CHARACTERIZATION OF THE SECOND INTERNAL TRANSCRIBED SPACER (ITS-2) rDNA REGION AMONG THE ELAPHOSTRONGYLINAE (NEMATODA: PROTOSTRONGYLIDAE)

BY

AMY JUNNILA ©

A THESIS

PRESENTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY

LAKEHEAD UNIVERSITY

THUNDER BAY, ONTARIO

SEPTEMBER, 2002

Your file Votre référence
ISBN: 0-612-83414-X
Our file Notre référence
ISBN: 0-612-83414-X

ABSTRACT

Four species of elaphostrongyline nematodes are known to infect cervids in North America. One species, *Parelaphostrongylus tenuis*, can cause neurologic disease (parelaphostrongylosis) in cervid hosts other than white tailed deer. Another, *Elaphostrongylus rangiferi*, can cause cerebrospinal elaphostrongylosis (CSE) in young, heavily infected caribou. The remaining species, *P. andersoni* and *P. odocoilei* do not cause neurologic disease but can cause verminuous pneumonia. Moreover, two European species, *E. cervi* and *E. alces*, are capable of infecting North American cervid populations if they enter with imported game animals, such as red deer. Differentiation of these species is problematic as they all produce first-stage larvae (L1) that are morphologically indistinguishable. This is a major concern for wildlife biologists who attempt to identify and limit the spread of pathogenic nematodes in North America.

This study improves upon existing methods of extraction and amplification of protostrongylid DNA by addressing the difficulties of obtaining DNA data from preserved as well as single nematodes, both adult and larval. A modified commercial kit extraction and purification procedure (QIAamp, Qiagen, Valencia, California) was developed and PCR parameters, such as cycling temperatures and times, were optimised to address these difficulties.

Second internal transcribed spacer (ITS-2) rDNA sequence data was obtained for all six elaphostrongyline species as well as an unidentified nematode from bighorn sheep in Washington. *Elaphostrongylus cervi* and *E. rangiferi* are both 585 base pairs (bp) long, *E. alces* is 575 bp, *P. tenuis* is 554 bp, *P. andersoni* is 545 bp, and *P. odocoilei* is 561 bp long. The unidentified nematode type 1 sequence from big horn sheep was 495 bp long. Identical ITS-2

sequences for *E. cervi* and *E. rangiferi* raises uncertainty regarding their distinct taxonomic status. Intraspecies variation was seen among the sequences of *Parelaphostrongylus,* but not among *Elaphostrongylus.*

Sequencing of the ITS-2 region also revealed RFLP recognition sites that were useful in distinguishing most species of elaphostrongyline nematodes and may, therefore, be useful in the development of routine diagnostic tests. Double digestion of individual *Parelaphostrongylus* spp. with Msl1 and Fok1 produced distinct banding patterns for all three species. Double digestion of *Elaphostrongylus* spp. with enzymes Mse1 and Fok1 distinguished *E. alces* from *E. cervi* and *E. rangiferi.*

Phylogenetic analysis using nucleotide sequences of the ITS-2 region generated an optimal tree with similar topology to earlier studies, which used morphological data as the basis for comparison. The genera *Elaphostrongylus* and *Parelaphostrongylus* were both monophyletic. The topology presented in this study suggests that the "muscle worms" (*P. andersoni* and *P. odocoilei*) are sister species and the "meningeal worm" (*P. tenuis*) is basal to this clade.

Elaphostrongylus alces was clearly resolved as a separate species from *E. cervi* and *E. rangiferi,* with *E. alces* diverging from *E. cervi* and *E. rangiferi* by 7%. *Elaphostrongylus cervi* and *E. rangiferi* sequences were identical, however, and their relationship could not be resolved. Variants 1 and 2 of each *Parelaphostrongylus* spp. differed by less than 1%.

Future studies may resolve the genetic differences between *E. cervi* and *E. rangiferi* by examining more potentially polymorphic regions of DNA, such as ITS-1 or other types of non-coding DNA, such as that found in the mitochondrial hypervariable region.

ACKNOWLEDGEMENTS

The funding for this project was provided by the Natural Sciences and Engineering Research Counsel of Canada and by the Lakehead University Graduate Scholarship.

First and foremost, I sincerely wish to thank my supervisor Dr. Murray W. Lankester for his guidance and insight throughout this project!

I also wish to thank Dr. J. El Molto, Director of the Lakehead University Paleo-DNA Lab for his assistance, his confidence, and for generously allowing me to use the DNA Lab facilities for this project over the past 2 years.

I am extremely grateful to the Paleo-DNA lab manager Dr. Carney Matheson and my fellow co-workers at the Paleo-DNA-Lab including Arlene Lahti, Renee Praymak, Curtis Hildebrandt, Chris Coker, and Steve Fratpietro for their support, both technical and moral, over the past two years. There is no better team out there!

This project would not have been possible without the hard work of Ahn Dao who performed countless Baermans on samples graciously donated by researchers in Canada, the U.S., and South America (too many to name!).

Thanks to my committee members: Dr. Janice M. Hughes for helping me "spin the toadies", Dr. Kam Leung for the "poo-poo" purification literature, and my external examiner Dr. Alvin Gajadhar for his time and expertise.

To the "Bio Cats" of the third floor: Rebecca Jeffery for taking the time to help with samples, "Trace" McIntosh for the Elk hair, Scott Cresey for taking the back seat from Sudbury to the Sault, and all of them for their advice and support! Last but not least, thanks to my number one #1, Franc, for keeping me sane until the end.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: GENERAL INTRODUCTION

Elaphostrongyline nematodes (Protostrongylidae: Elaphostrongylinae) are parasitic roundworms that mature in the central nervous system and skeletal muscles of cervids (Boev and Schultz, 1950). Four species are known to infect North American populations, and at least two others are found in Europe and Asia (Lankester, 2001). Due to the growing importance of game ranching, wild cervid relocation programs, and demand for exotic imports, it is imperative that both domestic and foreign parasites be accurately diagnosed to prevent the spread of disease.

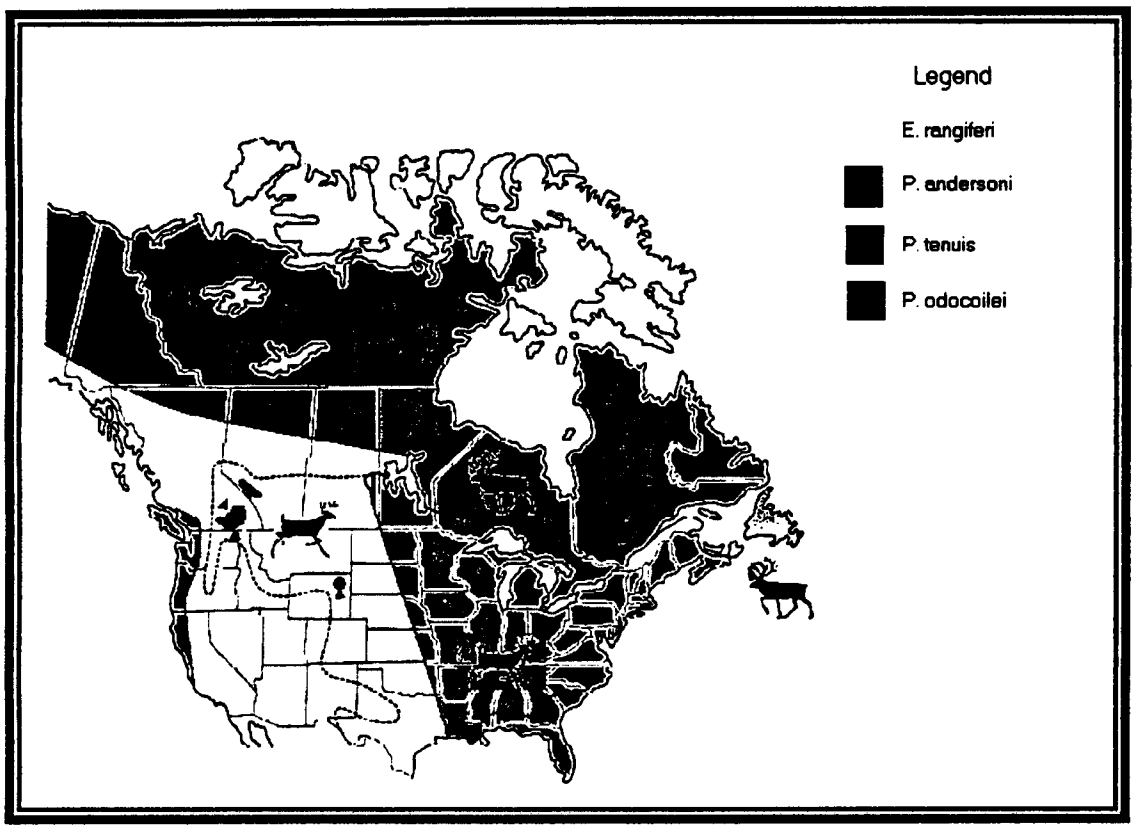
The goals of this study were to improve existing nematode DNA extraction procedures and to address the question of nematode species identification using molecular techniques, such as the polymerase chain reaction (PCR), DNA sequencing, and restriction fragment length polymorphism (RFLP). Furthermore, the DNA sequence data were used to assess phylogenetic relationships among six species of the genera *Elaphostrongylus* and *Parelaphostrongylus*.

Parelaphostrongylus tenuis (Doughtery, 1945) is a meningeal worm that was first described from white-tailed deer (*Odocoileus virginianus*). During the1960's and 70's, it was realized that *P. tenuis* causes severe neurologic disease (parelaphostrongylosis) in moose (*Alces alces*) and other wild cervids (Anderson 1964a, 1964b), wild and domestic bovids and camelids, and has been reported to cause paralysis in sheep (Anderson, 1963).

In eastern and central North America (Figure 1), *P. tenuis* occurs in white-tailed deer, their normal definitive hosts, and terrestrial gastropods, their intermediate hosts (Lankester, 2001). It is still not known why meningeal worm has not spread to the western areas of the continent even though white-tailed deer can be found there (Lankester, 2001).

The first-stage larvae (L1) of *P. tenuis* and other elaphostrongylines are passed in the

FIGURE 1. Distribution of elaphostrongyline nematodes in North America. Modified from Lankester (2001).



host's feces and gastropods become infected in the foot when they crawl over the feces. In the gastropod, the parasite develops to the second-stage larvae (L2), followed by the infective, or L3, stage (Lankester, 2001). Common intermediate hosts of *P. tenuis* include *Zonitoides* spp., *Discus cronkhitei*, and *Deroceras* spp. (Lankester and Peterson, 1996).

Animals become infected when they ingest the L3-containing gastropod accidentally with food (Lankester, 2001). The L3s penetrate the wall of the abomasum and in the case of *P. tenuis*, migrate to the dorsal horns of grey matter in the spinal cord where they develop to the fourth (L4) and fifth stages (Anderson, 1968). In white-tailed deer, the fifth stage sub-adults leave the spinal cord, move anteriorly in the subdural space, and enter the cranial venuous sinuses. In abnormal hosts, such as moose, *P. tennuis*' longer development time, larger size, and altered behaviour in the CNS are believed to be responsible for causing parelaphostrongylosis or "moose sickness" (Anderson, 1964a; 1964b). In white-tailed deer, adult nematodes reproduce and the host passes dorsal-spined larvae in the feces. In most abnormal hosts, however, either the worms or the host dies before larvae can be passed; although infected moose and elk (*Cervus elaphus canadensis*) will occasionally pass L1s (Lankester, 2001).

Parelaphostrongylus andersoni (Prestwood, 1972) is a muscle worm that was first described from white-tailed deer from the southeastern United States including Alabama, Arkansas, Georgia, Louisiana, and North and South Carolinas (Prestwood et al., 1974; Anderson and Prestwood, 1981; Forrester, 1992). The parasite is also widely distributed in northern Canada, being reported additionally in woodland caribou (*Rangifer tarandus caribou*) of Newfoundland, Labrador, northern Quebec, northwestern Ontario, central Manitoba, and central Northwest Territories (Lankester and Hauta, 1989; Lankester and Fong, 1989; 1998). Further reports of *P.*

andersoni suggest a discontinuous distribution across the range of white-tailed deer in North America with foci in Michigan (Pybus et al., 1990), southeastern and central British Columbia (Pybus and Samuel, 1981) and northeastern Wyoming (Edwards, 1995)(Figure 1).

Similarly to *P. tenuis*, gastropods are the intermediate hosts for *P. andersoni* (Anderson and Prestwood, 1981; Lankester and Fong, 1998) and infection of the final host occurs by accidental ingestion. Rather than moving to the central nervous system, *P. andersoni* matures in the host's backstraps (longissimus dorsi), and other skeletal muscles (Pybus, 1983; Pybus and Samuel, 1984). Paired adult worms mate and females deposit eggs into small veins. The eggs are carried to the lungs where they hatch as L1s, migrate up the bronchial tree, are swallowed, and passed in feces (Lankester, 2001). The eggs of the parasite are responsible for disease, not the adults as with meningeal worm. Animals with low-level infections generally exhibit no signs of disease. (Prestwood et al., 1974; Lankester and Hauta, 1989). Heavy infections, however, result in large numbers of eggs and larvae in the lungs, which can cause respiratory distress and pneumonia (Prestwood and Nettles, 1977; Anderson, 2000). Wildlife biologists are interested in identifying *P. andersoni* infections because of their potential to mask *P. tenuis* infection where the ranges of these parasites overlap. In general, *P. andersoni* does not cause serious disease (Lankester, 2001).

Another muscle worm, *P. odocoilei* (Hobmaier and Hobmaier, 1934) was first described from Columbian black-tailed deer (*Odocoileus columbianus*), however, it has also been reported in mule deer (*Odocoileus hemonius*), mountain goat (*Oreamnos americanus*), and caribou (*Rangifer tarandus caribou*) (Pybus et al., 1984; Gray and Samuel, 1986). The parasite appears to have a strictly western distribution being known in mountain goats of northern Washington (Pybus et al., 1984), black-tailed deer (*Odocoilelus coloumbianus*) of British Columbia (Pybus et

al., 1984), and in mule deer (*Odocoileus hemonius*) of Alberta and California (Hobmaier and Hobmaier, 1934; Samuel et al., 1985a) (Figure 1). It has recently been reported in Dall's sheep (*Ovis dalli dalli*) of the Yukon and Northwest Territories (Kutz et al., 2001).

The life history of *P. odocoilei* closely resembles that of *P. andersoni* (Lankester, 2001). *Parelaphostrongylus odocoilei* also requires a gastropod intermediate host, which is accidentally ingested by the final host with vegetation. Likewise, the worms eventually mature in the skeletal muscles of the back (Lankester, 2001). Heavy infections are responsible for accumulations of large numbers of eggs and larvae in the lungs (Hobmaier and Hobmaier, 1934). Mule deer are thought to be particularly susceptible (Lankester, 2001).

Species of the genus *Elaphostrongylus* (Cameron, 1931) are generally restricted to Eurasia. The only member of this genus present in North America is *E. rangiferi* (Mitskevitch, 1958, 1964) known in caribou of Newfoundland (Lankester and Fong, 1989) (Figure 1). This parasite can also be found in wild and domestic reindeer of northern Fennoscandinavia and Russia (Lankester, 2001). Infection with *E. rangiferi,* or "brain worm", causes cerebrospinal elaphostrongylosis (CSE), a neurologic disease that occurs most often in young, heavily infected caribou (Lankester and Fong, 1998). Signs of CSE include lack of fear, poor condition, lameness, poor coordination, ataxia, and weakness of the hindquarters (Lankester and Northcott, 1979; Lankester and Fong, 1998).

Similarly, L1s of *E. rangiferi* require a gastropod intermediate host to develop to the L3 stage, and the gastropods are consumed accidentally by the host with vegetation. The L3s reach adulthood and mate in the CNS (Handeland and Skorping, 1992), after which they migrate out of the cranium and spinal canal (Hemmingson et al., 1993; Handeland, 1994). Adults of this parasite

can be found on or among muscles of the chest, abdomen, or hindlimbs (Lankester and Northcott, 1979; Hemingsen et al., 1993).

Elaphostrongylus cervi, also called "tissue worm" (Lankester, 2001), was first described from red deer (*Cervus elaphus*) of Scotland (Cameron, 1931). To date, the taxonomy of this parasite remains controversial because there appears to be differences in pathogenicity across its range. In Fennoscandinavia, Europe, and New Zealand, infected hosts typically show no sign of disease; although in Asia, deer farms experience periodic epizootics that can cause considerable economic damage (Lankester, 2001). The form of *E. cervi* found in Siberian red deer was named *E. panticola*. However due to the lack of consistently diagnostic morphological differences between them, it is generally accepted that *E. panticola* is a synonym of *E. cervi* (Gibbons et al., 1991).

As with other elaphostrongyline nematodes, *E. cervi* requires a gastropod intermediate host in which to develop to the L3 stage, and the infected gastropod must be ingested with vegetation. The L3s migrate into the thoracic cavity and then to the CNS via lateral nerves (Olssen et al., 1998). Anderson (1968) believed that some development might have to take place in the nerve tissue of the CNS before the worms can mature and move out into the skeletal muscles.

Regulatory agencies in both Canada (Gajadhar et al., 1994) and Australia (Presidente 1986a, 1986b) have prevented the introduction of *E. cervi* at quarantine facilities. However, laboratory studies have shown that the North American species of terrestrial gastropods *Triodopsis multilineata* and *Deroceras reticulum* are suitable intermediate hosts (Gajadhar and Tessaro, 1995), thus, imported *E. cervi* could potentially become established in North America.

Elaphostrongylus alces (Steen et al., 1989), also called "brain worm" (Lankester, 2001), is

known only in moose from Norway, Sweden, and Finland (Steen et al., 1989; Gibbons et al., 1991). In this host, *E. alces* causes the neurological disease elaphostrongylosis and has been associated with the wasting and death of animals, particularly calves and yearlings (Steen et al., 1998).

Moose become infected by ingesting infected gastropods. The L1s undergo a tissue migration to the CNS as with *E. rangiferi* (Olsson et al., 1995). Unlike *E. rangiferi*, however, *E. alces* does not penetrate the dura (Steen 1991). Worms mature epidurally along the spinal canal and then leave to establish among muscles of the back and thighs. Phylogenetic analysis using DNA sequence data may help to clarify relationships among elaphostrongyline species.

Refinement and Standardization of Nematode DNA Extraction and Amplification Techniques

Prior to attempting DNA analysis on any sample, it is necessary to assess its condition, and to develop and employ standard methods of analysis so that DNA recovery can be maximized and work may be easily replicated. A number of studies exist documenting the extraction and amplification of the nematode second internal transcribed spacer (ITS-2), however, each study used a different DNA extraction method and PCR parameters to obtain product (Gasser et al., 1993; Divina et al., 2000; Dallas et al., 2000; Gajadhar et al., 2000). For example, the melting temperatures of PCR primers are generally used to set the annealing temperature during a PCR reaction. Most protocols generally recommend using an annealing temperature of 2-5 °C below the melting temperatures of the primers to achieve optimum specificity (Henegairu et al., 1997). The studies cited above used annealing temperatures ranging from 55 to 60°C. The present study set out to determine the optimum PCR cycling times and temperatures for amplification of the

elaphostrongyline ITS-2 rDNA region.

