de Gruijter *et al.* 2002; Hu *et al.* 2002a; Hu *et al.* 2002b), the study as described in this thesis sampled six different regions of the mt genome as well as a sufficient number of individuals. In order to address the question whether there is a role for the immune pressure of the host as a selective force on *C. oncophora* populations, the distribution and change of haplotypes present in two populations was determined (Chapter 5). The two populations differed. One was the laboratory population, propagated for over 30 years in the laboratory, while the second population was derived from a passage experiment through 'immune' hosts. For the passage experiment animals were primed with *C. oncophora* larvae and after initiation of infection treated with anthelmintics. The primary infection induces an immune response (Armour 1989; Kanobana *et al.* 2001) and upon challenge of these primed animals the worms encounter that acquired immunity. The eggs resulting from the challenge infections were cultured to infective larvae and afterwards used to challenge new primary infected animals. Through the process of propagating the parasites in immune hosts the egg output increased, see Fig. 1 chapter five. While it has been demonstrated that parasitic nematodes can adapt to host immunity and successfully infect immune hosts within as few as 3-8 generations the genetic status of these individual parasites is lacking (Wakelin and Goyal 1996). The increase in egg output during the challenge infection of the subsequent generations of the passage experiment could not be linked to a change in the genetic diversity of the mt

markers. The mode of action of a parasite is a function of their genes in combination with their environment. The demonstrated genotypic variation of the *C. oncophora* genome will produce phenotypic variation. Although phenotypic variation has an underlying genetic component it is not always the result of selection or mutation within a gene. Examples like altered gene activity, alternative splicing and the existence of gene family's can lead to phenotypic alterations (Jones *et al.* 2001). An important aspect of the latter is the efficient generation of genetic diversity (Ohta 2000), of which the immunoglobins are a good example of the diverse functions displayed by one gene family. Recently it has been demonstrated that the immuno dominant 14 kD protein isolated from excretory/secretory (E/S) products of *Cooperia punctata*, recognized after infection, belongs to a large gene family (Yatsuda *et al.* 2001). Although a lot of effort has been made to design vaccines on the basis of such 'recognized' proteins (Schallig *et al.* 1997; Frank *et al.* 1999; Frontera *et al.* 2003; Vercauteren *et al.* 2003), there are up to date no vaccines that are successful on this basis. This suggests that the use of multiple genes for altering antigen variation and thus escaping the immune response is a strategy employed by the worms. As a consequence this changing of gene activity will neither have an influence on the genetic structure nor genetic diversity of the population.

The outcome of the comparison of the genetic structure between the laboratory population and a population passaged using 'immune' animals could not establish that the increased egg output of the latter during subsequent generations, suggesting adaptation, was correlated with a change in the genetic structure on the basis of mt SNPs. The passaged population was as heterogeneous as the starting population and evidence of a genetic bottleneck whatsoever was lacking. The question whether the passaged population genetically changed cannot be answered. If any alteration did occur it certainly did not have an influence on the mt diversity of the population. Although no changes in the population structure were measured it still cannot be excluded that a selection of nuclear genotypes occurred, without affecting the population diversity. This is very plausible since the selection for drug resistance in *H. contortus* does not involve a decrease in genetic diversity (Otsen *et al.* 2001). It would be worthwhile to compare the polymorphisms studied in this thesis with *C. oncophora* populations having a different history. For instance the use of an isolate passaged in a different breed of cattle encompassing a different immune response. Possibly other populations encompass different genetic structures providing insight in its influence on genetic complexity of these populations. Evidently the genetic population structure is influenced by multiple factors related to the ability of gene flow like movement of the host population and population size of the parasites (Blouin *et al.* 1995). Therefore,

individuals derived from naturally infected hosts may be analysed and compared with the populations studied here. As was demonstrated in chapter three, different frequencies of haplotypes were found between the field and laboratory individuals. An analysis, with the mt markers used in chapter five, of a natural isolate will demonstrate if the laboratory population lost variability as a result of laboratory propagation. Moreover, for studying genetic adaptation it is interesting to include a natural isolate since these individuals encounter hosts that acquired immunity during their first year grazing season. It should be noted, however that the history of natural isolates usually is less well known than that of the laboratory maintained populations.