Gasser and colleagues (1993) reported difficulties obtaining pure *Trichostrongylus* spp. samples with sufficient template DNA to amplify by PCR; a general phenomenon that has also been reported elsewhere (Gasser, 2001). This failure to obtain amplifiable DNA from individual adults and larvae has been attributed to the presence of a tough cuticle (Gasser et al., 1993; Gasser, 2001). Furthermore, co-precipitating substances produced during extraction may inhibit subsequent PCR (Simpson et al., 1982; Gasser et al., 1993, Gasser, 2001).

Gajadhar and colleagues (2000) extracted and amplified DNA from adult elaphostrongylines, pooled L1s, and pooled L3s, however, their extraction protocol involved the use of organic solvents, including phenol and chloroform, and physical grinding of the nematodes that could result in shearing of the DNA (Gasser et al., 1993; Gasser, 2000). The protocols outlined in Gajadhar et al. (2000) are lengthy and did not address the problem of extracting DNA from individual larvae. Moreover, because of the use of organic solvents, laboratories lacking expensive fume hoods could not replicate the work. Other extraction procedures (Banerjee et al., 1995; Dallas et al., 2000) call for an even more lengthy proteinase K digestion in extraction buffer, which can require an 18 hour incubation step.

Several studies reported the use of the QIAamp *Tissue Kit* from Qiagen (Valencia, California), however, in all cases the extractions were conducted solely on fragments of adults or whole adult nematodes. No modifications to the manufacturer's procedure were cited in these studies, nor were the nematodes reported to be fixed, only frozen (Heise et al., 1999; Hoglund et al., 1999; Divina et al., 2000). Studies of preserved human tissues indicate fixative time and fluid may affect the ability to amplify DNA (Greer et al., 1991; Gall et al., 1993; Isola et al.,

1994; Coombs et al., 1999). Larval and adult nematodes are often fixed in preservatives such as formalin, ethanol, and glyceralcohol (Fagerholm, 1979), substances that could be responsible for the degradation of DNA and inhibition of the PCR (Greer et al., 1991; Coombs et al., 1999; Barnes et al., 2000).

First-stage protostrongylid larvae present a particular problem because the modified Baerman technique (Forrester and Lankester, 1997) lets particles of fecal material filter into the water along with the parasites. The resulting filtrate is a mixture of water, feces, and larvae. Humic substances and excess bacterial DNA contained in fecal material have been identified as potential inhibitors (Machiels et al., 2000) and must be removed from the sample prior to PCR.

This study focuses specifically on the ITS-2 region because ribosomal DNA serves as a taxonomic discriminator at the species and genus levels of micro-parasites (Newton et al., 1988a, 1988b; Hoste et al., 1995; Dallas et al., 2000; Divina et al., 2000; Gadjadhar et al., 2000). Furthermore, the ITS-2 region is part of a tandem repeat (Ellis et al., 1986) and, because it is present in more than one copy, the region may amplify better from degraded samples than would a single copy gene.

The present study seeks to refine and standardize previously published protocols, and to retrieve amplifiable DNA from formalin, ethanol, and glyceralcohol fixed nematodes. Samples in this study, fixed for as long as seven years, were tested for successful extraction and amplification in an attempt to adduce the possibility of obtaining DNA from still older, archival, fixed specimens. Standard phenol-chloroform methods employed in previous studies (Gasser et al., 1993; Gajadhar et al., 2000) were replaced by safer procedures reported in the literature (Newton et al., 1988a, 1988b; Hoste et al., 1995; Dallas et al., 2000; Divina et al., 2000) and here. A

commercial kit format (QIAamp) was the optimal extraction method, as this would allow for relatively inexpensive and rapid extraction, and simultaneous purification of large numbers of samples.

Identification of Elaphostrongyline Nematodes Using Molecular Techniques

The geographic ranges of North American elaphostrongylines overlap in some areas (Lankester, 2001). Hence, any identification technique, including DNA analysis, must consider the possibility of mixed-species infections by being sufficiently specific to screen both pools of larvae and individual larvae. The most likely areas in which mixed infections occur will be in zones where parasite distributions of the overlap. In Newfoundland, *P. andersoni* co-occurs with *E. rangiferi* (Lankester and Fong, 1998). On the west coast, the distribution of *P. andersoni* can overlap with *P. odocoilei* (Ballantyne and Samuel, 1984). In the southeastern United States, *P. andersoni* may co-occur with *P. tenuis* (Prestwood et al., 1974) (Figure 1).

Isolation of adult elaphostrongylines for morphological identification is problematic because retrieval from the brain and back muscles of their hosts requires a difficult necropsy involving a search through the back-straps (longissmus dorsi), CNS, and other skeletal muscles of infected animals (Lankester, 2001). The identification of first-stage larvae (L1) can also be problematic due to the fact that the six elaphostrongyline species produce morphologically indistinguishable larvae with a dorsal spine (Lankester, 2001). To further compound the problem, all members of the family Protostrongylidae, except *Protostrongylus* spp. produce dorsal-spined larvae. Examples of such species that may occur in wild ungulates are *Muelleruis capillaris*, *Umingmakstrongylus pallikukensis*, *Varestrongylus alpenae*, and *Orthostrongylus macrotis.*

The larvae of elaphostrongyline parasites are ideal candidates for DNA identification because the L1s are easily collected from the host's faeces (Forrester and Lankester, 1997), and the L3s can be recovered from enzymatically digested gastropod intermediate hosts (Lankester and Peterson, 1996; Lankester, 2001). A DNA test that identifies larvae would reduce the need to locate, euthanize, and necropsy infected animals.

In addition to four species currently found in North America, two European species – *E. alces* and *E. cervi* – were included in this study (1) to resolve the molecular-genetic relationship among *E. cervi, E. rangiferi, and E. alces,* and (2) because *E. cervi* could potentially be carried to North America in imported animals, such as red deer (Gajadhar et al., 1994).

Systematics of the Elaphostrongyline Nematodes

Previous studies examining the systematics of elaphostrongyline nematodes based on their morphology include a reconstruction of the genus *Parelaphostrongylus* by Carreno and Lankester (1994); an analysis of the relationships among elaphostrongyline nematodes, not including *E. rangiferi* or *E. alces* (Platt, 1984); an analysis of the morphological differences among *Elaphostrongylus* spp. (Gibbons et al., 1999), and a phylogeny of nematodes in the family Protostrongylidae, which included only *E. cervi* and *P. odocoilei* from the Elaphostrongylinae (Carreno and Hoberg, 1999).

The study by Platt (1984) found two equally parsimonius cladograms for *P. tenuis, P. andersoni*, and *P. odocoilei. Elaphostrongylus cervi* was the most pleisiomorphic species in the study and was used as the outgroup. The genus *Parelaphostrongylus* was monophyletic in both topologies, but the arrangement of taxa within the genus differed. One reconstruction paired *P.*

andersoni and *P. odocoilei* (muscle worms) with *P. tenuis* (meningeal worm), sister to that clade. A second cladogram united *P. tenuis* and *P. andersoni*, both parasites of white-tailed deer, in the internal clade with *P. odocoilei*, a parasite of mule deer, in the basal position. Platt (1984) suggested that grouping of parasites based on the location of the parasite within the host (first reconstruction) was a more likely scenario, and thus favoured the muscle worm alliance. It is now known that *P. andersoni* is widespread in caribou of northern Canada, perhaps more so than in white-tailed deer (Lankester, 2001), which would call into question the idea of a close evolutionary relationship between *P. andersoni* and *P. tenuis* based on the fact that both species parasitize white-tailed deer.

Carreno and Lankester (1994) also demonstrated that the genus *Parelaphostrongylus* is monophyletic with an internal clade comprising muscle worms *P. andersoni* and *P. odocoilei*, with the meningeal worm *P. tenuis* basal to this pair. *Elaphostrongylus rangiferi* was used as an out-group.

Gibbons et al. (1991) revisited the morphological characteristics of the elaphostrongyline nematodes and concluded that *E. panticola* was a synonym of *E. cervi*, but that *E. cervi*, *E. rangiferi*, and *E. alces* should be considered distinct species. These ideas are both phylogenetic inferences, however, no statistical analyses were carried out on qualitative characteristics, and no tree was given.

The present study uses sequence data to (i) test the previous taxonomic inference of *Parelaphostrongylus* based on the location of the parasite within the host, and (ii) resolve the relationships among *E. cervi*, *E. rangiferi*, and *E. alces* by relating them to the other elaphostrongyline nematodes.

Ribosomal DNA as a Species Identifier

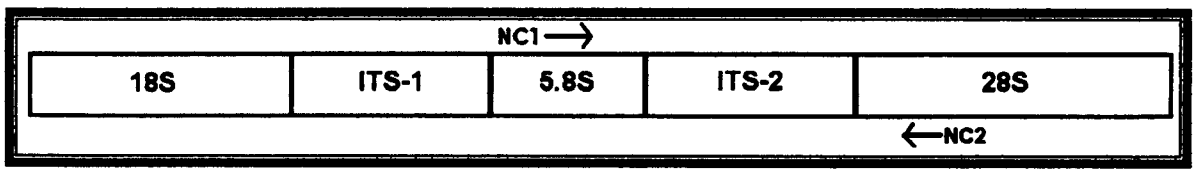
In eukaryotic organisms, there exists multiple copies of highly conserved rRNA genes that code for ribosomal RNAs. These genes are often arranged in a series (tandem repeat) where each gene is separated from the next by a stretch of non-coding DNA, known as spacer DNA. Spacer DNA is thought to play a role in the assembly of the primary RNA transcript, but it varies greatly in length and sequence depending on the organism in question (Alberts et al., 1994).

One complete tandem repeat on *Caenorhabditis elegans* chromosome 1 was sequenced by Ellis et al. (1986). This tandem repeat consisted of the external transcribed spacer (ETS) followed by (i) the 18s rRNA gene; (ii) the internal transcribed spacer 1 (ITS-1); (iii) the 5.8s rRNA gene; (iv) the internal transcribed spacer 2 (ITS-2); and (v) the 26s rRNA gene (homologous to 28s). Both the 5' and the 3' ends of the genes were found to have sequences that are conserved in several nematode species (Campbell et al., 1995; Gasser and Monti, 1997; Heise et al.,1999; Hung et al., 1999). Universal primers NC1 (5' ACGTCTGGTTCAGGGTTGTT 3') and (NC2 5' TTAGTTTCTTTTCCTCCGC 3') were developed from these regions of homology to amplify the ITS-2 region of nematodes (Ellis et al. 1986; Gadjadhar et al., 2000) (Figure 2).

Detecting Genetic Variation with Molecular Techniques

There are two general approaches to the problem of studying genes that may contain species-specific information. The first method, DNA-DNA hybridisation, relies on the premise that single-stranded DNA with sequence similarity (homology) of 60-70% will hybridize under appropriate conditions to form a stable, double-stranded molecule (Alberts et al., 1994; Potts, 1996). The unknown species DNA can be tested for sequence homology based on how much

FIGURE 2. Diagram of the linear organization of rRNA genes and their associated spacer DNA comprising a tandem repeat on *C. elegans* chromosome 1 (Ellis et al., 1986). The approximate locations of universal primers NC1 and NC2 are indicated with arrows.



DNA of each species has hybridised. This technique works well with large genes and genomic DNA. (Alberts et al., 1994; Potts, 1996).

For smaller genes, PCR based methods work especially well. In some cases, several taxa that contain the same gene can be amplified with a single primer pair – called universal primers – because the primer sequence exists in all taxa of interest. PCR and gel electrophoresis of DNA fragments amplified by universal primer sets can sometimes reveal visible differences in the size of PCR products produced from different taxa, which can be used as a practical diagnostic tool, however, this procedure (Bowels and McManus, 1993). In taxa that are closely related, PCR and gel electrophoresis may not be sufficient to reveal diagnostic size differences on an electrophoretic gel. In other words, the greater the sequence homology between taxa, the more difficult it is to distinguish them with PCR and gel electrophoresis alone. In such cases, researchers must go beyond PCR and use techniques such as DNA sequencing, restriction fragment length polymorphism (RFLP), an examine other regions of the DNA to distinguish closely related taxa (Gasser, 2001).

DNA sequencing determines the exact order of nucleotide bases (adenine, thymine, cytosine, and guanine) that make up a gene or fragment of DNA. Once the base sequence of an organism's DNA is known, it can be compared to that of other organisms. The species and relatedness of the organisms can then be investigated by phylogenetic analysis. Thus, DNA sequencing is the method that provides the finest resolution of all methods. In addition, recent technological advances in this area have resulted in the development of sequencing protocols that are less costly and time consuming. However, it is not always practical to sequence large numbers of samples in an attempt to identify species. If sequences can be obtained for the organisms of

interest, this information can then be used to identify differences in base composition to develop and refine less expensive and time consuming methods of identification such as restriction fragment length polymorphism (RFLP) and species specific PCR primers.

The RFLP technique builds on the tendency for certain bacterial enzymes to cut DNA at specific base combinations (either combinations of four, six or eight bases) within a sequence. If the enzyme recognition sequences are variable between species in their presence or absence, number of recognition sequence occurrences, or length of fragment produced, then the enzyme may be used to distinguish species. When restricted PCR products are run on an electrophoretic gel, the variable size and number of fragments provide reliable taxonomic identifiers. The RFLP technique, thus, detects genetic variation rapidly without the need for reading the base composition throughout the entire region of interest.

In this study, PCRs of the second internal transcribed spacer (ITS-2) region were carried out with universal nematode primers developed by Ellis et al. (1986) and previously used by Gajadhar et al. (2000). The six species of elaphostrongyline nematodes could not be identified by the size of their ITS-2 PCR products alone, so the ITS-2 regions of all six species were sequenced. The sequence data provided potential RFLP recognition sites that could be used to distinguish between the species despite the highly conserved nature of the ITS-2 region in these taxa.

CHAPTER 2: IMPROVED METHODS FOR THE EXTRACTION AND AMPLIFICATION OF ITS-2 rDNA FROM NEMATODES IN VARIOUS CONDITIONS

Sample Collection

First-stage dorsal-spined, protostrongylid larvae were isolated from faeces of individual cervids by Ahn Dao (2000), using the modified Baerman beaker technique (Forrester and Lankester, 1997). This method involves placing faeces in an envelope made from fibreglass window screening, which is submerged inside a water-filled beaker. Viable larvae pass through the screening, sink to the bottom of the beaker. After 6-24 hours, the water can be decanted and the larvae counted.

L3s were isolated by pepsin digest from gastropod intermediate hosts (Lankester and Peterson, 1996). L1s and L2s were killed by the pepsin digest and only viable L3s were recovered using this method (Lankester and Anderson, 1968).

Individual larvae were removed from pooled samples of approximately 20-25 L1s or L3s by dispensing some of the storage media containing parasites into a small Petri dish and examining the sample with a stereomicroscope at 40X magnification. Single larvae were pipetted into a 1.5ml microcentrifuge tube. Adult nematodes were recovered from hosts at necropsy. In most cases, the posterior ends of adult males were kept for morphological identification and the anterior portion of the worm was processed for DNA analysis.

DNA Extraction Protocol

DNA was extracted from individual L1s and L3s, pooled samples of approximately 10-25 L1s from individual cervids, pools of approximately 10-15 L3s, and anterior ends and middle

fragments of adult nematodes, both male and female (See Appendix). Pooled samples refer to a collection of larvae from an individual host. Some donated samples were fixed in ethanol or 10% glyceralcohol made with 70% ethanol. Other samples, which were originally received in water or Baerman filtrate, were transferred to 10% formalin, where they remained fixed for two months before testing for positive PCR amplification.

A nematode sample and approximately 100µl of its storage medium (See Appendix) were pipetted into a 1.5ml microcentrifuge tube and desiccated into a pellet in 20 minutes using a vacuum desiccator. DNA was extracted with QIAamp *Tissue Kit* from Qiagen (Valencia, California). The following modifications (underlined) were made to the manufacturers instructions:

I. Pelleted samples (including fecal particles) were washed twice with 100µl TE buffer (10mM Tris – HCl, 1mM EDTA, pH 8.0) prior to QIAamp extraction, centrifuged for 5 min at full speed (~14,000 rpm), and the supernatant removed and discarded. Residual TE did not affect subsequent steps.

II. 180µl of Buffer ATL was added to the pellet and pulse vortexed for approximately 10 seconds.

III. 20µl of proteinase K was added to the sample/Buffer ATL mixture and incubated at 70°C for 3 hours and 1 additional hour at 95°C.

IV. After incubation, 20µl of RNAse and 200µl of Buffer AL were added to the sample mixture and pulse vortexed for approximately 10 seconds.

V. 200µl of molecular biology grade (96%) ethanol (Sigma, St. Louis, Missouri) was added to the sample mixture and pulse vortexed for approximately 10 seconds.

VI. The mixture from step V (including the precipitate) was carefully applied to the QIAamp spin column and 500µl buffer AW1 was added. The solution was mixed well. The column was centrifuged at 8000rpm for 1 minute. The QIAamp spin column was placed in a new 2ml collection tube and the tube containing the filtrate was discarded.

VII. The QIAamp spin column was opened carefully and 500µl AW2 was added and the solution was mixed well. The column was centrifuged at full speed (~14,000 rpm) for 1 minute. The tube containing the filtrate was discarded.

VIII. The QIAamp spin column was placed in a new 2ml collection tube and centrifuged at full speed for 1 minute to eliminate buffer AW2 carry-over. The tube containing the filtrate was discarded.

IX. The QIAamp spin column was placed in a new 2ml centrifuge tube (to contain the final elutant) and 200µl of buffer AE was added and allowed to incubate at room temperature for 2 minutes. The column was centrifuged at 8000 rpm for 1 minute.

X. Step IX was repeated twice for a total of 600µl of purified DNA extract.

XI. For long-term storage, the DNA was eluted in buffer AE and placed at -20°C as per the manufacturer's recommendations.

In addition, one hair root sample from each of muskox (*Ovibos moschatus*), elk (*Cervus elaphus canadensis*), white-tailed deer (*Odocoileus virginianus*), and moose (*Alces alces*) were extracted with this modified QIAamp procedure to ensure negative PCR amplification with the parasite primers NC1/NC2 (Ellis et al., 1986) used in this study.

DNA Quantification and Absorbance at 260nm and 280nm

Samples were quantified and purity was checked using the Gene Quant II spectrophotometer (Pharmecia Biotech). QIAamp purified extract was diluted 1:10, 1:100, and 1:1000 in Buffer AE. For each dilution, 75µl of extract was pipetted into the cuvette and the absorbance and concentration values were read three times. The average concentration and absorbance readings of samples were calculated (Table 1). Elaphostrongyline ITS-2 PCR product of known concentration (200 ng/µl) was added to control samples of low, moderate, and high levels of fecal material, 10% formalin, 70% ethanol, 95% ethanol, and glyceralcohol (without any nematodes), and the absorbance and concentration of DNA was checked.

The A260/A280 absorbance ratios (indicator of purity) of the controls and of some samples were low indicating that impurities were likely present. An additional purification with p30 (BioRad Corporation) size exclusion columns and recheck of the A260/A280 absorbance ratio was carried out in these cases.

Polymerase Chain Reaction (PCR) Protocol

Initial PCR's on QIAamp extracted nematodes were carried out according to the method outlined by Gajadjar et al. (2000), however, this combination of procedures produced excess DNA template and the PCR was optimized accordingly.

The Universal primers NC1 and NC2, were constructed commercially, HPLC purified, and lyophilized by Operon Technologies (Almeda, California). Primers were resuspended in sterile, double distilled water to a concentration of 10µM each before use.

PCR reactions were carried out in a sterile hood physically separated from the extraction

area, and the purification area to prevent cross contamination of sample DNA. A master mix of reagents was made containing 0.25 mM each of dATP, dCTP, dGTP, dTTP (New England Biolabs, Beverly, Massachusetts), 2 mM $MgSO_4$ (New England Biolabs), 1X bovine serum albumin (New England Biolabs), 1X thermopol reaction buffer containing 2 mM $MgSO_4$ (New England Biolabs), 0.2 μM primer (Operon Technologies), 2.5 units (U) of Deep Vent® DNA (exo-) polymerase (New England Biolabs), and sterile, double distilled water (Sigma, St. Louis, Missouri). Purified DNA extract was added at a volume of 1 μl to 49 μl of the master mix. All PCR preparations were carried out on ice to prevent the premature activation of Deep Vent® (exo-). Annealing temperature is among the most important parameters in a PCR reaction, therefore, the optimal annealing temperature for NC1/NC2 PCR reactions was determined by carrying out a gradient PCR in an Eppendorf Master Cycler Gradient thermocycler. Possible annealing temperatures from 53°C to 64°C were tested. Cycling times of 15 and 30 seconds and one minute were tested over the course of several PCRs each with differing denaturation, annealing, and extension times. The optimal PCR parameters were as follows: (i) DNA was subjected to hot start at 96°C for 5 minutes, followed by (ii) 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 30 seconds; and (iii) final hold at 4°C. High yield samples sometimes produced large, non-specific PCR artifacts that could interfere with sequencing. In these cases, purified sample was diluted 1:5 or 1:10 before use in PCR.

PCR amplification results were checked on a 5% polyacrylamide gel. The gels were stained for 25 minutes in an ethidium bromide solution consisting of 5μl ethidium bromide in 100 ml 1X TBE buffer (Tris, Boric Acid, EDTA). All gels were visualized on a transilluminator, and

photographed with a Polaroid Gel Cam (Polaroid Corp., Cambridge, Massachusetts).

Results

Adult nematodes, adult fragments, and pooled samples of 25 larvae generally produced extracts with higher amounts of DNA than samples of a single larva (Table 1). Samples that were fixed in 10% formalin yielded amounts of DNA similar to those from samples stored in water. However, samples in both 70% and 95% ethanol and in glyceralcohol yielded lower amounts of DNA than those in water or formalin (Table 1). Samples containing ethanol and glyceralcohol had low A260/A280 ratios indicating that impurities were present in the DNA extract. The Gene Quant II could not provide a DNA concentration value (read as concentration of 0 ng/μl) for theses samples until additional purifications with p30 columns were carried out. The concentration of control samples of unpurified storage media with 200 ng/μl PCR product added could not be read (0 ng/μl).

All samples (See Appendix) showed positive ITS-2 amplification bands on polyacrylamide gels. Some samples originally contained extremely high amounts of fecal material and potential PCR inhibitors such as ethanol and glyceralcohol, and additional purification with p30 gel filtration cartridges (BioRad Corporation, Hercules, California) was required before they would amplify. No amplification of host sample DNA was detected in any PCR amplification.

PCR reactions carried out on QIAamp extracted nematodes, using the parameters outlined by Gajadhar et al. (2000), produced excess template, and extremely high levels of high molecular weight non-specific PCR product (Figure 3), therefore, the PCR procedure was modified to compensate for the high amount of DNA obtained by QIAamp extraction. The results of the

TABLE 1. Preservation conditions and time since collection in relation to the purity indicator (A260/A280 ratio) of selected samples. A sample with a ratio number at or below 1.8 is considered to contain impurities.

Sample #	Sample ID	Stage/Type	Storage Media	Collection Date	Concentration (ng/ul)	A260/A280 Ratio
1a	*E. alces*	L1- Single	Baerman filtrate*	1997	2.003	2.132
2a	*E. alces*	L1- Single	Baerman filtrate*	1997	3.300	2.222
3a	*E. cervi*	L1- Single	Baerman filtrate***	Jun.00	3.000	2.593
4a	*E. cervi*	L1- Single	Baerman filtrate***	Jun.00	4.112	1.899
5	*E. rangiferi*	Adult	H20	Apr.01	30.200	1.195
6	*E. rangiferi*	Adult	H20	Apr.01	27.626	2.043
7	*E. rangiferi*	Adult	H20	Apr.01	25.595	2.045
8	*E. rangiferi*	Adult	Glyceralcohol	Apr.01	0a / 5.574b	1.121a / 1.992b
9	*E. rangiferi*	Adult	Glyceralcohol	Apr.01	0a / 10.001b	1.021a / 2.210b
10a	*E. rangiferi*	L1 - Single	Baerman filtrate*	N/A	4.280	2.293
11	*O. macrotis*	Adult	Glyceralcohol	2001	0a / 9.159b	1.339a / 2.001b
12	*O. macrotis*	Adult	H20	2001	19.650	1.189
13	*O. macrotis*	L3 - Pooled	H20	2001	21.200	1.176
14	*P. andersoni*	Adult	H20	Apr.01	20.001	2.155
15	*P. andersoni*	Adult	H20	Apr.01	23.430	2.176
17	*P. andersoni*	L1 - Pooled	Baerman filtrate*	Feb.00	22.008	2.211
18	*P. andersoni*	L1 - Pooled	Baerman filtrate*	Sep.99	27.612	2.035
21	*P. odocoilei*	L1 - Pooled	Baerman filtrate*	N/A	20.869	2.179
23	*P. odocoilei*	Adult	10% Formalin	1990	23.537	2.168
24	*P. odocoilei*	Adult	10% Formalin	1990	22.265	2.196
25	*P. odocoilei*	Adult	10% Formailn	1990	27.380	2.178
26	*P. odocoilei*	Adult	H20	1990	24.052	2.170
27	*P. odocoilei*	Adult	H20	1990	19.436	2.204
28	*P. odocoilei*	Adult	H20	1990	20.675	2.034
32	*P. tenuis*	Adult	70% Ethanol	Jun.99	9.530	1.230

[a] Reading taken before additional purification with p30 columns,

[b] Reading taken after additional purification with p30 columns, * low fecal debris, ** moderate fecal debris,

*** high fecal debris

TABLE 1 continued. Preservation conditions and time since collection in relation to the purity indicator (A260/A280 ratio) of selected samples. A sample with a ratio number at or below 1.8 is considered to contain impurities.

Sample #	Sample ID	Stage/Type	Storage Media	Collection Date	Concentration (ng/ul)	A260/A280 Ratio
33	*P. tenuis*	Adult	70% Ethanol	Jun.99	11.381	1.521
34	*P. tenuis*	Adult	70% Ethanol	Jun.99	0a / 1.115b	1.166a / 1.541b
35	*P. tenuis*	Adult	70% Ethanol	Jun.99	0a / 8.808	1.105a / 2.232b
36	*P. tenuis*	Adult	70% Ethanol	Jun.99	0a / 0.995b	1.292a / 2.321b
40	*P. tenuis*	Adult	Glyceralcohol	Jul.94	0a / 7.775b	1.111a / 1.190b
41	*P. tenuis*	Adult	Glyceralcohol	Jul.94	0a / 12.130b	1.027a / 2.234b
42	*P. tenuis*	Adult	Glyceralcohol	Jul.94	0a / 9.954b	1.18a / 2.312b
43	*P. tenuis*	Adult	Glyceralcohol	Jul.94	0a / 6.250b	1.184a / 2.222b
45	*V. alpenae*	Adult	Glyceralcohol	2001	0a / 7.600b	1.595a / 1.994b
47	Putative *P. tenuis*	L1 - Pooled	H20	Feb.00	6.955	1.598
47a	Putative *P. tenuis*	L1- Single	H20	Feb.00	4.619	1.400
48a	Putative *P. tenuis*	L3 - Single	70% Ethanol	Feb.00	0.862	1.253
48b	Putative *P. tenuis*	L3 - Single	70% Ethanol	Feb.00	4.400	1.339
50a	Unident.1	L1- Single	Baerman filtrate**	Mar.00	3.160	1.810
51a	Unident.2	L1- Single	Baerman filtrate**	Mar.00	3.327	1.796
58	Unident.9	L1 - Pooled	Baerman filtrate*	Feb.00	17.755	1.799
59a	Unident.10	L1- Single	Baerman filtrate**	Dec.99	5.931	2.199
65a	Unident.16	L1- Single	Baerman filtrate**	Feb.00	7.132	2.164
66	Unident.17	L1 - Pooled	Baerman filtrate*	Mar.00	23.705	2.137
67a	Unident.18	L1- Single	Baerman filtrate*	Nov.99	0.824	2.128
72a	Unident.23	L1- Single	Baerman filtrate*	Nov.99	5.616	2.108
73	Unident.24	L1 - Pooled	Baerman filtrate*	Nov.99	22.576	2.081
74	Unident.25	L1 - Pooled	Baerman filtrate*	Nov.99	20.022	2.004
75	Unident.26	L1 - Pooled	Baerman filtrate*	Nov.99	24.105	1.986
76a	Unident.27	L1- Single	Baerman filtrate*	Mar.00	2.221	1.937
83a	Unident.34	L1- Single	Baerman filtrate*	Aug.98	1.062	2.122

[a] Reading taken before additional purification with p30 columns,

[b] Reading taken after additional purification with p30 columns, * low fecal debris, ** moderate fecal debris, *** high fecal debris

FIGURE 3. Gel photograph (5% polyacrylamide) demonstrating excess ITS-2 PCR product and extremely high levels of high molecular weight non-specific product using parameters outlined by Gajadhar et al. (2000) on QIAamp extracted nematodes. Lanes 1 to 4: adult *E. rangiferi* (#5, #6, #7, #8). Lanes 5 and 6: *E. cervi* L1's (#3, #4) Lanes 7 and 8: adult *P. odocoilei* #26 and #27, Lane 9: negative extraction reagent control, Lane 10: negative PCR reagent control, Lane 11: molecular size marker pBR322/MspI digest.

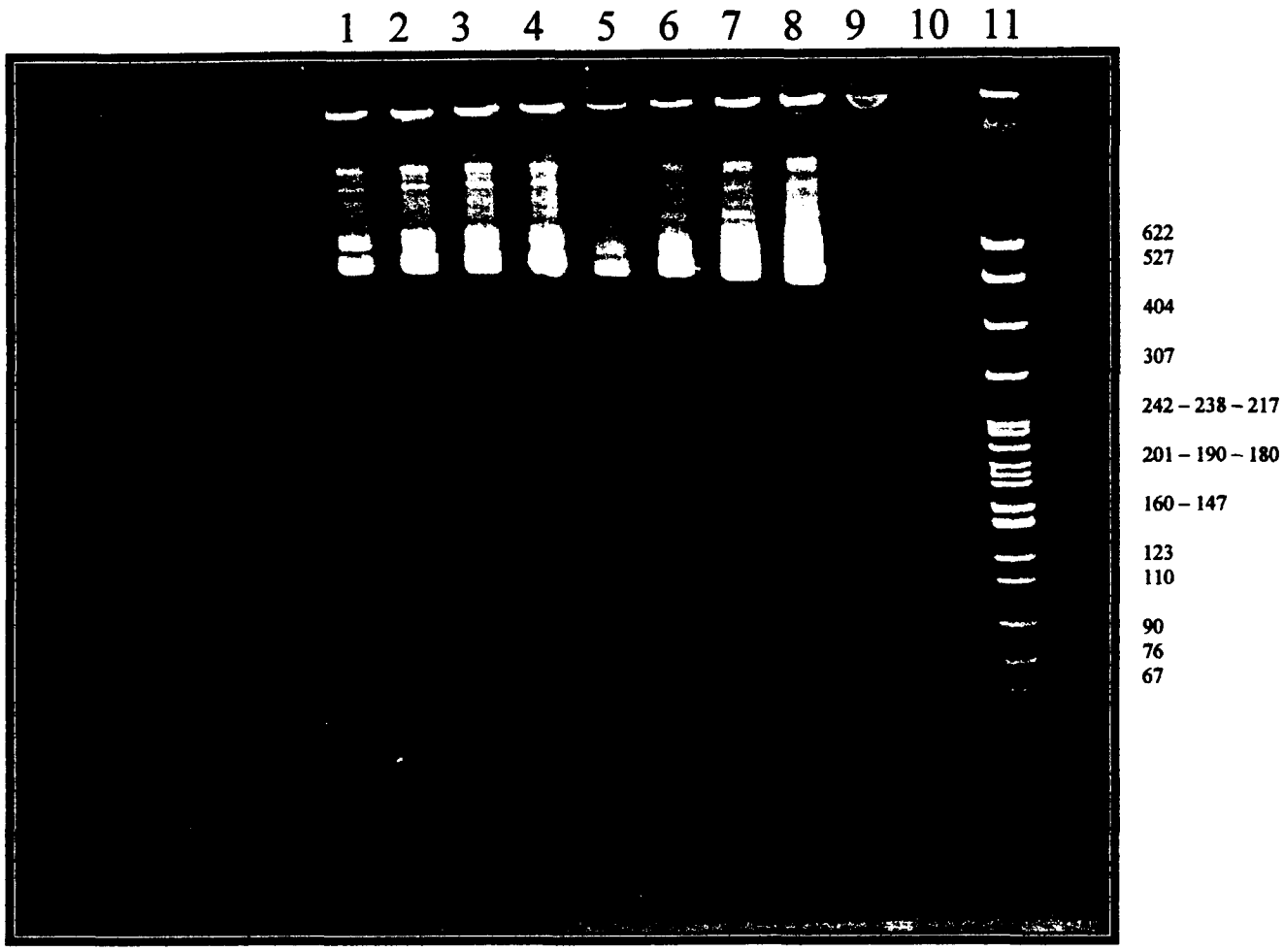
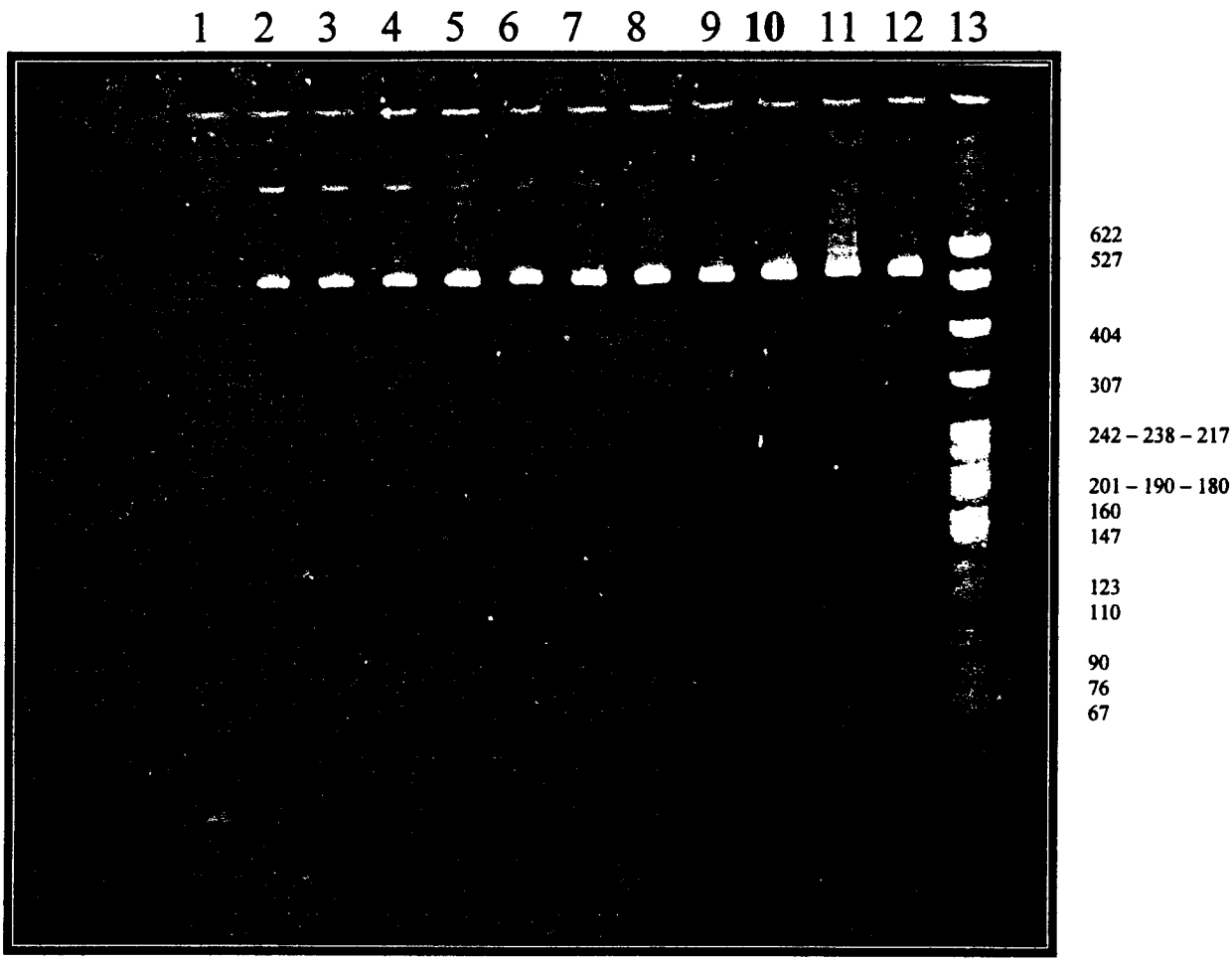


FIGURE 4. A representative gradient PCR (primers NC1 and NC2) used to determine optimum annealing temperature (range: 53.0°C to 64.0°C). The sample used to determine optimal annealing temperature was adult *P. odocoilei* (#27) cycled using the following temperatures:Lane 1: 64.0°C, Lane 2: 63.8°C, Lane 3: 63.1°C, Lane 4: 62.3°C, Lane 5: 61.1°C, Lane 6: 59.6°C, Lane 7: 57.7°C, Lane 8: 56.1°C, Lane 9: 54.9°C, **Lane 10: 54.0°C**, Lane 11: 53.3°C, Lane 12: 53.0°C, Lane 13: molecular size marker pBR322/Msp1 digest (New England Biolabs). Optimal temperature is shown in bold. Polyacrylamide gel concentration is 5%.



gradient PCR, using *P. odocoilei* (sample 27) as the test specimen, are shown in Figure 4. The optimal annealing temperature for subsequent reactions was determined to be 54°C. At this temperature, there are no PCR artifacts or "satellite bands." In gradient PCR's, all other elaphostrongyline nematodes amplified well with a 54°C annealing temperature (data not shown).