The lack of genetic discrepancy between the laboratory and passaged population can also be a consequence of the experimental setup. For instance the time span normally involved in co-evolution between parasites and hosts is longer than the nine generations applied in the passage experiment. Further, the animals that were used, all being as heterogeneous as the worm populations, will influence the outcome of the experiment. The host immune defence in dealing with parasite infections employs several strategies (Balic *et al.* 2000). As a result the passage experiment had many variables which could not be controlled. And the use of five animals for each generation in the passage experiment may have been too little. Since the animals are polymorphic encompassing different immune responses, selection of the parasites is complicated and may obstruct the selection of one uniform adapted population. Perhaps a genetically homogenous host population should be used with a defined homogeneous immune response. In that way it is clear that any differences seen in the parasite population would not have been caused by the heterogeneity of the host. On the other hand the use of an outbred host population has the advantage that the results can more easily be extrapolated to the current field situation.

Conclusion

In conclusion, the genetic diversity of the laboratory population that has been used for research over the last 30 years was comparable with that of related parasitic nematodes (Blouin *et al.* 1995). But a full understanding of population diversity and genetic structures will probably be obtained only if natural parasite populations are included as well as that host genetic diversity is taken into account. Since the parasite population itself exhibits heterogeneity and has shown a capacity to adapt in the face of strong selection pressure (i.e. anthelmintics), successful control programs should not only rely on one procedure. Although recently it has been suggested on the basis of modeling that the risk parasites will evolve and adapt to genetically resistant hosts is smaller than other control strategies

(Bishop and Stear 2003). As long as the knowledge is lacking how some parasites are able to escape the host immune response and cope with other changing environments the sole dependence on genetically resistant animals is not the approach to control gastrointestinal nematode infections. In addition, it is not likely that resistance against all gastrointestinal parasites will be achieved (Gasbarre *et al.* 2001). Therefore, both anthelmintic treatment and pasture management will remain necessary for future parasite control.

REFERENCES

Anderson T.J., (2001) The dangers of using single locus markers in parasite epidemiology: *Ascaris* as a case study. *Trends Parasitol*, 17; 183-188.

Armour J., (1989) The influence of host immunity on the epidemiology of trichostrongyle infections in cattle. *Vet Parasitol*, 32; 5-19.

Balic A., Bowles V.M. and Meeusen E.N., (2000) The immunobiology of gastrointestinal nematode infections in ruminants. *Adv Parasitol*, 45; 181-241.

Bishop S.C. and Stear M.J., (2003) Modeling of host genetics and resistance to infectious diseases: understanding and controlling nematode infections. *Vet Parasitol*, 115; 147-166.

Blouin M.S., Yowell C.A., Courtney C.H. and Dame J.B., (1995) Host movement and the genetic structure of populations of parasitic nematodes. *Genetics*, 141; 1007-1014.

Blouin M.S., Yowell C.A., Courtney C.H. and Dame J.B., (1998) Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Mol Biol Evol*, 15; 1719-1727.

Callaghan M.J. and Beh K.J., (1994) A middle-repetitive DNA sequence element in the sheep parasitic nematode, *Trichostrongylus colubriformis*. *Parasitology*, 109; 345-350.

de Gruijter J.M., Polderman A.M., Zhu X.Q. and Gasser R.B., (2002) Screening for haplotypic variability within *Oesophagostomum bifurcum* (Nematoda) employing a single-strand conformation polymorphism approach. *Mol Cell Probes*, 16; 185-190.

Fisher M.C. and Viney M.E., (1996) Microsatellites of the parasitic nematode *Strongyloides ratti*. *Mol Biochem Parasitol*, 80; 221-224.

Frank G.R., Wisnewski N., Brandt K.S., Carter C.R., Jennings N.S. and Selkirk M.E., (1999) Molecular cloning of the 22-24 kDa excretory-secretory 22U protein of *Dirofilaria immitis* and other filarial nematode parasites. *Mol Biochem Parasitol*, 98; 297-302.