Discussion

The difficulties of extracting DNA from single L1s and L3s (See Chapter 1) have been overcome by developing the methods outline above. The sensitivity and versatility of this technique also allows amplification of DNA from fixed archival samples that may have been previously passed over for study due to their preservation condition and time spent in storage, which increases the feasibility of obtaining viable ITS-2 sequences from more antiquated nematode collections. Individual and pooled L1s, L3s, adults, and fragments of adults suspended in 95% ethanol, 70% ethanol, glyceralcohol, 10% formalin, or distilled water can be extracted and amplified using this technique.

The nitrogenous bases in nucleotides have a light wavelength absorption maximum of about 260 nm. In contrast to nucleic acids, proteins have a UV absorption maximum of 280 nm due to tryptophan residues. The absorbance of a DNA sample at 280 nm gives an estimate of the protein contamination of the sample. The ratio of the absorbance at 260 nm/ absorbance at 280 nm is a measure of the purity of a DNA sample; it should be between 1.65 and 1.85. Some samples in this study had high A260/A280 ratios after additional purification with p30 size exclusion columns, however, the contaminating proteins did not affect the ability to amplify DNA. The A260/A280 absorbance ratios did not correlate with DNA concentration, and were more

dependent on the presence of humic substances and other PCR inhibitors within the sample.

Given that ethanol is widely used in molecular biology procedures, the poor amplification of the ethanol fixed material versus the good amplification of the formalin-fixed specimens seems to be a surprising result. However, poor penetration of the ethanol could leave the interior of the sample biologically active and therefore subject to degradation by cellular nucleases which can remain active for a considerable amount of time, whereas the protein cross-linking effect created by formaldehyde fixation may avoid this process (Greer et al. 1990; Barnes et al. 2000). Furthermore, cell wall disruption caused by protein denaturation and ethanol leaching of lipid components could allow DNA to migrate into the surrounding medium and this phenomenon has been reported elsewhere (Greer et al. 1990; Barnes et al. 2000).

The QIAamp method utilizes a combination of extraction by digestion with proteinase K (PK) and purification with a silica-based spin column (QIAamp® *Tissue Kit* manufacturers instructions, 2000). It has been suggested that silica-based purification can remove PCR inhibitors (Yang et al., 1998). Moreover, the QIAamp method removes potentially interfering RNA and poses no risk to researchers because it does not involve the use of toxic organic substances. This technique has the advantage over those used by Gasser and colleagues (1993) and Gajadhar and colleagues (2000) by having extraction and purification combined in one simplified procedure. In addition, the optimal PCR cycling times and temperatures were found by carrying out a gradient PCR, which tested 12 different temperatures simultaneously. The assay specificity achieved using gradient optimized PCR parameters significantly reduces "satellite bands" reported in some PCR reactions (Gajadhar et al., 2000). An annealing temperature of 60°C and extension time of one minute, as used in Gajadhar et al. (2000), resulted in template overload and high molecular weight

non-specific product that interfered greatly with subsequent sequencing attempts.

Furthermore, this rapid technique can increase laboratory sample throughput. This researcher found that batches of 25 samples can be extracted, purified, amplified, and sequenced in less than two working days. The protocol outlined above is more easily reproduced than those previously mentioned, and should enhance the ability of laboratories to accurately and rapidly screen larval samples, an important factor in the management of cervid parasites.

Extraction with QIAamp generally recovers high concentrations of DNA when only a small quantity (1µl) of sample is used in each 50µl reaction. In addition, the DNA extract can be stored long term at –20°C for later analysis. In some pooled samples, 1µl of extract was too concentrated and required dilution of 1:5 or 1:10 before adding 1µl to the PCR reaction. Concentrations of DNA template that were too high produced high molecular weight non-specific product that interfered in sequencing and restricting the sample.

Other changes from the protocol used by Gajadhar et al. (2000) were to the PCR annealing temperature and time, and choice of polymerase used to amplify the DNA. The annealing temperature was changed to 55°C based on sample amplification using gradient PCR because 55°C consistently did not produce "satellite bands." The annealing time was also lowered to 30 seconds to reduce the possibility of PCR mis-priming that may result initially in non-specific product. Deep Vent® DNA (exo-) Polymerase (New England Biolabs) was chosen for this study because (i) it has a lower error rate than *Taq* polymerase, (ii) does not have the tendency to insert A's at truncated locations in the sequence, and (iii) has a longer half-life than *Taq* polymerase (Jannasch et al., 1992). Use of Deep Vent® (exo-) Polymerase may reduce the number of polymerase-generated errors, especially when amplifying DNA from preserved, potentially

degraded specimens.

In summary, there are several improvements provided by this protocol over previously published methods (Gasser et al., 1993; Hoste et al., 1995; Dallas et al., 2000; Divina et al., 2000; Gajadhar et al., 2000). The use of the modified QIAamp procedure eliminates the use of organic solvents and therefore, poses little health risk to researchers. The procedure saves time by incorporating extraction and purification in one simple procedure. It eliminates the grinding step included in many published procedures which avoids mechanical shearing of DNA by tools, such as zirconium beads (Gajadhar et al., 2000), glass rods, mortar and pestles (Gasser et al., 1993), and other types of tissue pulverizers. The PCR parameters used were specifically optimized to amplify the nematode species of interest. The modified extraction and PCR protocols outlined in this study address the difficulties of extracting and amplifying DNA from single larvae and preserved nematodes, problems that have not been addressed in detail by previous methods.

CHAPTER 3: IDENTIFICATION OF 6 SPECIES OF ELAPHOSTRONGYLINE NEMATODES BY POLYMERASE CHAIN REACTION (PCR) AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

ITS-2 Sequencing Protocol

First-stage and third-stage larvae (approximately 10-25 pooled larvae and individual larva specimens), and adults (anterior ends, middle fragments, and whole adults) of known and unknown identification, were extracted using the modified QIAamp procedure and amplified with ITS-2 primers (See Chapter 2). ITS-2 lengths (bp) for species not sequenced in this study were estimated using a Kodak 1D gel doc system that automatically compares PCR product to a molecular size marker. ITS-2 PCR product was purified with QIAquik (Qiagen, Valencia, California). Simulated mixed infections were created by adding 1µl of *P. andersoni* DNA extract to 1µl of either *P. tenuis*, *P. odocoilei*, or *E. rangiferi* DNA extract and 1µl of the resulting mixture was used in a PCR using NC1 and NC2 primers. These mixed samples were used to test the utility of the RFLP sites in cases of double infection.

Sequencing PCRs were set up using 4µl/reaction of 0.8µM of the universal primers NC1 (forward) or NC2 (reverse) primer (Ellis et al., 1986). A Big Dye Terminator Sequencing Kit (Perkin Elmer Corporation, Foster City, California) provided a master mix of all necessary sequencing PCR reagents. Master mix was added at a volume of 8µl/reaction, and sample PCR product (10ng/µl) was added at a volume of 8µl/reaction. Sequencing reactions were carried out for 25 cycles under the following parameters: denaturation at 96°C for 30 seconds, annealing at 50°C for 15 seconds, extension at 60°C for four minutes, and final hold at 4°C. Sequencing PCRs were purified with AGCT columns (Edge Biostystems) before each strand was sequenced on an

ABI 3100 genetic analyzer (Perkin Elmer Corporation, Foster City, California). Forward and reverse strands from each sample were sequenced independently in three separate trials to confirm results.

Choosing Samples for Sequencing

ITS-2 sequences were obtained from samples positively identified by adult male morphology or sample identity was known because the larvae (L1s and L3s) were obtained from an experimentally infected host (See Appendix). In most cases, two individual L1s or L3s from pooled larval samples were sequenced and/or restricted separately to determine if intraspecific variation was present within the pooled sample and to check for the presence of mixed infections. An additional 2-5 larval nematodes were placed in 1.5 ml microcentrifuge tubes and frozen at –86°C for future examination. The remainder of the pooled samples (approx. 10-25 larvae) were extracted together.

In total, six samples of *P. andersoni*, nine samples of *P. odocoilei*, sixteen samples of *P. tenuis*, six samples of *E. rangiferi*, two samples of *E. cervi* L1s, and two samples of *E. alces* L1s, all of unequivocal species identification, from individual hosts, were sequenced and used to determine diagnostic RFLP sites. Unidentified nematode L1s from bighorn sheep (*Ovis canadensis*) were also sequenced because their ITS-2 region was found to be close in size to that of the elaphostrongylines.

The sequences were aligned with Clustal W (1.18) Multiple Alignment Software and restriction maps were created with Sequencher™ software. After the restriction sites were determined, they were tested on ITS-2 fragments from the six species as described below.

Restriction Fragment Length Polymorphism (RFLP) Protocol

ITS-2 PCR products were concentrated from a volume of 50 µl to 20 µl in Nanosep (Pall Filtron) spin columns according to the manufacturer's instructions. In two 0.5 µl microcentrifuge tubes, the Nanosep concentrated samples were divided into two10 µl duplicates, one sample for digestion and one sample for an uncut control. *Parelaphostrongylus* spp. samples to be restricted were double-digested with a combination of 0.5 µl enzyme Msl I (New England Biolabs), 0.5 µl enzyme Fok I (New England Biolabs), and 2.0 µl NEB buffer 2. *Elaphostrongylus* spp. samples to be restricted were double-digested with a combination of 0.5µl enzyme Mse I (New England Biolabs), 0.5µl enzyme Fok I (New England Biolabs), and 2.0µl NEB buffer 2. All three enzymes used in this study have 100% activity in NEB buffer 2. ITS-2 PCR products were restricted overnight in a heated, dry bath at 37°C. The presence or absence of restriction sites and variation in size of the fragments were inferred from the size of the migrating bands on a 5% polyacrylamide gel. Mixed infection digestions contained several clustered fragments with sizes less than 300bp. Therefore, visualization of RFLP bands was attempted on 7% polyacrylamide gels to better resolve the location of many similarly-sized generated by digestion.

Results

Sequencing with primers NC1 and NC2 provided the ITS-2 lengths for the known species: *P. andersoni* 545 base pairs (bp), *P. odocoilei* is 562 bp, *P. tenuis* is 554 bp, *E. rangiferi* and *E. cervi* are both 585 bp, and *E. alces* is 575 bp (Figure 5). ITS-2 sequences obtained in this study revealed intraspecific sequence variation within each of the three *Parelaphostrongylus* spp., with each species comprising two consistently distinct ITS-2 types (Figure 5). The genus

FIGURE 5. Sequence alignment of six species of elaphostrongyline nematodes using primers NC1 and NC2. Sequence variants of *Parelaphostrongylus* spp. and the putative protostrongylid sp. are included. Primer NC1 (Ellis et al., 1986) is indicated in underlined green uppercase letters and NC2 (Ellis et al., 1986) is indicated in underlined green lowercase letters. Primer PTP1 (Gajadhar et al., 2000) is indicated in underlined blue uppercase letters and PTP2 (Gajadhar et al., 2000) is indicated in underlined blue lowercase letters. Primer ECP1 (Gajadhar et al., 2000) is indicated in underlined uppercase magenta letters and ECP1R (Gajadhar et al., 2000) is indicated in lowercase magenta letters. Sequence identity: PT1 and PT2 - *P. tenuis* var.1 and var. 2, PO1 and PO2 – *P. odocoilei* var. 1 and var. 2, PA1 and PA2 – *P. andersoni* var. 1 and var.2, ER – *E. rangiferi*, EC – *E. cervi*, EA – *E. alces*, PMU – putative protostrongylid sp. Note that primers ECP1 and ECP1R overlap in the center.

FIGURE 5. Clustal W (1.8)
Multiple Sequence Alignment

```
PT1      #1   ACGTCTGGTTCAGGGTTGTT GAAGAAAT:TCTACTCTAGT
PT2      #1   ACGTCTGGTTCAGGGTTGTT GAAGAAAT:TCTACTCTAGT
PO1      #1   ACGTCTGGTTCAGGGTTGTT GAAGAAAT:TCTACTCTAGT
PO2      #1   ACGTCTGGTTCAGGGTTGTT GAAGAAAT:TCTACTCTAGT
PA1      #1   ACGTCTGGTTCAGGGTTGTT GAAGAAAT:TCTACTCTAGT
PA2      #1   ACGTCTGGTTCAGGGTTGTT GAAGAAAT:TCTACTCTAGT
ER       #1   ACGTCTGGTTCAGGGTTGTT GAAGAGAGATCTACTCTAGT
EC       #1   ACGTCTGGTTCAGGGTTGTT GAAGAGAGATCTACTCTAGT
EA       #1   ACGTCTGGTTCAGGGTTGTT GAAGAAAT:TCTGCTCTAGT
PMU      #1   ACGTCTGGTTCAGGGTTGTT AA::AAGTTAATATTATGGT
                                   • •• •••••• •• • •

PT1      #41  CAAGTTGCATATGCA:ATAA ACATAG::AGGAAAAACATG
PT2      #41  CAAGTTGCATATGCA:ATAA ACATAG::AGGAAAAACATG
PO1      #41  CAAGTTGCATATGCA:ATAA ACATAG::AGGAAAAACATG
PO2      #41  CAAGTTGCATATGCA:ATAA ACATAG::AGGAAAAACATG
PA1      #41  CAAGTTGCATATGCA:ATAA ACATAG::AGGAAAAACATG
PA2      #41  CAAGTTGCATATGCA:ATAA ACATAG::AGGAAAAACATG
ER       #41  CGAG::ATAAACATAGAAGA TCATCGTAAGATAGAAGATA
EC       #41  CGAG::ATAAACATAGAAGA TCATCGTAAGATAGAAGATA
EA       #41  CGAATCATATATGCATATCA TCATCATCATATGTACGATA
PMU      #41  CG:::CATATATACA:ATAT ATGTATACTGCTATATATTG
                •••••••  • ••• • ••• ••• ••••••••••• ••• •

PT1      #81  GATGTATGACATG:::::::  ::::::::::A::TATAA:::
PT2      #81  GATGTATGACATG:::::::  ::::::::::T::TATAA:::
PO1      #81  AATGTATGACATG:::::::  ::::::::::A::TATAA:::
PO2      #81  AATGTATGACATG:::::::  ::::::::::A::TATAA:::
PA1      #81  GATGTGTGACATG:::::::  ::::::::::A::TATAA:::
PA2      #81  GATGTGTGACATG:::::::  ::::::::::A::TATAA:::
ER       #81  T::::ATGACATG:::::::  ::::::::::ATATATC::::
EC       #81  T::::ATGACATG:::::::  ::::::::::ATATATC::::
EA       #81  TGCATATAATATGCATAGAA GAAAGATAGATATATGACCT
PMU      #81  T:::TATGACATG:::::::  ::::::::::::::GATG::::
                •••••• • •                 •••• •••••
```

FIGURE 5. Clustal W (1.8)
Multiple Sequence Alignment

```
PT1      #121   ::::TATCTATGCTTTTCTA TG:TATGCGACGACTAGATT
PT2      #121   ::::TATCTATGCTTCTCTA TG:TATGCGACGACTAGATT
PO1      #121   ::::TATCTATGCTTTTCTA TG:TATGCGACGACTAGATT
PO2      #121   ::::TATCTATGCTTCTCTA TG:TATGCGACGACTAGA:T
PA1      #121   ::::TATCTATGCTTTTCTA TG:TATGCGACGACTAGATT
PA2      #121   ::::TATCTATGCTTCTCTA TG:TATGCGACGACTAGATT
ER       #121   ::::TATCTA::TACTTCTA TG:TATACGACGACTA:ATT
EC       #121   ::::TATCTA::TACTTCTA TG:TATACGACGACTA:ATT
EA       #121   GATGTATCTAG:CGCTTCTA TGATGTACAACGACTA:ATT
PMU      #121   ::::::::::::::CGGTTATA TG:::TCGAACGGT:::ACT
                ____ ______ _ ___ ___ _____ _

PT1      #161   TGTCAAACGGTATtc:::gtc aagacgat:::::::::gatt
PT2      #161   TGTCAAACGGTATtc:::gtc aagacgat:::::::::gatt
PO1      #161   TGTCAAACGGTATtc:::gtc aagacgat:::::::::gatt
PO2      #161   TGTC:AACGGTATtc:::gtc aagacgat:::::::::gatt
PA1      #161   TGTCAAACGGTATtc:::gtc aagacgat:::::::::gatt
PA2      #161   TGTCAAACGGTATtc:::gtc aagacgat:::::::::gatt
ER       #161   TGTCAAACGGTATTCgcgtc aaaacgatagacggacgATT
EC       #161   TGTCAAACGGTATTCgcgtc aaaacgatagacggacgATT
EA       #161   TGTCAAGCGGTATTC::GTC AAGATGAT::::GGACGATT
PMU      #161   CGCGTTATCGTCATC::GTT GACGTGTG:::::::CAATT
                _ _______ __ __ _ _ ___ ____________

PT1      #201   ccCGTTTTAGTGAAGAATTA ::CGACAATGGCAACATTTA
PT2      #201   ccCGTTTTAGTGAAGAATTA ::CGACAATGGCAACATTTA
PO1      #201   ccCGTTTTAGTGAAGAATTA C::GACAATGGCAACATTTA
PO2      #201   ccCGTTTTAGTGAAGAATTA C::GACAATGGCAACATTTA
PA1      #201   ccCGTTTTAGTGAAGAATTA ::CGACAATGGCAACATTTA
PA2      #201   ccCGTTTTAGTGAAGAATTA ::CGACAATGGCAACATTTA
ER       #201   CCCGTTTTAGTGAAGAATTT ::GAACGATAGCAAAATATA
EC       #201   CCCGTTTTAGTGAAGAATTT ::GAACGATAGCAAAATATA
EA       #201   CCCGTTTTAGTGAAGAATTT TTGAACGATAGCAAAATTTA
PMU      #201   CCCGTTTCAGAAAAAGAATA T::AATGATAGCAACATGTG
                _ __ __ _ _ ____ __ _ _ _ _
```

FIGURE 5. Clustal W (1.8)
Multiple Sequence Alignment

```
PT1     #241   ATAATAATGTACATACAG:T  GAATATGTA:TG::::TATT
PT2     #241   ATAATAATGTACATACAG:T  GAATATGTA:TG::::TATT
PO1     #241   GTAATAATGTACATACAG:T  GAATATGTA:TGGATGTATT
PO2     #241   GTAATAATGTACATACAG:T  GAATATGTA:TGGATGTATT
PA1     #241   ATAATAATGTACATACAG:T  AAATATGTA:TG::::TATT
PA2     #241   ATAATAATGTACATACAG:T  AAATATGTA:TG::::TATT
ER      #241   ATAATAACGTACATATGG:T  AAATATGTATTATTGTTATT
EC      #241   ATAATAACGTACATATGG:T  AAATATGTATTATTGTTATT
EA      #241   ATAATAACGTACATATGGAT  AGATATGTACTATTGTTATT
PMU     #241   ATAATA:TGTA:::::::::T  AAATATATA:CA::::TGTT
                •       ••    ••••••••   ••      •   •••••••  •

PT1     #281   ATTATACCGAATATA:ATAT  ATGATGATATGTGC::::CT
PT2     #281   ATTATACCGAATATA:ATAT  ATGATGATATGTGC::::CT
PO1     #281   ATTATACCGAATATA:ATAT  ATGATGATATGTGC::::CT
PO2     #281   ATTATACCGAATATA:ATAT  ATGATGATATGTGC::::CT
PA1     #281   ATTATACCGAATATA:ATAT  ATGATGATATGTGC::::CT
PA2     #281   ATTATACCGAATATA:ATAT  ATGATGATATGTGC::::CT
ER      #281   ATTATACCGAATATA:ATAT  ATGATGATGTGTGCGTATCT
EC      #281   ATTATACCGAATATA:ATAT  ATGATGATGTGTGCGTATCT
EA      #281   ATTATACCGAATATA:ATAT  ATGATGATGTGTGC::::CT
PMU     #281   GTCAAGCTAAATGTATATAT  GTGCAGCCGTATGC::::::
                • • •• ••    •   •      •   •• ••• •    ••••••

PT1     #321   ATGCTAGTGATGTTGTTACC  AC::::::CATCGTTAATGT
PT2     #321   ATGCTAGTGATGTTGTTACC  AC::::::CATCGTTAATGT
PO1     #321   ATGCTAGTGATGTTGTTACC  AC::::::CATCGTTAATGT
PO2     #321   ATGCTAGTGATGTTGTTACC  AC::::::CATCGTTAATGT
PA1     #321   ATGCTAGTGATGTTGTTACC  AC::::::CATCGTTAATGT
PA2     #321   ATGCTAGTGATGTTGTTACC  AC::::::CATCGTTAATGT
ER      #321   ATGCTAGTGATGTTGTTATT  ACGACGATCATCGTTAATGC
EC      #321   ATGCTAGTGATGTTGTTATT  ACGACGATCATCGTTAATGC
EA      #321   ATGCTAGTGATGTTGTTATT  ACGA:::TCATCGTTAATGC
PMU     #321   :AGCTAGTGCTCTCATTGCT  ACA::::CTGTCGTCGATTT
                ••        •  •  ••   •••    ••••••••     ••   ••
```

FIGURE 5. Clustal W (1.8)
Multiple Sequence Alignment

```
PT1     #361   TGATTATCAAT:GGGTATCA TTGAAAATCATGAATA::::
PT2     #361   TGATTATCAAT:GGGTATCA TTGAAAATCATGAATA::::
PO1     #361   TGATTATCAAT:GGGTATCA TTGAAAATCATGAATA::::
PO2     #361   TGATTATCAAT:GGGTATCA TTGAAAATCATGAATA::::
PA1     #361   TGATTATCAAT:GGGTATCA TTGAAAATCATGAATA::::
PA2     #361   TGATTATCAAT:GGGTATCA TTGAAAATCATGAATA::::
ER      #361   TGATTATCAAT:GGGTATCA TTGAAAATCATGAATAATAG
EC      #361   TGATTATCAAT:GGGTATCA TTGAAAATCATGAATAATAG
EA      #361   TGATTATCAAT:GGGTATCA TTGAAAATCGTGAATA:TGG
PMU     #361   TGTTCTTCAATCGGGTATCG TTGAAGATCATGACG:::::
                 • ••      •         •          •    •    ••  ••••

PT1     #401   :TCAATCGAAAAAAAAAAA::  :AC:::AGAATCATTGATGA
PT2     #401   :TCGATCGAAAAAAAAAAA::  :AC:::AGAATCATTGATGA
PO1     #401   :TAAATCGAAAAAAAAAAA::  :AAAAGAGAATCATTGATGA
PO2     #401   :TAAATCGAAAAAAAAAAA::  :AAAAGAGAATCATTGATGA
PA1     #401   :TAAATCGAAAAAAAAAA:::  :::::::GAATCATTGATGA
PA2     #401   :TAAATCGAAAAAAAAAA:::  :::::::GAATCATTGATGA
ER      #401   ATAAATC:TATGAAAAAATA AAAAAACAATACATTGACGA
EC      #401   ATAAATC:TATGAAAAAATA AAAAAACAATACATTGACGA
EA      #401   ATAAATCCTATGGAAAAA::  ::AAAACAACTCATTGACGA
PMU     #401   :::::::::::::::::::: :::TTGATGTTAATCGTCGA
                •  ••    ••  •••      ••  ••••••••••••   •  ••

PT1     #441   TGGATGACATGTATTCGACG  G:::TAATAACAA:AGCTAT
PT2     #441   TGGATGACATGTATTCGACG  G:::TAATAACAA:AGCTAT
PO1     #441   TGGATGACATGTATTCGACG  G:::TAATAACAA:AGCTAT
PO2     #441   TGGATGACATGTATTCGACG  G:::TAATAACAA:AGCTAT
PA1     #441   TGGATGACATGTATTCGACG  G:::TAATAACAA:AGCTAT
PA2     #441   TGGATGACATGTATTCGACG  G:::TAATAACAA:AGCTAT
ER      #441   TGGATGACATGTGTTATAGA  G:::TAATAACGC:GACTAT
EC      #441   TGGATGACATGTGTTATAGA  G:::TAATAACGC:GACTAT
EA      #441   TGGATGACATGTGTTATAGA  GGAGTAATAACGC:GGCTAT
PMU     #441   TAGTTGACGTGTGTTGCTCA  G:::TAATGATGATGACTAT
                • •      •    •   •••••   •••     •  ••••••
```

FIGURE 5. Clustal W (1.8)
Multiple Sequence Alignment

```
PT1     #481   TAACAC::TAGACATAGATA ::ATGCATGTT::GAGCACA
PT2     #481   TAACAC::TAGACATAGATA ::ATGCATGTT::GAGCACA
PO1     #481   TAACAC::TAGACATAGATA ::ATGCAAGTT::GAGCACA
PO2     #481   TAACAC::TAGACATAGATA ::ATGCAAGTT::GAGCACA
PA1     #481   TAACAC::TAGACATAGATA ::ATGCAAGTT::GAGCATA
PA2     #481   TAACAC::TAGACATAGATA ::ATGCAAGTT::GAGCACA
ER      #481   TAACACACTAAACATAGATA CAATGCATGTTAAGCGCACA
EC      #481   TAACACACTAAACATAGATA CAATGCATGTTAAGCGCACA
EA      #481   TAACAC::::::::::::TA ::::::::::::::::GCATA
PMU     #481   AAACAC::TAGAC:TGAATA T::::::::::::::::GCATA

                •      ••   •  •  ••    ••      •   ••  •   •

PT1     #521   AAATTCTCATCATCATTATT ATTATGTTTACAATCACTTG
PT2     #521   AAATTCTCATCATCATTATT ATTATGTTTACAATCACTTG
PO1     #521   AAATTCTCATCATCATTATT ACTAGGTTTACAATCACTTG
PO2     #521   AAATTCTCATCATCATTATT ACTAGGTTTACAATCACTTG
PA1     #521   AAATTTTCATCATCATTAT: :::::GTTTACAATCACTTG
PA2     #521   AAATTTTCATCATCATTAT: :::::GTTTACAATCACTTG
ER      #521   TAATTCTCATCACTATTATT :::::ATATTTGATCACTTG
EC      #521   TAATTCTCATCACTATTATT :::::ATATTTGATCACTTG
EA      #521   AAATTCTCATCA:::TTATT :::::ATATTTAATCACTTG
PMU     #521   CATCGTTGCATATATATAAT :::::ACATTTAATT:CTTG

                • ••••  ••••  ••••   ••  •••••••• •••   ••

PT1     #561   TAAAACGCAACCTGAACTCA GATGTGATTACCCGCTGAAC
PT2     #561   TAAAAAGCAACCTGAACTCA GATGTGATTACCCGCTGAAC
PO1     #561   TAAAACGCAACCTGAACTCA GATGTGATTACCCGCTGAAC
PO2     #561   TAAAACGCAACCTGAACTCA GATGTGATTACCCGCTGAAC
PA1     #561   TAAAACGCAACCTGAACTCA GATGTGATTACCCGCTGAAC
PA2     #561   TAAAACGCAACCTGAACTCA GATGTGATTACCCGCTGAAC
ER      #561   TAAAACGCAACCTGAACTCA GACGTGATTACCCGCTGAAC
EC      #561   TAAAACGCAACCTGAACTCA GACGTGATTACCCGCTGAAC
EA      #561   TAAAACGCAACCTGAACTCA GACG:GATTACCCGCTGAAC
PMU     #561   TATAATGCAACCTGAACTCA GACGTGAATACCCGCTGAAC

                 •  •              •  •  •
```

FIGURE 5. Clustal W (1.8)
Multiple Sequence Alignment

```
PT1     #601   TTAAGCATATCATTTagcgg  aggaaaagaaactaa
PT2     #601   TTAAGCATATCATTTagcgg  aggaaaagaaactaa
PO1     #601   TTAAGCATATCATTTagcgg  aggaaaagaaactaa
PO2     #601   TTAAGCATATCATTTagcgg  aggaaaagaaactaa
PA1     #601   TTAAGCATATCATTTagcgg  aggaaaagaaactaa
PA2     #601   TTAAGCATATCATTTagcgg  aggaaaagaaactaa
ER      #601   TT:AGCATATCATTTagcgg  aggaaaagaaactaa
EC      #601   TT:AGCATATCATTTagcgg  aggaaaagaaactaa
EA      #601   TTAAGCATATCATTTagcgg  aggaaaagaaactaa
PMU     #601   TTAAGCATATCATTTagcgg  aggaaaagaaactaa
```

Elaphostrongylus did not exhibit intraspecific variation. ITS-2 bands produced from amplification of simulated mixed infections did not resolve on gels, resembling single infection bands. A minor size difference in the initial ITS-2 PCR product was observed between the elaphostrongyline genera, however, species within *Elaphostrongylus* and *Parelaphostrongylus* were not readily distinguishable by gel electrophoresis (Figure 6). This result was also reported by Gajadhar et al. (2000).

Based on the DNA sequences (Figure 5), species-level diagnostic restriction sites were found at several positions. The RFLP sites distinguished *P. tenuis*, *P. andersoni*, and *P. odocoilei* (Figure 7). The sites could also distinguish *E. alces* from *E. cervi* and *E. rangiferi*, but could not distinguish between *E. cervi* and *E. rangiferi* (Figure 7). Mixed infections of *P. odocoilei* + *P. andersoni*, *E. rangiferi* + *P. andersoni*, and *P. tenuis* + *P. andersoni* could usually be detected during sequencing as overlapping sequence electropherograms, but the mixtures listed above produced a combination of RFLP fragment patterns that had unresolved bands. In addition, some smaller fragments ran off the polyacrylamide gels (data not shown). Restriction maps for *Parelaphostrongylus* spp. and *Elaphostrongylus* spp. are shown in Figures 8 and 9, respectively.

PCR with the universal primers NC1 and NC2 and gel electrophoresis revealed three types of unidentified infections of dorsal-spined larvae (Figure 10). Unidentified type 1 was found to be 495 base pairs by sequencing, unidentified type 2 was approximately 330 base pairs based on comparison with size standard pBR322/MspI digest, and unidentified type 3 was approximately 220 base pairs based upon comparison with the same size standard (Figure 10). Only one natural double infection was detected by PCR with NC1 and NC2. One band was consistent with *Parelaphostrongylus* spp., the other was the 220 bp unidentified type 3 infection. The

FIGURE 6. Gel photograph (5% polyacrylamide) showing ITS-2 bands for elaphostrongyline nematodes. Lane 1: molecular size marker pBR322/Msp1 digest, Lane 2: negative PCR control, Lane 3: *E. cervi* single larvae (#3b), Lane 4: *E. cervi* single larvae (#4b), Lane 5: *P. andersoni* single larvae (#16b), Lane 6: *P. odocoilei* single larvae (#20b), Lane 7: *P. odocoilei* single larvae (#21b), Lane 8: *P. odocoilei* pooled larvae (#20), Lane 9: *P. tenuis* pooled larvae (#44), Lane 10: *E. alces* pooled larvae (#1), Lane 11: *E. alces* pooled larvae (#2), Lane 12: *P. andersoni* pooled larvae (#16), Lane 13: *E. rangiferi* pooled larvae (#10), Lane 14: *P. andersoni* pooled larvae(#17), Lane 15: *E. cervi* pooled larvae (#3).

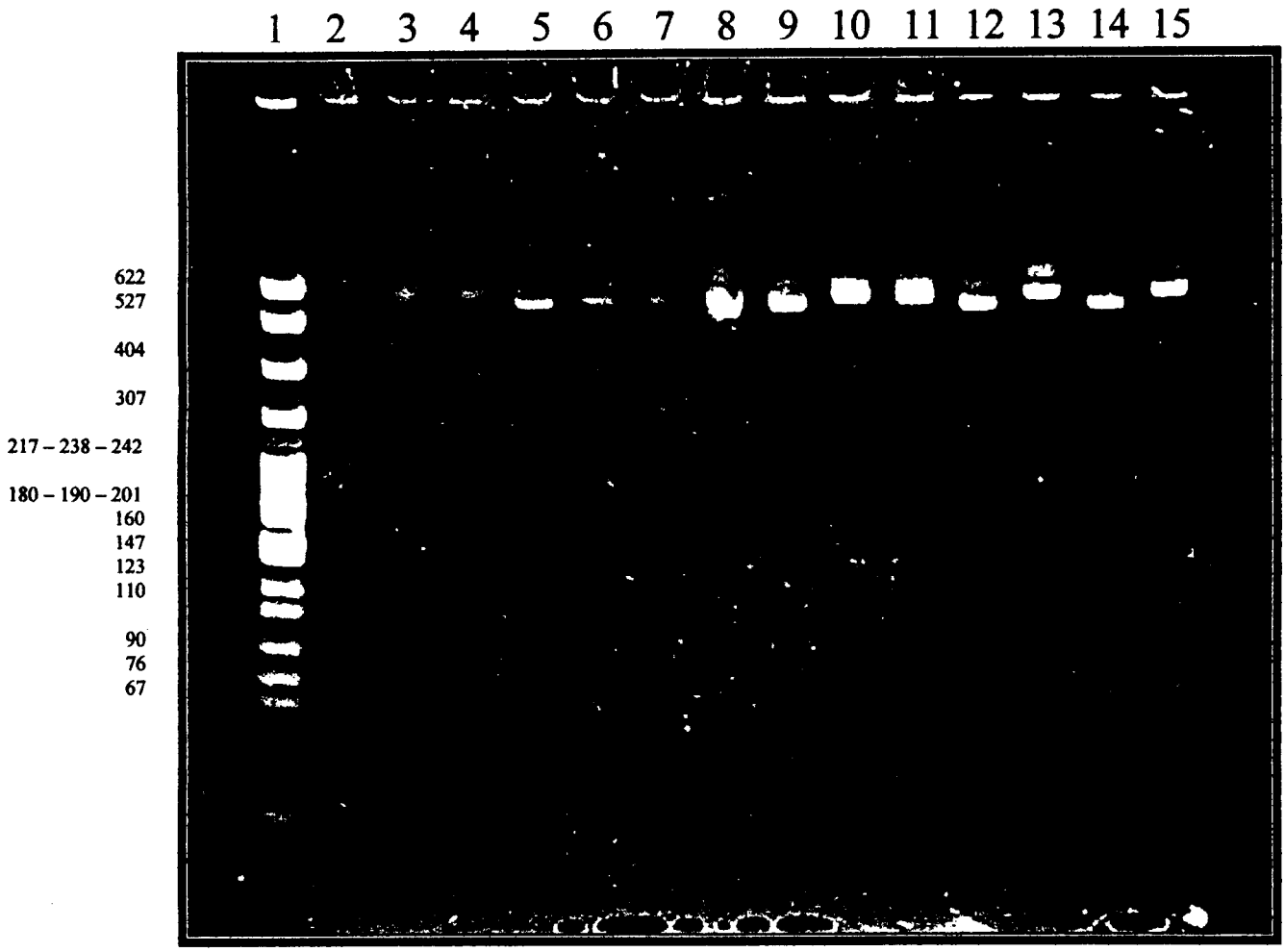


FIGURE 7. Double digestion of elaphostrongyline nematode ITS-2 fragments. Lane 1: molecular size marker pBR233/Msp1, Lane 2: *E. alces* (# 1), Lane 3: *E. cervi* (# 3), Lane 4: *E. rangiferi* (# 5), Lane 5: *P. tenuis* (#29), Lane 6: *P. odocoilei* (# 22), Lane 7: *P. andersoni* (#14).

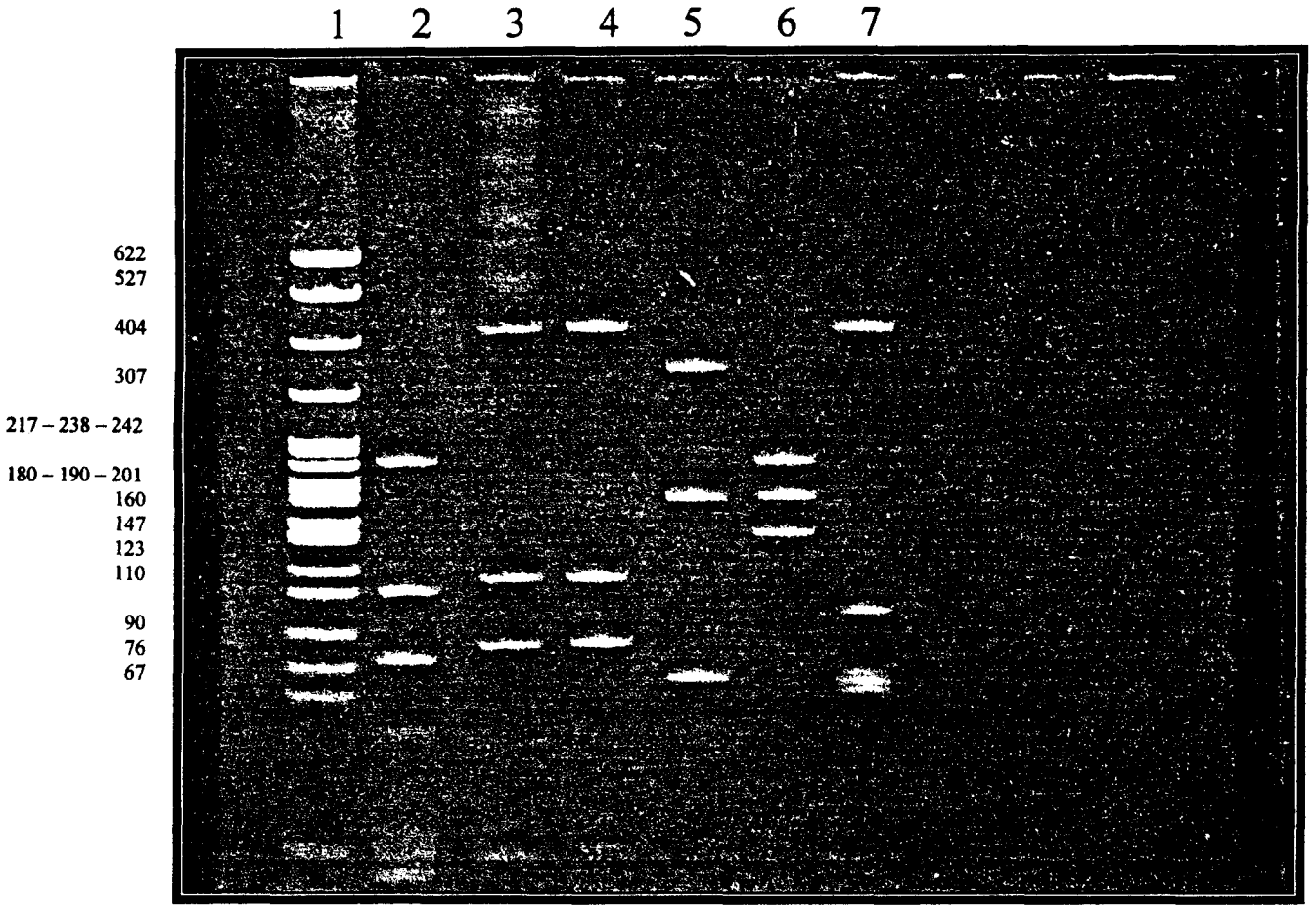
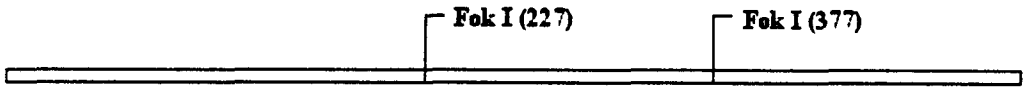


FIGURE 8. Restriction maps of *Parelaphostrongylus* spp. depicting digestion with Msl I and FokI. Numbers in brackets indicate nucleotide positions where the enzyme cuts the DNA.