Frontera E., Roepstorff A., Gazquez A., Reina D., Serrano F.J. and Navarrete I., (2003) Immunohistochemical distribution of antigens in liver of infected and immunized pigs with *Ascaris suum*. *Vet Parasitol*, 111; 9-18.

Gasbarre L.C., Leighton E.A. and Sonstegard T., (2001) Role of the bovine immune system and genome in resistance to gastrointestinal nematodes. *Vet Parasitol*, 98; 51-64.

Hoekstra R., Criado-Fornelio A., Fakkeldij J., Bergman J. and Roos M.H., (1997) Microsatellites of the parasitic nematode *Haemonchus contortus*: polymorphism and linkage with a direct repeat. *Mol Biochem Parasitol*, 89; 97-107.

Hu M., Chilton N.B., Zhu X. and Gasser R.B., (2002a) Single-strand conformation polymorphism-based analysis of mitochondrial cytochrome c oxidase subunit 1 reveals significant substructuring in hookworm populations. *Electrophoresis*, 23; 27-34.

Hu M., Hoglund J., Chilton N.B., Zhu X. and Gasser R.B., (2002b) Mutation scanning analysis of mitochondrial cytochrome c oxidase subunit 1 reveals limited gene flow among bovine lungworm subpopulations in Sweden. *Electrophoresis*, 23; 3357-3363.

Janssen P., Coopman R., Huys G., Swings J., Bleeker M., Vos P., Zabeau M. and Kersters K., (1996) Evaluation of the DNA fingerprinting method AFLP as an new tool in bacterial taxonomy. *Microbiology*, 142; 1881-1893.

Jones S.J., Riddle D.L., Pouzyrev A.T., Velculescu V.E., Hillier L., Eddy S.R., Stricklin S.L., Baillie D.L., Waterston R. and Marra M.A., (2001) Changes in gene expression associated with developmental arrest and longevity in *Caenorhabditis elegans*. *Genome Res*, 11; 1346-1352.

Kanobana K., Vervelde L., Van Der Veer M., Eysker M. and Ploeger H.W., (2001) Characterization of host responder types after a single *Cooperia oncophora* infection: kinetics of the systemic immune response. *Parasite Immunol*, 23; 641-653.

Ohta T., (2000) Evolution of gene families. *Gene*, 259; 45-52.

Okimoto R., Macfarlane J.L., Clary D.O. and Wolstenholme D.R., (1992) The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. *Genetics*, 130; 471-498.

Otsen M., Hoekstra R., Plas M.E., Buntjer J.B., Lenstra J.A. and Roos M.H., (2001) Amplified fragment length polymorphism analysis of genetic diversity of *Haemonchus contortus* during selection for drug resistance. *Int J Parasitol*, 31; 1138-1143.

Schallig H.D., van Leeuwen M.A., Verstrepen B.E. and Cornelissen A.W., (1997) Molecular characterization and expression of two putative protective excretory secretory proteins of *Haemonchus contortus*. *Mol Biochem Parasitol*, 88; 203-213.

Vercauteren I., Geldhof P., Peelaers I., Claerebout E., Berx G. and Vercruysse J., (2003) Identification of excretory-secretory products of larval and adult *Ostertagia ostertagi* by immunoscreening of cDNA libraries. *Mol Biochem Parasitol*, 126; 201-208.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J. and Kuiper M., (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res*, 23; 4407-4414.

Wakelin D. and Goyal P.K., (1996) *Trichinella* isolates: parasite variability and host responses. *Int J Parasitol*, 26; 471-481.

Wolstenholme D.R., (1992) Animal mitochondrial DNA: structure and evolution. *Int Rev Cytol*, 141; 173-216.

Yatsuda A.P., De Vries E., Vieira Bressan M.C. and Eysker M., (2001) A *Cooperia punctata* gene family encoding 14 kDa excretory-secretory antigens conserved for trichostrongyloid nematodes. *Parasitology*, 123; 631-639.