P. tenuis

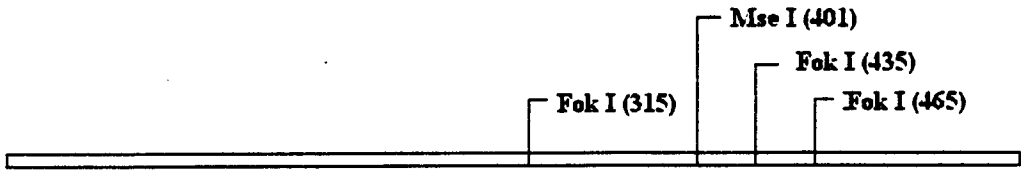
P. odocoilei



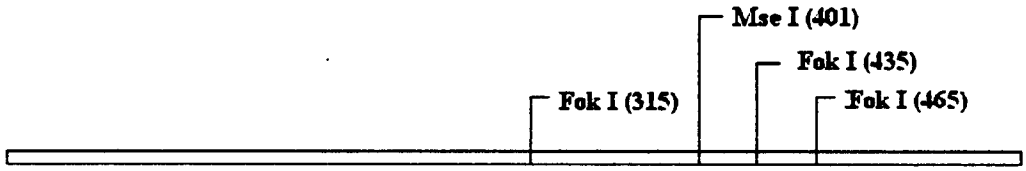
P. andersoni

FIGURE 9. Restriction maps of *Elaphostrongylus* spp. depicting digestion with enzymes Fok I and Mse I. Numbers in brackets indicate nucleotide positions where the enzyme cuts the DNA.

E. rangiferi



E. cervi



E. alces

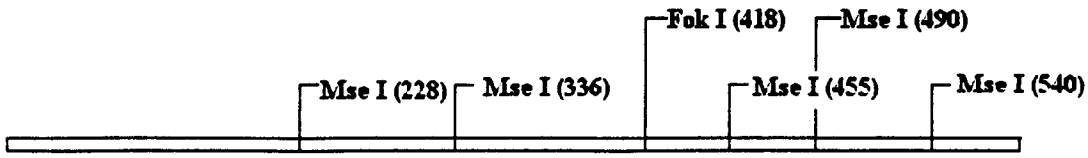
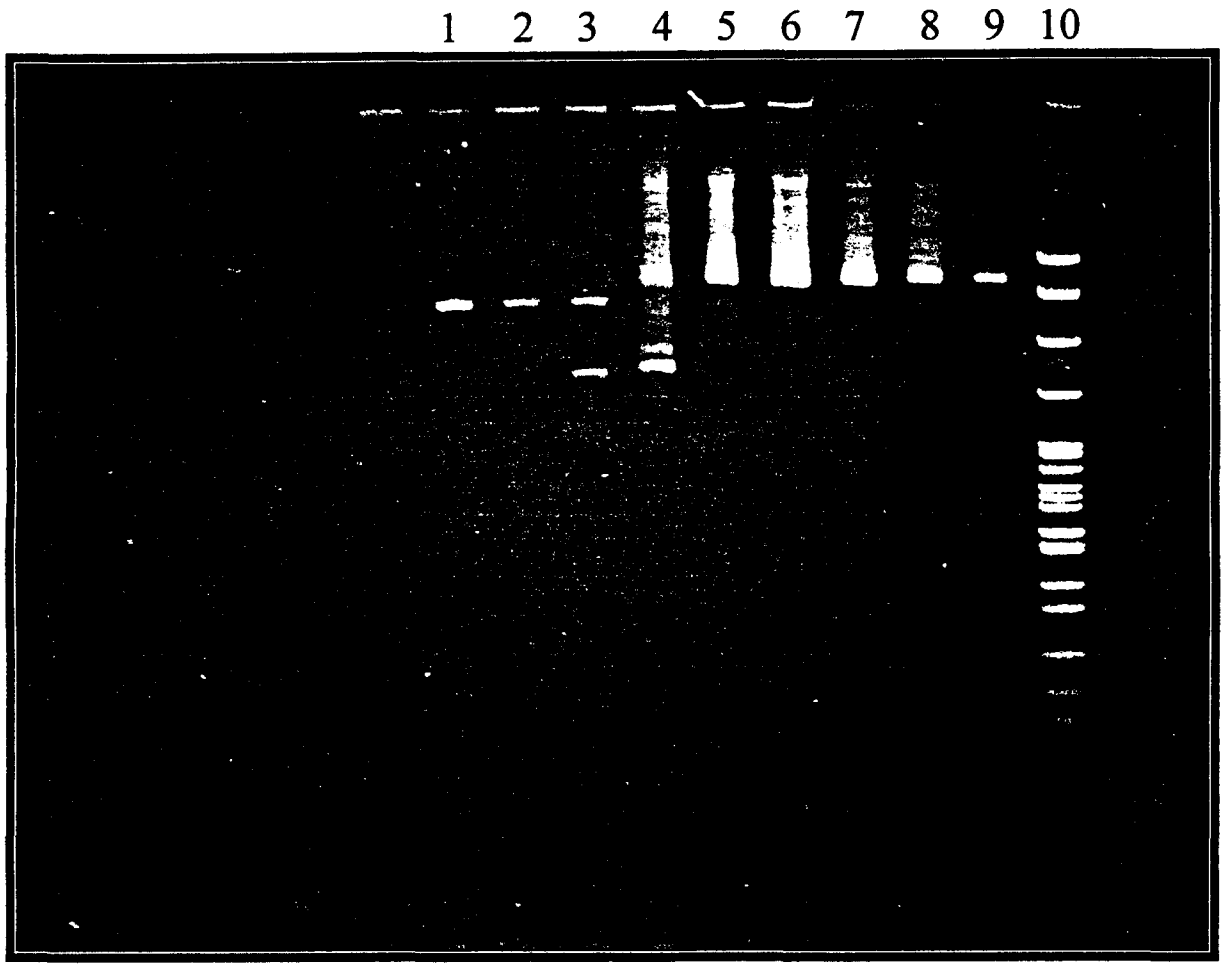


FIGURE 10. Gel photograph (polyacrylamide 5%) of NC1/NC2 PCR product from dorsal-spined larvae of unidentified protostrongylid nematodes. Lanes 1 and 2: Unidentified type 1 (#51a and #52a). Lane 3: natural mixed infection of unidentified type 1 and unidentified type 3 (#53). Lane 4: natural mixed infection of *P. odocoilei* and an unidentified type 3 nematode (#50). Lanes 5 to 9: elaphostrongyline nematodes for comparison. Lane 10: molecular size marker pBR322/Msp1.



Parelaphostrongylus spp. band was identified as *P. odocoilei* by RFLP. *Orthostrongylus macrotis* samples (#11, #12, and #13) each amplified one weak band of approximately 720bp. *Varestrongylus alpenae* (sample #45) amplified one slightly shorter band weak band of approximately 700bp. Nematode samples that were neither *Elaphostrongylus* nor *Parelaphostrongylus* were immediately recognized on gels because their ITS-2 products varied greatly in size; in some cases by more than one hundred bases (Figure 10).

Discussion

The RFLP test described above achieved the main goal of this study, which was to distinguish the four species of elaphostrongyline nematodes found in North America. Intraspecific variation occurs within the Parelaphostrongylinae, but it does not affect the RFLP test, as the cut sites apparently are not located at base positions so far seen to be polymorphic within species. The test also targeted specifically the mixed infections that wildlife biologists can expect based on the distribution of these parasites in North America. No natural mixed infections were detected with the RFLP. However, artificial mixed infections produced crowded and difficult to interpret RFLP patterns where small cut fragments frequently ran off the end of the gel. The optimal solution for accurately determining mixed infection composition may lie in the ability to identify individual larvae from the mixed infection. This would avoid crowded gels with many restriction fragments of less than 300bp, which require running a higher concentration of polyacrylamide gel.

Intraspecific variation was detected within the genus *Parelaphostrongylus*. Interestingly, *P. tenuis* from eastern North America and *P. tenuis* from Costa Rica have identical ITS-2 sequences (*P. tenuis* variant 1), but putative *P. tenuis* material from Marquette, Michigan (#46 and

47) differed from the other *P. tenuis* samples by two transitions and two transversions (*P. tenuis* variant 2) (Figure 5). These were pooled samples of dorsal-spined L1s (Garvon, 2001, per. Comm.). They were 256-302 μm in length, which is somewhat shorter than measurements published for *P. tenuis* and *P. andersoni* (for a review see Lankester, 2001); although, alcohol shrinkage could have been responsible for their shorter length. Alternatively, they are approaching the shorter length of *Varestrongylus* L1s (Grey et al., 1985b). The samples from Marquette, Michigan were presumed to be *P. tenuis* and were named *P. tenuis* variant 2 based on the sequence length being identical to *P. tenuis* variant 1 (554bp) and differing only by 2 transitions and 2 transversions. Other specimens whose sequence matched *P. tenuis* variant 2 were two L3 specimens (#48a and #48b), digested from *Triodopsis* sp. that were experimentally infected with L1s passed from white-tailed deer in the vicinity of Marquette (Garvon, 2001 per. comm.). The first L3 (#48a) measured 735 μm, which is somewhat small for *P. tenuis* and *P. andersoni* (900-1080 μm), but large for *Varestrongylus alpenae*. The second L3 (#48b) measured 935 μm, which is more consistent with *P. tenuis* L3s (Lankester, 2001). In addition, two L3s digested from *A. alternata*, 835 and 875 μm long, were also identified as *P. tenuis* variant 2. The slightly short measurement may be the result of alcohol shrinkage.

P. odocoilei ITS-2 sequences from mule deer in Alberta, Montana, and California were uniform (*P. odocoilei* variant 1). *P. odocoilei* variant 2 sequence from mule deer near Penticton, British Columbia differed from *P. odocoilei* variant 1 by 1 transition. It is interesting to note that this experimental infection was established with L1s from mule deer in the vicinity of Penticton, with adult *P. odocoilei* in muscles; however, the larvae in the feces were longer (range 395-460 μm) than lengths published for this species (Hobmaier and Hobmaier, 1934). Gray and Samuel

(1986) found similarly long dorsal-spined larvae (415 +/- 17 μm) in woodland caribou from northeastern Alberta.

Two variants of *P. andersoni* were also discovered by sequencing the ITS-2 region. *P. andersoni* variant 1 was found among all *P. andersoni* samples from caribou in Newfoundland, Canada. *P. andersoni* variant 2 differed from *P. andersoni* variant 1 by two transitions, and was the sequence found in the single source of L1s from mainland Canada (woodland caribou near Detour Lake, ON).

The ITS-2 region is a spacer DNA and is, therefore, more free to mutate than a functional RNA gene. This could explain the presence of intra-species variation seen among *Parelaphostrongylus* spp. The genotypic variants of *Parelaphostrongylus*, with the exception of *P. andersoni* variant 1 and 2, do not seem to correspond to geographic separation of the parasites (Figure 1; See Appendix) and may have arisen as a result of microenvironmental factors. Alternatively, the variants could represent different strains exhibiting different levels of virulence. Further sequencing studies of coding regions in the Protostrongylidae should be conducted to test this hypothesis. Species of the genus *Elaphostrongylus* did not exhibit intraspecific variation, but in the case of *E. cervi* and *E. alces*, this could be due to limited sample size. Only two fecal samples of these nematode species from single experimentally infected hosts were available for study.

Three consistently distinct ITS-2 types, shorter than those from *V. alpenae*, *O. macrotis* (above) and the elaphostrongylines, were amplified from some unidentified L1s (See Appendix). Unidentified type 1 is 495 bp long and was found in bighorn sheep and muskox in Washington and Alaska, respectively. Unidentified type 2 is approximately 330 bp and was found in elk from

Montana and one big horn sheep in Washington. Unidentified type 3 is 220 bp and was found with *P. odocoilei* in a black-tailed deer in Washington. A BLAST search revealed that none of the nematodes sequenced in this study matched the previously published *Dictyocaulus* ITS-2 sequences (Genbank accession number: U37718). Furthermore, there are no other published protostrongylid ITS-2 sequences among the Genbank entries.

An attempt was made here to obtain and sequence *Orthostrongylus macrotis* and *Varestrongylus alpenae* because they are parasites of cervids that also produce dorsal-spined larvae. *Orthostrongylus macrotis* and *V. alpenae* were each found to produce weak ITS-2 bands of approximately 700 bp, a length too large to match with any of three unidentified types of nematodes or elaphostrongylines. There was insufficient ITS-2 PCR product to sequence, but on polyacrylamide gels *O. macrotis* and *V. alpenae* had bands consistently larger than any other species in this study. There could be several reasons for the poor amplification of *O. macrotis* and *V. alpenae.* Failed extractions, altered primer annealing sites, fixation time, fluid, and sub-optimal PCR conditions could all result in poor amplification. Although, the analysis protocols outlined in chapter 2 were optimized to account for these variables, it is not known why the amplification product in these samples was weak.

In future studies, positively identified adults of all potentially co-occurring nematodes should be obtained and sequenced to positively identify the three unidentified types of infections (See Appendix) occurring in wild ungulate feces.

The estimated ITS-2 region lengths given by Gajadhar et al. (2000) can be replaced by precise sizes determined from the DNA sequences as follows: *Elaphostrongylus cervi* and *E. rangiferi* 585 bp in length, *E. alces* 575 bp, *P. tenuis* 554 bp, *P. andersoni* 545 bp, and *P.*

odocoilei 561 bp (Figure 5). Furthermore, as reported by Gajadhar et al. (2000), the entire ITS-2 region (amplified with NC1 and NC2) of *Umingmakstrongylus pallikukensis* was 445 bp long. However, they further stated that the amplification band for the combination of NC2 (universal reverse) and ECP1R (internal to ITS-2) was 699bp, which is larger than the entire ITS-2 region itself. Similarly, Epe and colleagues (1997) reported that *Dictyocaulus viviparus* has a total ITS-2 length of 457 base pairs (Genbank accession number: U37718), but Gajadhar et al. (2000) reported two ITS-2 bands with sizes of 728 bp and 782 bp generated from NC2 and ECP1R (Figure 5) amplification of *Dictyocaulus* sp. If these results were correct, a subset of ITS-2 would be larger than the entire region. The ITS-2 sequence for *Dictyocaulus* sp. entered into Genbank (Genbank accession number: U37718) may be incomplete. The NC1/NC2 primer combination was used in the study (Epe et al., 1997) but neither primer appears in the sequence. It is imperative that these inconsistencies be resolved in future studies so that potentially co-occurring infections can be properly identified.

In conclusion, automated DNA sequencing is the best method by which to identify elaphostrongyline L1s and L3s, both single and pooled samples. Sequencing has the advantage of higher specificity over RFLP methods. It is also superior to species-specific primer methods, which do not identify sequence variants. Mixed infection sequences are easily identified on ABI Prism 3100 generated electropherograms because they look like overlapping electropherogram peaks. Due to recent technological advances, automated DNA sequencing is the method of choice for identifying useful genetic markers because it is now more rapid (2 hours for 16 sequence reads) and relatively inexpensive (at present, $5.00 per sample).

CHAPTER 4: PHYLOGENY OF 6 SPECIES OF ELAPHOSTRONGYLINE NEMATODES INFERRED FROM THEIR ITS-2 SEQUENCES

Materials and Methods

Sequences of the ITS-2 region for *P. tenuis*, *P. andersoni*, *P. odocoilei*, *E. rangiferi*, *E. cervi*, and *E. alces*, were obtained using the protocols outlined in Chapter 2. Sample #51, #51a, #51b, #52, #53, #54, and #55 were collected from bighorn sheep in Washington by B. Foreyt (Appendix), and their 495bp ITS-2 region was sequenced. This sequence was tentatively identified by staff at the Canadian Food Inspection Agency, Centre for Animal Parasitology, Saskatchewan (Steeves-Gurnsey, 2002: per. comm.), as that of a putative non-elaphostrongyline protostrongylid. This sequence was chosen as the outgroup to root the tree because published *Dictyocaulus* sp. sequences are incomplete (See Chapter 3). The sequence divergence between the putative protostrongylid sp. and the elaphostrongylines was less than the divergence between *Dictyocaulus viviparus* and the elaphostrongylines (data not shown), suggesting that the putative protostrongylid sp. is a closer genetic relative to the elaphostrongylines than *Dictyocaulus* spp.

Modeltest 3.0 (Posada and Crandall, 1998) software was used to determine the model of nucleotide substitution that best fit the data. Modeltest software also provides empirical values for nucleotide frequencies and substitution rates, and estimates the gamma distribution parameter of rate heterogeneity and the proportion of invariant sites. Trees were constructed by the maximum likelihood (ML) method using PAUP (Phylogenetic Analysis Using Parsimony) 4.0 (Swofford, 1998) with the Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution (Hasegawa, Kishino, and Yano, 1985) incorporating a gamma distribution value provided by Model Test. Four gamma rate categories were implemented. Sequence divergences between taxa were computed

using the HKY85 distance.