CHAPTER SEVEN

SUMMARY

Cooperia oncophora is one of the most common intestinal parasitic nematodes of cattle in temperate climates worldwide contributing to serious production losses. It is considered as a mild pathogen which can be effectively controlled with anthelmintics. However, this control strategy is threatened by the development of anthelmintic resistance. Although resistance in cattle nematodes emerged just recently and is not yet common, it is a widespread phenomenon in nematode parasites of sheep. For the latter it has been proven that resistance can develop within 5–8 generations following the introduction of a drug. Molecular analysis of the most commonly encountered types of resistance, such as those to benzimidazoles, showed that a small resistant subpopulation was present within the susceptible population from the onset. This observation raises important questions like how much genetic variability is present in the starting population? And to what extent does the evolution to anthelmintic resistance result in changes in polymorphism within a population? As a consequence of the increased occurrence of anthelmintic resistance an alternative for controlling gastrointestinal nematodes could be the use of animals that are genetically resistant to worm infections. However, like the use of anthelmintic drugs, this creates a different environment in which the parasites have to live. It is generally accepted that parasites possess a variety of mechanisms for escaping or modulating the host immune response. Hence, it is of interest to know how a parasite population reacts if it encounters a host that is immune either through breeding or vaccination.

To address these questions for *C. oncophora* populations, molecular markers were needed. Since *C. oncophora* infections are not a major threat to the livestock industry research addressing *C. oncophora* genetics has lagged behind. Therefore little sequence information was available and the first aim within the project was to characterize genetic markers. Therefore the majority of this thesis is dedicated to the search for markers within the genome of *C. oncophora*. An ideal marker must have sufficient variation, be reliable and be simple to generate and interpret. The amplified fragment length polymorphism (AFLP) analysis, revealed an enormous amount of genetic differences between individuals from the same population (Chapter 2). The within population variation disclosed by the AFLP was too high for simple interpretation and large population screening experiments. Although a different approach of the AFLP, using cDNA, demonstrated less polymorphism it failed when applied on individual worms. Since direct determination of possible genomic regions under selection was not feasible, neutral markers situated on the mitochondrial genome were assessed. Initially a PCR-RFLP experiment, performed on the partial mitochondrial

encoded cytochrome oxidase subunit I gene (*cox*1), confirmed the existence of between and within population variation of *C. oncophora* (Chapter 3). It was demonstrated that variation in two restriction sites was present in two populations and that the distribution differed significantly among the two. The frequencies of the variant polymorphisms demonstrated a distinction between a laboratory maintained population and a field isolate of *C. oncophora*. Because the polymorphisms were identified in one of the most conserved genes of the mt genome, it indicated that the mt genome indeed contains sufficient variation for identification of genetic markers. Consequently the complete mt genome of *C. oncophora* was sequenced. The characteristics of the composition and the polymorphisms identified in the *C. oncophora* mt genome are described in Chapter 4. From 426 identified polymorphisms a selection was made which subsequently was used in a population study to address the distribution of the variation within the laboratory population. An additional population, derived from a passage experiment through immune hosts, was tested to establish whether this population had undergone a change in the level of diversity induced by the encountered immune pressure of the host. Although a phenotypic change was observed in the passaged population, genetic differences based on the mt polymorphisms between this population and the laboratory maintained population could not be demonstrated. However it cannot be concluded that no genetic changes had occurred. It remains possible that changes on the nuclear genome occurred, which did not affect the level of mt genome diversity of the population.

Although the research described in this thesis could not demonstrate genetic changes in a parasite population under the influence of host immunity, it has contributed to describing the enormous genetic variability that exists within animal parasitic nematodes. In particular the delineation of the complete mt genome sequence, with an additional substantial number of its polymorphisms, will contribute to further studies on the genetic population structures of nematode species.

SAMENVATTING (VOOR NIET INGEWIJDEN)

Cooperia oncophora is een parasiet die voorkomt in de dunne darm van runderen. De levenscyclus van *C. oncophora* (zie Fig. 1., hoofdstuk 1) speelt zich gedeeltelijk af in het rund, hier vindt de geslachtelijke voortplanting plaats waarna de eieren met de mest (faeces) op de weide terecht komen. Uit de eieren ontwikkelen zich de larfjes die na twee keer vervellen een nieuw rund kunnen infecteren, wanneer deze het gras dat besmet is eet. *C. oncophora* behoort tot de nematoda (wormen) en samen met andere parasitaire wormen zijn ze verantwoordelijk voor een jaarlijks verlies van honderden miljoenen euro's in de veehouderij in Nederland. Het verlies wordt veroorzaakt doordat infecties met deze parasitaire wormen van invloed zijn op de ontwikkeling van de koe, met als gevolg lagere vlees- en melk-productie. De aandacht binnen het onderzoek van parasitaire wormen heeft zich de laatste decennia voornamelijk gericht op de identificatie van de verschillende soorten omdat de uiterlijke kenmerken van de verschillende wormen niet altijd toereikend zijn. Ook de levenscyclus van parasieten maakt het meestal onmogelijk te onderzoeken of uiterlijk dezelfde wormen ook daadwerkelijk met elkaar kunnen voortplanten waardoor ze tot dezelfde soort behoren. Daardoor is veel onderzoek gericht op het identificeren en bepalen van de relaties tussen soorten aan de hand van hun erfelijke materiaal. De erfelijke informatie is opgeslagen in het DNA wat is opgebouwd uit vier verschillende bouwstenen (basen of nucleotiden) die worden aangeduid met de letters A, T, C en G. De volgorde van de basen (sequentie) kan in de coderende delen (genen) vertaald worden in een eiwit. De sequentie kan verschillen tussen individuen en daarmee kunnen onderlinge relaties tussen de verschillende individuen maar ook tussen soorten worden vastgesteld.

Een toename in moleculair genetisch onderzoek binnen de parasitologie kan ook worden toegeschreven aan de ontwikkeling van resistentie tegen ontwormingsmiddelen (anthelmintica) bij verschillende parasiet soorten. Wat in eerste instantie voornamelijk voorkwam bij schaap-wormen werd later ook, zij het minder frequent, waargenomen bij runder-wormen. Onderzoek heeft aangetoond dat de ontwikkeling van resistentie een genetische achtergrond heeft. Bijvoorbeeld bij resistentie tegen de benzamidazolen, een klasse van anthelmintica, is aangetoond dat één verandering in het gen dat codeert voor de microtubili (essentiele bouwstenen voor de cel) de worm resistent maakt tegen deze anthelmintica. Tevens is ook vastgesteld dat de ontwikkeling van resistentie heel snel kan plaatsvinden omdat het restistente gen al in de populatie aanwezig is waardoor deze na enkele generaties bij het merendeel van de individuen in de populatie voorkomt. Het probleem met resistentie ontwikkeling is dat de worminfecties op den duur niet meer

behandeld kunnen worden met anthelmintica. Met als gevolg dat er gezocht moet worden naar alternatieven om de wormen te bestrijden. Ook de maatschappelijke bewustwording van de chemische residuen die sommige van de anthelmintica in het vlees en of de melk achterlaten speelt een rol om onderzoek te doen naar alternatieve bestrijdingsmethoden.