Results

The base frequencies for the ITS-2 alignment of sequences used in this analysis were as follows: 34.75% A, 14.35% C, 19.20% G, and 31.69% T. The sequence divergences between all taxa are presented in Table 2. The optimal tree is presented in Figure 11. For simplicity, only variant one sequences from each taxon were represented as their sequences had divergences of less than 1% (Table 2). Results suggest that the genus *Parelaphostrongylus* is monophyletic. An internal clade contains the muscle worms *P. andersoni* and *P. odocoilei*, with meningeal worm *P. tenuis* basal to this pair. Species of the genus *Elaphostrongylus* formed a separate clade with *E. rangiferi* and *E. cervi* forming an internal clade, and *E. alces* as the basal species. The unidentified protostrongylid nematode was the most divergent species (Table 2). The monophyly of the Elaphostrongylinae is implied by these results, but cannot be confirmed, as *E. panticola* was not included in this study.

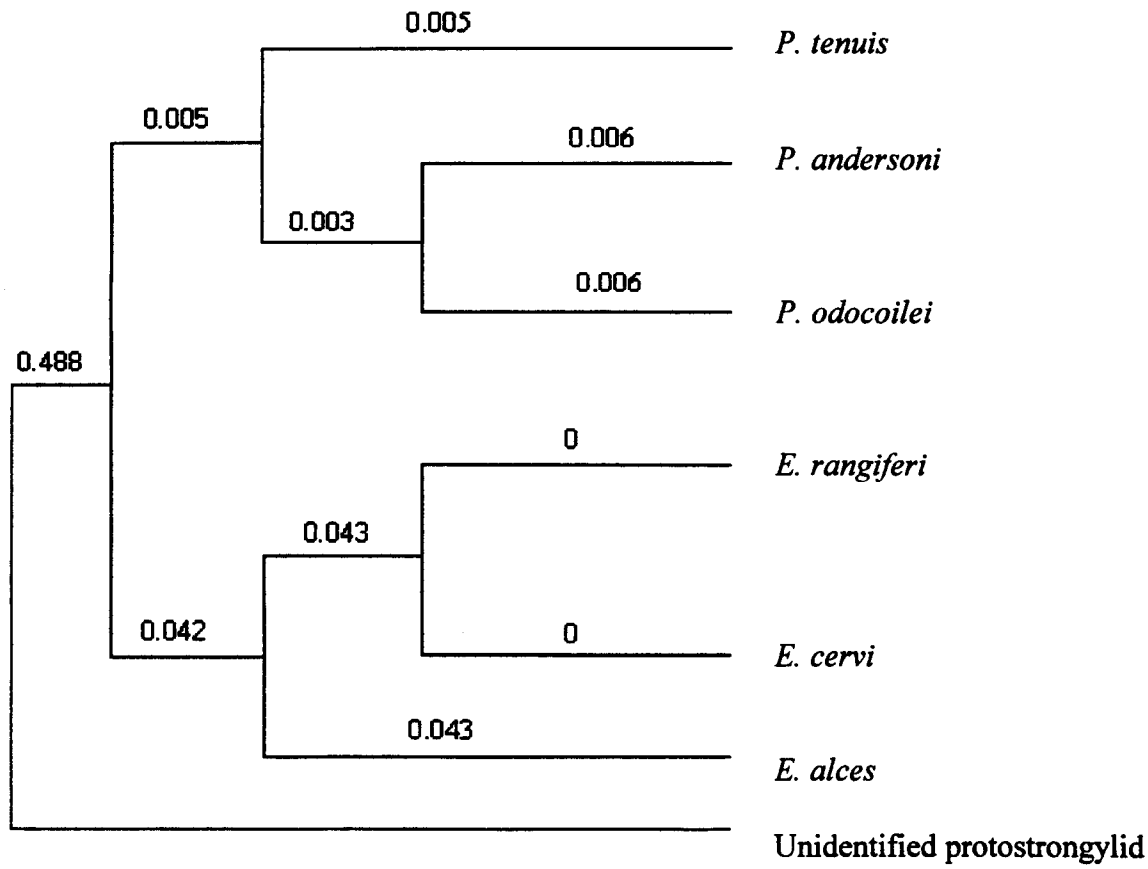
Discussion

The molecular data produced an optimal tree with similar topology to cladograms generated from morphological data by Platt (1984) and Carreno and Lankester (1994). The tree presented here places the "muscle worms" together with the "meningeal worm" as their sister group. This arrangement is biologically sound because *P. tenuis* survives best in its normal host, white-tailed deer, while *P. odocoilei* and *P. andersoni* can survive sufficiently in several suitable hosts (Lankester, 2001). Moreover, *P. tenuis* is known to cause severe neurological disease in abnormal hosts, while *P. odocoilei* and *P. andersoni* generally do not (Lankester, 2001).

TABLE 2. HKY85 distance matrix depicting percentage of sequence divergence between taxa in this study. Percentages are given as decimals.

	1	2	3	4	5	6	7	8	9	10
1. *P. tenuis*										
2. *P. tenuis 2*	0.00726									
3. *P. odocoilei*	0.01844	0.01474								
4. *P. odocoilei 2*	0.02025	0.01285	0.0018							
5. *P. andersoni*	0.01482	0.01111	0.00938	0.01122						
6. *P. andersoni 2*	0.01858	0.01109	0.01314	0.01122	0.00369					
7. *E. rangiferi*	0.15735	0.14801	0.15895	0.156	0.15009	0.15022				
8. *E. cervi*	0.15735	0.14801	0.15895	0.156	0.15009	0.15022	0			
9. *E. alces*	0.17454	0.1647	0.1766	0.1735	0.16472	0.15968	0.07826	0.07826		
10. *Mullerius sp.*	0.4233	0.42011	0.4304	0.43199	0.4225	0.41432	0.43243	0.43243	0.39404	

FIGURE 11. Optimal topology depicting phylogenetic relationships among six species of elaphostrongyline nematodes. Branch lengths are indicated at the nodes. The unidentified protostrongylid nematode sequence was used to root the tree.



Elaphostrongylus alces was clearly resolved as a separate species from *E. cervi* and *E. rangiferi,* with *E. alces* diverging from *E. cervi* and *E. rangiferi* by 7% of its sequence (Table 2). However, *E. cervi* and *E. rangiferi* could not be distinguished from each other. The ITS-2 sequences for these taxa were identical (Figure 5; Table 2). Controversy surrounds *E. rangiferi's* taxonomic status relative to *E. cervi,* and the ITS-2 rDNA sequence was not sufficient to distinguish them. Kutzer and Prosl (1975) considered both *E. rangiferi* and *E. panticola* to be synonyms of *E. cervi.* Other researchers argue that *E. cervi* and *E. rangiferi* are separate species (Steen et al. 1989; Gibbons et al. 1991) based on subtle differences morphological characteristics and differences in hosts affected.

Morphological and molecular phylogeny, current geographic distribution, and host specificity combined make it tempting to suggest that *Parelaphostrongylus* spp. originated in the definitive host *Odocoileus* (Platt, 1984; Carreno and Lankester, 1994). A phylogenetic analysis using mitochondrial DNA (Cronin, 1991) suggested that *Rangifer* is a monophyletic group separate from the *Cervinae* and *Odocoileinae*. If this is correct, *Elaphostrongylus* may have speciated in *Rangifer* and *Parelaphostrongylus* speciated in *Odocoileus*. Platt (1984) believed that the speciation of *P. tenuis* occurred prior to the origin of extant *Odocoileus* spp., and that subsequently *P. andersoni* and *P. odocoilei* co-speciated along with the definitive hosts *O. virginianus and O. hemonius*, respectively. Lankester and Hauta (1989) found that *P. andersoni* was widespread in caribou of North America, and suggested that *Rangifer* might be the original host rather than white-tailed deer. The presence of *P. andersoni* in North American caribou can be explained by the parasites switching hosts when the distributions of caribou and white-tailed deer overlap (Carreno and Lankester, 1994). However, if *P. andersoni* speciated with *Rangifer* in the

old world, we would expect to find *P. andersoni* in Europe (Carreno and Lankester, 1994). To date, no *P. andersoni* has been found in European cervids, however, more study, including DNA analysis, may be required to find them.

The identity and origins of a common ancestor between *Elaphostrongylus* spp. remain unclear as there is no variation between *E. cervi* and *E. rangiferi,* and *E. panticola* was not available for study. However, this study suggests that *E. alces* is distinct species from *E. cervi* and *E. rangiferi*, and that *E. cervi* and *E. rangiferi* likely share a Eurasian progenitor.

The common ancestor of *Parelaphostrongylus* spp. probably existed in an ancestral *Odocoileus*, perhaps with muscle worms and meningeal worms segregated in the host. Speciation of *P. tenuis* from the muscle worm progenitor may have occurred due to habitat specialization and physical isolation of meningeal worm from muscle worm in the same ancestral host. The further speciation of *P. odocoilei* and *P. andersoni* may have occurred upon speciation of mule deer and white-tailed deer hosts, respectively. It is thought that these two deer may have speciated due to allopatry (Tamarin 1996), perhaps as a result of isolation on both sides of the North American prairies (Carreno and Lankester, 1994). This scenario was supported by a comparison of phylogenies of the hosts with that of the parasites (Carreno and Lankester, 1994), and it is consistent with the results presented in this study.

APPENDIX. Summary of information about nematodes used as sources of DNA for this study.

Sample #	Sample ID	Stage/ Type	Host	ITS-2 Length(bp)	ID Method	Molecular ID	Infection Type	Location	Donor	Collection Date	Storage Medium
1	*E. alces*	L1-Pooled	Moose	575	Sequenced	*E. alces*	Expt'l.	Sweden	M. Steen [a]	1997	Baerman Filtrate*
1a	*E. alces*	L1-Single	Moose	575	Sequenced	*E. alces*	Expt'l.	Sweden	M. Steen [a]	1997	Baerman Filtrate*
1b	*E. alces*	L1-Single	Moose	575	Sequenced	*E. alces*	Expt'l.	Sweden	M. Steen [a]	1997	Baerman Filtrate*
2	*E. alces*	L1-Pooled	Moose	575	Sequenced	*E. alces*	Expt'l.	Sweden	M. Steen [a]	1997	Baerman Filtrate*
2a	*E. alces*	L1-Single	Moose	575	Sequenced	*E. alces*	Expt'l.	Sweden	M. Steen [a]	1997	Baerman Filtrate*
2b	*E. alces*	L1-Single	Moose	575	Sequenced	*E. alces*	Expt'l.	Sweden	M. Steen [a]	1997	Baerman Filtrate*
3	*E. cervi*	L1-Pooled	Red Deer	585	Sequenced	*E. cervi*	Expt'l.	Center for Animal Parasitology, Saskatoon	A. Gajadhar [b]	Jun.00	Baerman Filtrate***
3a	*E. cervi*	L1-Single	Red Deer	585	Sequenced	*E. cervi*	Expt'l.	Center for Animal Parasitology, Saskatoon	A. Gajadhar [b]	Jun.00	Baerman Filtrate***
3b	*E. cervi*	L1-Single	Red Deer	585	Sequenced	*E. cervi*	Expt'l.	Center for Animal Parasitology, Saskatoon	A. Gajadhar [b]	Jun.00	Baerman Filtrate***
4	*E. cervi*	L1-Pooled	Red Deer	585	Sequenced	*E. cervi*	Expt'l.	Center for Animal Parasitology, Saskatoon	A. Gajadhar [b]	Jun.00	Baerman Filtrate***
4a	*E. cervi*	L1-Single	Red Deer	585	Sequenced	*E. cervi*	Expt'l.	Center for Animal Parasitology, Saskatoon	A. Gajadhar [b]	Jun.00	Baerman Filtrate***
4b	*E. cervi*	L1-Single	Red Deer	585	Sequenced	*E. cervi*	Expt'l.	Center for Animal Parasitology, Saskatoon	A. Gajadhar [b]	Jun.00	Baerman Filtrate***
5	*E. rangiferi*	Adult	Caribou	585	Sequenced	*E. rangiferi*	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	Apr.01	H20
6	*E. rangiferi*	Adult	Caribou	585	Sequenced	*E. rangiferi*	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	Apr.01	H20
7	*E. rangiferi*	Adult	Caribou	585	Sequenced	*E. rangiferi*	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	Apr.01	H20
8	*E. rangiferi*	Adult	Caribou	585	Sequenced	*E. rangiferi*	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	Apr.01	Glyceralcohol
9	*E. rangiferi*	Adult	Caribou	585	Sequenced	*E. rangiferi*	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	Apr.01	Glyceralcohol
10	*E. rangiferi*	L1-Pooled	Caribou	585	Sequenced	*E. rangiferi*	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	N/A	Baerman Filtrate*
10a	*E. rangiferi*	L1-Single	Caribou	585	Sequenced	*E. rangiferi*	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	N/A	Baerman Filtrate*
10b	*E. rangiferi*	L1-Single	Caribou	585	Sequenced	*E. rangiferi*	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	N/A	Baerman Filtrate*
11	*O. macrotis*	Adult	Mule Deer	aprox.720	PCR	Putative *O. macrotis*	N/A	Alberta, Parasite Collection	A. Shostak [d]	2001	Glyceralcohol
12	*O. macrotis*	Adult	Mule Deer	aprox.720	PCR	Putative *O. macrotis*	N/A	Alberta	M. Pybus [f]	2001	H20
13	*O. macrotis*	L3 - Pooled	Mule Deer	aprox.720	PCR	Putative *O. macrotis*	N/A	Alberta	M. Pybus [f]	2001	H20
14	*P. andersoni*	Adult	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	Apr.01	H20
15	*P. andersoni*	Adult	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Avalon Herd, Newfoundland	M. Lankester [c]	Apr.01	H20
16	*P. andersoni*	L1-Pooled	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Bay de Verde, Newfoundland	M. Lankester [e]	Feb.00	Baerman Filtrate*
16a	*P. andersoni*	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Bay de Verde, Newfoundland	M. Lankester [e]	Feb.00	Baerman Filtrate*
16b	*P. andersoni*	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Bay de Verde, Newfoundland	M. Lankester [e]	Feb.00	Baerman Filtrate*
17	*P. andersoni*	L1-Pooled	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Bay de Verde, Newfoundland	M. Lankester [e]	Feb.00	Baerman Filtrate*
17a	*P. andersoni*	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Bay de Verde, Newfoundland	M. Lankester [e]	Feb.00	Baerman Filtrate*
17b	*P. andersoni*	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Bay de Verde, Newfoundland	M. Lankester [e]	Feb.00	Baerman Filtrate*
18	*P. andersoni*	L1-Pooled	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Cape Shore, Newfoundland	M. Lankester [e]	Sep.99	Baerman Filtrate*
18a	*P. andersoni*	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Cape Shore, Newfoundland	M. Lankester [e]	Sep.99	Baerman Filtrate*
18b	*P. andersoni*	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Cape Shore, Newfoundland	M. Lankester [e]	Sep.99	Baerman Filtrate*
19	*P. andersoni*	L1-Pooled	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Cape Shore, Newfoundland	M. Lankester [e]	Sep.99	Baerman Filtrate*
19a	*P. andersoni*	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Cape Shore, Newfoundland	M. Lankester [e]	Sep.99	Baerman Filtrate*
19b	*P. andersoni*	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 1	Wild	Cape Shore, Newfoundland	M. Lankester [e]	Sep.99	Baerman Filtrate*
20	*P. odocoilei*	L1-Pooled	Mule Deer	561	Sequenced	*P. odocoilei* var 1	Wild	Alberta	M. Pybus [f]	2000	Baerman Filtrate**
20a	*P. odocoilei*	L1-Single	Mule Deer	561	Sequenced	*P. odocoilei* var 1	Wild	Alberta	M. Pybus [f]	2000	Baerman Filtrate**
20b	*P. odocoilei*	L1-Single	Mule Deer	561	Sequenced	*P. odocoilei* var 1	Wild	Alberta	M. Pybus [f]	2000	Baerman Filtrate**

[a] Steen et al. 1997
[b] Gajadhar et al. 1994
[c] Lankester and Fong 1998
[d] Dr. A. Shostak, University of Alberta, Edmonton
[e] Ball and Lankester, 2001
[f] M. Pybus, Alberta Fish and Wildlife Division
[g] R. Carreno U.C. Davis, California
[h] W. Peterson, Libby, Montana
[i] Lankester and Samuel (Unpublished)
[j] Carreno et al. 2001

APPENDIX continued. Summary of information about nematodes used as sources of DNA for this study.