Er zijn meerdere scenarios die onderzocht worden voor alternatieve bestrijding zoals het gebruik van schimmels die de larfjes doden. Maar ook het toedienen van nutrienten in de voeding van de runderen waardoor deze beter in staat is om de wormen kwijt te raken is een aanpak die onderzocht wordt. De beste oplossing zou natuurlijk de ontwikkeling van een vaccin zijn maar dit wordt bemoeilijkt doordat de wormen een scala aan verschillende mogelijkheden hebben om de afweerreaktie (immuun respons) van de gastheer te omzeilen. Een ander verschil met de ons hedendaagse bekende vaccins is dat deze gericht zijn tegen eencellige organismen, zoals bacteriën en virussen terwijl de worm een meercellig, en dus complexer organisme is wat de ontwikkeling van een vaccin moeilijker maakt. Behalve dat de wormen verschillend op het afweer systeem kunnen reageren is er nog de waarneming dat ook de gastheer een rol speelt hoe een infectie met de parasiet verloopt. Zo zijn er runderen die, nadat ze proefondervindelijk besmet zijn met de parasiet, nauwelijks eieren uitscheiden met de faeces. Dit komt omdat deze dieren een erfelijke immuniteit tegen de parasiet bezitten. Ten tweede zijn er dieren die wel eieren uitscheiden maar dat maar voor een korte periode doen en vervolgens een bepaalde mate van bescherming hebben opgebouwd tegen een nieuwe infectie. Tot slot zijn er dieren die niet of slecht in staat zijn om de wormen onder controle te krijgen en een bepaalde mate van eieren blijven uitscheiden. Deze dieren ontwikkelen geen immuniteit en het zijn deze dieren die verantwoordelijk zijn voor het weiland en het in stand houden van de parasiet populatie. Deze waarnemingen zijn van belang bij het ontwikkelen van een andere strategie in het voorkomen van schade door wormen en dat is het selecteren van dieren (gastheren) die van nature meer resistent zijn tegen infecties. Het selecteren van dieren in de veehouderij is niet nieuw. Kenmerken zoals melkproductie en vleesproductie zijn al eerder met selectieve fokprogramma's doorgevoerd in de bestaande runderpopulatie. Een nadeel van het fokken van alleen de dieren die een genetische bescherming hebben tegen worminfecties is het gevaar dat de worm zich zou kunnen aanpassen aan het veranderde milieu (de resistente gastheer) zodra de worm daarmee in aanraking komt en uiteindelijk in staat zou blijken een aanpassing te kunnen maken om de resistente dieren alsnog succesvol te infecteren. Het behoeft geen uitleg dat deze situatie niet wenselijk is. Dat de worm in staat zou zijn zich aan te passen aan resistente gastheren is voor sommige soorten, hoewel in beperkte mate, al beschreven.

Het doel van dit proefschrift was te onderzoeken of *C. oncophora* in staat zou zijn zich aan te passen aan de afweer van de gastheer en indien dit zou blijken was de vraag of deze verandering dan veroorzaakt werd door een genetische aanpassing binnen de wormpopulatie. Om een antwoord te kunnen geven op deze vraagstelling is het nodig dat er markers in het DNA (genoom) worden gevonden die gebruikt kunnen worden om genetische veranderingen te meten. De locatie van deze markers kan verschillen, zo bestaat er het DNA in de kern (nucleair genoom) en het DNA in de mitochondriën (mitochondriaal genoom). Behalve de locatie verschillen ze ook in overerving, zo krijgt elk individu een copie van beide ouders (diploïd) als het gaat om de genetische informatie van het nucleaire genoom, terwijl het mitochondriale (mt) genoom wordt doorgegeven via de moeder (haploïd). Verder bevinden zich op het mt genoom vrijwel alleen coderende DNA gebieden in tegenstelling tot het nucleaire genoom dat veel meer niet coderende gebieden bevat. In het algemeen bevatten niet coderende gebieden veel meer variatie dan coderende genen. De genen die op het mt genoom liggen zijn vergelijkbaar voor alle organismen en zijn een essentieel onderdeel voor de energie voorziening van de cel. Voor het identificeren van DNA markers is het nodig dat er substantiële variatie (polymorfisme of heterogeniteit) bestaat in het DNA. Voor het onderzoek naar polymorfisme binnen populaties of individuen bestaan verschillende technieken. Omdat voor *C. oncophora* uitermate weinig DNA sequenties bekend waren, moest er in eerste instante gezocht worden naar de beste techniek voor het in kaart brengen van de genetische diversiteit van *C. oncophora*.

Twee technieken voor het onderzoeken van genetische diversiteit worden beschreven in hoofdstuk 2 en 3. De eerste is een techniek waarbij de analyse plaatsvindt over het totale genoom. Dit onthulde veel genetische variatie tussen individuen uit dezelfde populatie. De hoeveelheid variatie was zo enorm dat deze te complex en daardoor niet bruikbaar was voor het screenen van honderden wormen. Daarom werd het onderzoek voortgezet op een beperkter deel van het totale genoom, n.l. het mt genoom. In eerste instantie is een deel van een gen dat het minst veranderlijk is in andere verwante wormen onderzocht en hierin bleek inderdaad variatie voor te komen die bruikbaar was om onderscheid te maken tussen een populatie wormen die in het laboratorium gebruikt wordt en een populatie wormen die buiten op het weiland aanwezig was. De volgende stap in het onderzoek was het in kaart brengen van het volledige mt genoom van *C. oncophora* en omdat dit tegelijk van meerdere individuen gebeurde konden de DNA sequenties afkomstig van de verschillende individuen gebruikt worden voor het opsporen van de genetische variatie binnen deze populatie. De mate en karateristieken van de gevonden variatie is beschreven in hoofdstuk 4. Vervolgens is een selectie gemaakt van de plaatsen die sterk verschilden tussen de individuen om

gebaseerd op die variatie twee populaties te onderzoeken. Één populatie wordt al bijna 30 jaar in het laboratorium gebruikt voor onderzoek. De andere populatie was afkomstig van een experiment waarbij de wormen in kalfjes werden doorgezet die al een immuniteit hadden kunnen opbouwen omdat ze daarvoor een milde infectie hadden doorgemaakt. Dit experiment is gedaan om te benaderen wat er zou kunnen gebeuren indien er op genetisch resistente gastheren geselecteerd wordt. Twee van de mogelijkheden die zich kunnen voordoen bij het doorzetten van de wormen in gastheren die al een afweer hebben kunnen opbouwen, zijn mutatie en/of selectie van het erfelijke materiaal. Mutaties zijn veranderingen die plaatsvinden in het DNA (vervangen van een base of het verwijderen of invoegen van nucleotiden). Door mutaties kunnen er andere kenmerken (fenotypen) ontstaan. Deze veranderingen vormen de basis voor evolutie. Selectie is een proces waarbij individuen met een slechter fenotype voor dat mileu in het nadeel zijn ten opzichte van individuen met een beter fenotype. Indien er geen verschillen bestaan vindt er ook geen selectie plaats. Door selectie ontstaan ook geen nieuwe fenotypen. Het resultaat van selectie is dat niet alle individuen binnen de populatie in gelijke mate bijdragen aan de volgende generatie. Selectie is ook de drijvende kracht om mutaties die ongunstig zijn voor de soort geleidelijk uit de populatie te verwijderen. De resultaten van het onderzoek waarbij de twee verschillende populaties worden vergeleken is beschreven in hoofdstuk 5. Hieruit bleek dat voor de populatie die negen generaties een immuun afweer van de gastheer had moeten doorstaan dit vooralsnog, op basis van mitochondriale variatie, geen gevolgen had op de genetische structuur van deze wormen.

Hoewel er nog geen concreet antwoord kan gegeven worden op de vraag of *C. oncophora* zich genetisch zal aanpassen wanneer selectief gefokte runderen met een betere afweer worden gebruikt zijn de verkregen resultaten over de diversiteit van *C. oncophora* een waardevolle bijdrage aan de kennis op populatie genetica van parasitaire wormen.

CURRICULUM VITAE

Ik, Margreet van der Veer, werd geboren op 6 juli 1970 in Arnhem. De middelbare schooltijd heb ik doorgebracht in Zevenaar, eerst aan de openbare MAVO en vervolgens aan het Andreas scholengemeenschap voor het verkrijgen van het HAVO diploma. Hierna ben ik in 1989 in Eindhoven gaan wonen om daar de studie algemene microbiologie te volgen aan de Hogeschool van Eindhoven. Na een onderzoeksstage van een jaar, bij het RIVM te Bilthoven, naar de genetische variatie bij *Borrelia burgdorferi*, de veroorzaker van de ziekte van Lyme, studeerde ik in 1994 af. In september van datzelfde jaar ben ik begonnen met de verkorte opleiding biologie aan de Universiteit Utrecht. Voor de afstudeerrichting ethologie heb ik een stage van 9 maanden naar het zwermen van de angelloze bijensoort *Tetragonisca angustula* in het tropisch regenwoud van Costa Rica uitgevoerd. Als tweede afstudeer richting heb ik moleculaire biologie gekozen, waarvoor ik wederom 9 maanden stage heb gelopen bij het RIVM te Bilthoven. Het betrof een onderzoek naar genetische variatie in *Mycobacterium tuberculose* stammen. Na het schrijven van een sciptie over signaaltransductie in cellen die aanzet tot geprogrammeerde celdood studeerde ik in december 1998 af. In mei 1999 ben ik gestart met het onderzoek zoals beschreven in dit proefschrift bij de vakgroep Parasitologie en Tropische Diergeneeskunde aan de Faculteit Diergeneeskunde van de Universiteit Utrecht.

Ik moet vandaag wormen vissen...
-Is dat moeilijk?-
Soms een beetje lastig met die kleine hengeltjes onder de microscoop..
-Wat voor aas gebruik je dan voor het vissen van die wormen?-
Ik gebruik geen aas, ze zijn dood...
-Maar als ze niet meer leven dan bijten ze toch niet??-

Schatje bedankt (!), natuurlijk niet alleen voor dit soort relativerende en ontnuchterende gesprekken die soms zóóóóó hard nodig waren maar voor alles, dikke kus. Het is niet te geloven maar het is AF!!!
Justine mijn lieve vriendin, dierbaar op alle fronten. Al zoveel samen meegemaakt dus dit kan er ook nog wel bij. Gaan we naar Costa Rica? René wil denk ik ook wel mee...
Hé René, ongelooflijke ....., iedereen vraagt zich af wat daar nou zou moeten staan, maar dat houden we maar voor onszelf. Bedankt voor de ehh, het ehh, tja waarvoor eigenlijk? ;-)
Geraldine, hartverwarmend, altijd heb jij er het volste vertrouwen in gehad dat het zou lukken, ja het is gelukt! (pfffffff), bedankt voor de aanmoedigingen van de zijlijn! Ik heb nu weer tijd (en zin) om mee te juichen.
Helga, moppie, thnx voor het in elkaar knallen (om in vaktermen te spreken) van m'n voorkantje, echt geweldig maar dat wist je al... Gaan we snel weer kattekwaad uithalen?
Leo, Ingrid en Sandra, partners in crime bij het RIVM, heel fijn dat ik bij jullie terecht kon voor de onmisbare analyses en chat sessies, al was het in de sneeuw een rot eind fietsen ik had het er graag voor over.
Sas, m'n fietsvriendin en Heli m'n grote voorbeeld dat stoppen ook een optie was, maatjes bij de 'buren' ik ben de hekkesluiter dus nooit meer een 'aio overleg'. Nu moeten we een ander excuus bedenken om nog een fles open te trekken, tijd om in het kookboek te duiken?
Kirezi, ja ik weet (nu zeker) hoe het voelt.... en ik ben blij dat ik het het lief en leed met je heb kunnen delen. Gelukkig is Breda niet zo ver want een privé dierenarts en goede vriendin is onbetaalbaar. Leer je me nog eens diagonaal lezen?
Alle mensen van stal, bedankt voor de goede verzorging van mn kalfjes, Patricia geef je Jaapie en die schele afentoe nog een dikke knuffel van me?
Erik, succes met het strikken van de das en veel plezier in het lab als we (de aio's die weer eens een gecorrigeerd manuscript terug wilde terwijl je geen tijd had, een maand is (te) lang hoor!, deze zin ook!) straks weg zijn.
Lief zusje, ik zal het nooit meer doen, die dieren pesten...
Pap en Mam het is aan jullie opgedragen en niet voor niets! Al die jaren hebben jullie me daarin (weer een nieuwe studie??) gesteund. Eindelijk is ze klaar!
En de rest van de familie (ja jij ook Carry), vriendjes en vriendinnetjes (dikke kus), en collegaas allemaal heel erg bedankt voor de bijdrage die jullie op je eigen manier hebben geleverd om dit proefschrift tot stand te laten komen.

Hargreet

"You have made your way from worm to man, and much in you is still worm"
Friedrich Nietzsche (1844-1900)