Sample #	Sample ID	Stage/ Type	Host	ITS-2 Length(bp)	ID Method	Molecular ID	Infection Type	Location	Donor	Collection Date	Storage Medium
21	*P. odocoilei*	L1-Pooled	Mule Deer	561	Sequenced	*P. odocoilei* var 1	Wild	California	R. Carreno[g]	N/A	Baerman Filtrate[e]
21a	*P. odocoilei*	L1-Single	Mule Deer	561	Sequenced	*P. odocoilei* var 1	Wild	California	R. Carreno[g]	N/A	Baerman Filtrate[e]
21b	*P. odocoilei*	L1-Single	Mule Deer	561	Sequenced	*P. odocoilei* var 1	Wild	California	R. Carreno[g]	N/A	Baerman Filtrate[e]
22	*P. odocoilei*	L1-Pooled	Mule Deer	561	Sequenced	*P. odocoilei* var 1	Wild	Montana	W. Peterson[h]	2000	Baerman Filtrate[e]
22a	*P. odocoilei*	L1-Single	Mule Deer	561	Sequenced	*P. odocoilei* var 1	Wild	Montana	W. Peterson[h]	2000	Baerman Filtrate[e]
22b	*P. odocoilei*	L1-Single	Mule Deer	561	Sequenced	*P. odocoilei* var 1	Wild	Montana	W. Peterson[h]	2000	Baerman Filtrate[e]
23	*P. odocoilei*	Adult	Mule Deer	561	Sequenced	*P. odocoilei* var 2	Expt'l.	Penticton, British Columbia	M. Lankester[i]	1990	10% Formailn
24	*P. odocoilei*	Adult	Mule Deer	561	Sequenced	*P. odocoilei* var 2	Expt'l.	Penticton, British Columbia	M. Lankester[i]	1990	10% Formailn
25	*P. odocoilei*	Adult	Mule Deer	561	Sequenced	*P. odocoilei* var 2	Expt'l.	Penticton, British Columbia	M. Lankester[i]	1990	10% Formailn
26	*P. odocoilei*	Adult	Mule Deer	561	Sequenced	*P. odocoilei* var 2	Expt'l.	Penticton, British Columbia	M. Lankester[i]	1990	H20
27	*P. odocoilei*	Adult	Mule Deer	561	Sequenced	*P. odocoilei* var 2	Expt'l.	Penticton, British Columbia	M. Lankester[i]	1990	H20
28	*P. odocoilei*	Adult	Mule Deer	561	Sequenced	*P. odocoilei* var 2	Expt'l.	Penticton, British Columbia	M. Lankester[i]	1990	H20
29	*P. tenuis*	Adult	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Wild	Parque Nacional de Santa Rosa, Costa Rica	R. Carreno[j]	Jun.99	70% Ethanol
30	*P. tenuis*	Adult	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Wild	Parque Nacional de Santa Rosa, Costa Rica	R. Carreno[j]	Jun.99	70% Ethanol
31	*P. tenuis*	Adult	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Wild	Parque Nacional de Santa Rosa, Costa Rica	R. Carreno[j]	Jun.99	70% Ethanol
32	*P. tenuis*	Adult	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Wild	Parque Nacional de Santa Rosa, Costa Rica	R. Carreno[j]	Jun.99	70% Ethanol
33	*P. tenuis*	Adult	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Wild	Parque Nacional de Santa Rosa, Costa Rica	R. Carreno[j]	Jun.99	70% Ethanol
34	*P. tenuis*	Adult	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Wild	Parque Nacional de Santa Rosa, Costa Rica	R. Carreno[j]	Jun.99	70% Ethanol
35	*P. tenuis*	Adult	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Wild	Parque Nacional de Santa Rosa, Costa Rica	R. Carreno[j]	Jun.99	70% Ethanol
36	*P. tenuis*	Adult	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Wild	Parque Nacional de Santa Rosa, Costa Rica	R. Carreno[j]	Jun.99	70% Ethanol
37	*P. tenuis*	Adult	Moose	554	Sequenced	*P. tenuis* var 1	Wild	Sleeping Giant Park, Ontario	F. Francis	Oct.94	Glyceralcohol
38	*P. tenuis*	Adult	Moose	554	Sequenced	*P. tenuis* var 1	Wild	Ontario	M. Lankester	Jul.94	Glyceralcohol
39	*P. tenuis*	Adult	Moose	554	Sequenced	*P. tenuis* var 1	Wild	Ontario	M. Lankester	Jul.94	Glyceralcohol
40	*P. tenuis*	Adult	Moose	554	Sequenced	*P. tenuis* var 1	Wild	Ontario	M. Lankester	Jul.94	Glyceralcohol
41	*P. tenuis*	Adult	Moose	554	Sequenced	*P. tenuis* var 1	Wild	Ontario	M. Lankester	Jul.94	Glyceralcohol
42	*P. tenuis*	Adult	Moose	554	Sequenced	*P. tenuis* var 1	Wild	Ontario	M. Lankester	Jul.94	Glyceralcohol
43	*P. tenuis*	Adult	Moose	554	Sequenced	*P. tenuis* var 1	Wild	Ontario	M. Lankester	Jul.94	Glyceralcohol
44	*P. tenuis*	L1-Pooled	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Expt'l.	N/A	M. Lankester	2000	Baerman Filtrate[e]
44a	*P. tenuis*	L1-Single	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Expt'l.	N/A	M. Lankester	2000	Baerman Filtrate[e]
44b	*P. tenuis*	L1-Single	White-tailed-deer	554	Sequenced	*P. tenuis* var 1	Expt'l.	N/A	M. Lankester	2000	Baerman Filtrate[e]
45	*V. alpenae*	Adult	Mule Deer	aprox.700	PCR	Putative *V. alpenae*	N/A	Alberta, Parasite Collection	A. Shostak[d]	2001	Glyceralcohol
46	Putative *P. tenuis*	L1-Pooled	White-tailed-deer	554	Sequenced	*P. tenuis* var 2	Wild	Presque Isle Park, Marquette, Michigan	J. Garvon	Feb.00	H2O
47	Putative *P. tenuis*	L1-Pooled	White-tailed-deer	554	Sequenced	*P. tenuis* var 2	Wild	Presque Isle Park, Marquette, Michigan	J. Garvon	Feb.00	H20
47a	Putative *P. tenuis*	L1-Single	White-tailed-deer	554	Sequenced	*P. tenuis* var 2	Wild	Presque Isle Park, Marquette, Michigan	J. Garvon	Feb.00	H20
47b	Putative *P. tenuis*	L1-Single	White-tailed-deer	554	Sequenced	*P. tenuis* var 2	Wild	Presque Isle Park, Marquette, Michigan	J. Garvon	Feb.00	H20
48a	Putative *P. tenuis*	L3-Single	*Triodopsis*	554	Sequenced	*P. tenuis* var 2	Wild	Presque Isle Park, Marquette, Michigan	J. Garvon	Feb.00	70% Ethanol
48b	Putative *P. tenuis*	L3-Single	*Triodopsis*	554	Sequenced	*P. tenuis* var 2	Wild	Presque Isle Park, Marquette, Michigan	J. Garvon	Feb.00	70% Ethanol
49a	Putative *P. tenuis*	L3-Single	*Anguispira alternata*	554	Sequenced	*P. tenuis* var 2	Wild	Presque Isle Park, Marquette, Michigan	J. Garvon	Feb.00	70% Ethanol

[a] Steen et al. 1997

[b] Gajadhar et al. 1994

[c] Lankester and Fong 1998

[d] Dr. A. Shostak, University of Alberta, Edmonton

[e] Ball and Lankester, 2001

[f] M. Pybus, Alberta Fish and Wildlife Division

[g] R. Carreno U.C. Davis, California

[h] W. Peterson, Libby, Montana

[i] Lankester and Samuel (Unpublished)

[j] Carreno et al. 2001

APPENDIX continued. Summary of information about nematodes used as sources of DNA for this study.

Sample #	Sample ID	Stage/ Type	Host	ITS-2 Length(bp)	ID Method	Molecular ID	Infection Type	Location	Donor	Collection Date	Storage Medium
49b	Putative *P. tenuis*	L3-Single	*Anguispira alternata*	554	Sequenced	*P. tenuis* var 2	Wild	Presque Isle Park, Marquette, Michigan	J. Garvon	Feb 00	70% Ethanol
50	Unident.1	L1-Pooled	Black-tailed-deer	220 and 561	PCR & RFLP	Unident. 3 & *P. odocoilei*	Wild	Washington	B. Foreyt	Mar.00	Baerman Filtrate**
50a	Unident.1	L1-Single	Black-tailed-deer	561 only	RFLP	*P. odocoilei*	Wild	Washington	B. Foreyt	Mar.00	Baerman Filtrate**
50b	Unident.1	L1-Single	Black-tailed-deer	561 only	RFLP	*P. odocoilei*	Wild	Washington	B. Foreyt	Mar 00	Baerman Filtrate**
51	Unident.2	L1-Pooled	Big Horn Sheep	495	Sequenced	(Unident. Type 1)	Wild	Washington	B. Foreyt	Mar 00	Baerman Filtrate**
51a	Unident.2	L1-Single	Big Horn Sheep	495	Sequenced	(Unident. Type 1)	Wild	Washington	B. Foreyt	Mar.00	Baerman Filtrate**
51b	Unident.2	L1-Single	Big Horn Sheep	495	Sequenced	(Unident. Type 1)	Wild	Washington	B. Foreyt	Mar.00	Baerman Filtrate**
52	Unident.3	L1-Pooled	Big Horn Sheep	495 and 330	Sequenced	(Unident. Type 1 and 2)	Wild	Washington	B. Foreyt	Mar.00	Baerman Filtrate**
53	Unident.4	L1-Pooled	Big Horn Sheep	495	Sequenced	(Unident. Type 1)	Wild	Washington	B. Foreyt	Mar.00	Baerman Filtrate**
54	Unident.5	L1-Pooled	Big Horn Sheep	495	Sequenced	(Unident. Type 1)	Wild	Washington	B. Foreyt	Mar.00	Baerman Filtrate**
55	Unident.6	L1-Pooled	Big Horn Sheep	495	Sequenced	(Unident. Type 1)	Wild	Washington	B. Foreyt	Mar 00	Baerman Filtrate**
56	Unident.7	L1-Pooled	Big Horn Sheep	495	Sequenced	(Unident. Type 1)	Wild	Washington	B. Foreyt	Mar.00	Baerman Filtrate**
57	Unident 8	L1-Pooled	Elk	330	PCR	Unidentified type 2	Wild	Keeler MTN, Montana	D. Dorman	Mar.00	Baerman Filtrate**
57a	Unident.8	L1-Single	Elk	330	PCR	Unidentified type 2	Wild	Keeler MTN, Montana	D. Dorman	Mar.00	Baerman Filtrate**
57b	Unident.8	L1-Single	Elk	330	PCR	Unidentified type 2	Wild	Keeler MTN, Montana	D. Dorman	Mar.00	Baerman Filtrate**
58	Unident.9	L1-Pooled	Moose	554	RFLP	*P. tenuis*	Wild	Minnesota	G. Huschile	Feb 00	Baerman Filtrate*
59	Unident.10	L1-Pooled	Muskox	495	PCR	(Unident. Type 1)	Wild	Kotzeube, Alaska	J. Daw	Dec.99	Baerman Filtrate**
59a	Unident.10	L1-Single	Muskox	495	PCR	(Unident. Type 1)	Wild	Kotzeube, Alaska	J. Daw	Dec.99	Baerman Filtrate**
59b	Unident.10	L1-Single	Muskox	495	PCR	(Unident. Type 1)	Wild	Kotzeube, Alaska	J. Daw	Dec.99	Baerman Filtrate**
60	Unident.11	L1-Pooled	Muskox	495	PCR	(Unident. Type 1)	Wild	Kotzeube, Alaska	J. Daw	2001	Baerman Filtrate**
61	Unident.12	L1-Pooled	Muskox	495	PCR	(Unident. Type 1)	Wild	Kotzeube, Alaska	J. Daw	2001	Baerman Filtrate**
62	Unident.13	L1-Pooled	Muskox	495	PCR	(Unident. Type 1)	Wild	Kotzeube, Alaska	J. Daw	2001	Baerman Filtrate**
63	Unident.14	L1-Pooled	Muskox	495	PCR	(Unident. Type 1)	Wild	Kotzeube, Alaska	J. Daw	2001	Baerman Filtrate**
64	Unident.15	L1-Pooled	Elk	495	PCR	(Unident. Type 1)	Wild	Keeler MTN, Montana	D. Dorman	2001	Baerman Filtrate**
65	Unident.16	L1-Pooled	Black-tailed-deer	545	RFLP	*P. andersoni*	Wild	Disk Island, Alaska	D. Crowley	Feb 00	Baerman Filtrate**
65a	Unident.16	L1-Single	Black-tailed-deer	545	RFLP	*P. andersoni*	Wild	Disk Island, Alaska	D. Crowley	Feb.00	Baerman Filtrate**
65b	Unident.16	L1-Single	Black-tailed-deer	545	RFLP	*P. andersoni*	Wild	Disk Island, Alaska	D. Crowley	Feb.00	Baerman Filtrate**
66	Unident.17	L1-Pooled	Mule Deer	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Mar.00	Baerman Filtrate*
67	Unident.18	L1-Pooled	Caribou	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*
67a	Unident.18	L1-Single	Caribou	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*
67b	Unident.18	L1-Single	Caribou	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*
68	Unident.19	L1-Pooled	Caribou	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate**
69	Unident.20	L1-Pooled	Caribou	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*
70	Unident.21	L1-Pooled	Caribou	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*
71	Unident.22	L1-Pooled	Caribou	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*
72	Unident.23	L1-Pooled	Mountain Goat	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*
72a	Unident.23	L1-Single	Mountain Goat	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*
71b	Unident.23	L1-Single	Mountain Goat	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov 99	Baerman Filtrate*
73	Unident.24	L1-Pooled	Mountain Goat	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*
74	Unident.25	L1-Pooled	Mountain Goat	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov.99	Baerman Filtrate*

[a] Steen et al. 1997
[b] Gajadhar et al. 1994
[c] Lankester and Fong 1998
[d] Dr. A. Shostak, University of Alberta, Edmonton
[e] Ball and Lankester, 2001
[f] M. Pybus, Alberta Fish and Wildlife Division
[g] R. Carreno U.C. Davis, California
[h] W. Peterson, Libby, Montana
[i] Lankester and Samuel (Unpublished)
[j] Carreno et al. 2001

APPENDIX continued. Summary of information about nematodes used as sources of DNA for this study.

Sample #	Sample ID	Stage/ Type	Host	ITS-2 Length(bp)	ID Method	Molecular ID	Infection Type	Location	Donor	Collection Date	Storage Medium
75	Unident.26	L1-Pooled	Mule Deer	561	RFLP	*P. odocoilei*	Wild	Caw Ridge, Alberta	M. Pybus[f]	Nov 99	Baerman Filtrate[a]
76	Unident.27	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Lac Joseph Herd, Quebec	S. Coutier	Mar.00	Baerman Filtrate[a]
76a	Unident.27	L1-Single	Caribou	545	RFLP	*P. andersoni*	Wild	Lac Joseph Herd, Quebec	S. Coutier	Mar 00	Baerman Filtrate[a]
76b	Unident.27	L1-Single	Caribou	545	RFLP	*P. andersoni*	Wild	Lac Joseph Herd, Quebec	S. Coutier	Mar 00	Baerman Filtrate[a]
77	Unident.28	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Lac Joseph Herd, Quebec	S. Coutier	Mar.00	Baerman Filtrate[a]
78	Unident.29	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Lac Joseph Herd, Quebec	S. Coutier	Mar 00	Baerman Filtrate[a]
79	Unident.30	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Aishihik Lake, Yukon	R. Ward	Feb.00	Baerman Filtrate[a,a]
80	Unident.31	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Mink Creek, British Columbia	E. Jenkins	Mar.99	95% Ethanol
81	Unident.32	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Dease River, British Columbia	E. Jenkins	Mar 99	95% Ethanol
82	Unident.33	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Watson Lake, British Columbia	E. Jenkins	Apr.99	95% Ethanol
83	Unident.34	L1-Pooled	Caribou	545	Sequenced	*P. andersoni* var 2	Wild	Detour Lake, Ontario	F. Mallory	Aug.98	Baerman Filtrate[a]
83a	Unident.34	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 2	Wild	Detour Lake, Ontario	F. Mallory	Aug 98	Baerman Filtrate[a]
83b	Unident.34	L1-Single	Caribou	545	Sequenced	*P. andersoni* var 2	Wild	Detour Lake, Ontario	F. Mallory	Aug 98	Baerman Filtrate[a]
84	Unident.35	L1-Pooled	Caribou	545	Sequenced	*P. andersoni* var 2	Wild	Detour Lake, Ontario	F. Mallory	Aug 98	Baerman Filtrate[a]
85	Unident.36	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Detour Lake, Ontario	F. Mallory	Aug 98	Baerman Filtrate[a]
86	Unident.37	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Detour Lake, Ontario	F. Mallory	Aug.98	Baerman Filtrate[a]
87	Unident.38	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Detour Lake, Ontario	F. Mallory	Aug.99	Baerman Filtrate[a]
88	Unident.39	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Detour Lake, Ontario	F. Mallory	Aug.99	Baerman Filtrate[a]
89	Unident.40	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Detour Lake, Ontario	F. Mallory	Aug.99	Baerman Filtrate[a]
90	Unident.41	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Detour Lake, Ontario	F. Mallory	Aug.99	Baerman Filtrate[a]
91	Unident.42	L1-Pooled	Caribou	545	RFLP	*P. andersoni*	Wild	Detour Lake, Ontario	F. Mallory	Aug.99	Baerman Filtrate[a]

[a] Steen et al. 1997
[b] Gajadhar et al. 1994
[c] Lankester and Fong 1998
[d] Dr. A. Shostak, University of Alberta, Edmonton
[e] Ball and Lankester, 2001
[f] M. Pybus, Alberta Fish and Wildlife Division
[g] R. Carreno U.C. Davis, California
[h] W. Peterson, Libby, Montana
[i] Lankester and Samuel (Unpublished)
[j] Carreno et al. 2001

LITERATURE CITED

ALBERTS, B., D. BRAY, J. LEWIS, M. RAFF, K. ROBERTS, AND J.D.WATSON. Molecular Biology of the Cell, 3rd ed. 1294 pp.

ANDERSON, R.C. 1963. The incidence, development, and experimental transmission of *Pneumostrongylus tenuis* Doughtery (Metastrongyloidea: Protostrongylidae) of the meninges of the white-tailed deer (*Odocoileus virginianus borealis*) in Ontario. Canadian Journal of Zoology 41:775-792.

ANDERSON, R.C. 1964a. Motor ataxia and paralysis in moose calves infected experimentally with *Pneumostrongylus tenuis* (Nematoda: Metastrongyloidea). Northeastern Wildlife Conference, Hartford, Connecticut, January 21, pp. 1-7.

ANDERSON, R.C. 1964b. Neurologic disease in moose infected experimentally with *Pneumostrongylus tenuis* from white-tailed deer. Veterinary Pathology 1:289-322.

ANDERSON R.C. 1968. The pathogenesis and transmission of neurotropic and accidental nematode parasites of the central nervous system of mammals and birds. Helminthological Abstracts 37:191-210.

ANDERSON, R.C. 2000. Nematode parasites of vertebrates: Their development and transmission, 2nd ed. Cambridge: C.A.B. International, University Press, Cambridge. 650 pp.

ANDERSON, R.C., AND A.K. PRESTWOOD. 1981. Lungworms. In Diseases and Parasites of White-Tailed Deer. Ed. W. R. Davidson, F.A. Hayes, V.F. Nettles, and F.E. Kellog. Miscellaneous Publications Number 7. Tallhassee, FL. Tall Timbers Research Station, pp.266-317.

BALLANTYNE, R.J., AND W.M. SAMUEL. 1984. Diagnostic morphology of three species of *Parelaphostrongylus* (Nematoda, Metastrongyloidea). Journal of Parasitology 70(4):602-604.

BANERJEE, S.K., W.F. MAKDISI, A.P. WESTON, S.M. MITCHELL, AND D.R. CAMPBELL. 1995. Microwave-based DNA extraction from paraffin-embedded tissue for PCR amplification. BioTechniques 18(5):768-773.

BARNES, I., J. HOLTON, D. VAIRA, M. SPIGELMAN, AND M. THOMAS. 2000. An assessment of the long-term preservation of the DNA of a bacterial pathogen in ethanol-preserved archival material. Journal of Pathology 192:554-559.

BOEV, S.N., AND R.S. SCHULTZ. 1950. Reconstruction of the systematics of nematodes of the family Protostrongylidae Leiper, 1926. Dokl. Akad. Nauk. SSSR. 70:355-358. (In Russian)

BOWLES, J. AND D.P. McMANUS. 1993. Rapid discrimination of *Echinococcus* species and strains using a polymerase chain reaction-based RFLP method. Molecular and Biochemical Parasitology 57:231-240.

CAMERON, T.W.M. 1931. On two new species of nematodes from the Scottish red deer. Journal of Helminthology 9:213-216.

CAMPBELL, A. J.D., R.B. GASSER, AND N.B. CHILTON. 1995. Differences in a ribosomal DNA sequence of *Strongylus* species allows identification of single eggs. International Journal for Parasitology 25(3):359-365.

CARRENO, R.A., L.A. DURDEN, D.R. BROOKS, A. ABRAMS, AND E. P. HOBERG. 2001. *Parelaphostrongylus tenuis* (Nematoda: Protostrongylidae) and other parasites of White-Tailed Deer (*Odocoileus virginianus*) in Costa Rica. Comparative Parasitology 68(2):177-184.

CARRENO, R.A. AND E.P. HOBERG. 1999. Evolutionary relationships among the Protostrongylidae (Nematoda: Metastronyloidea) as inferred from morphological characters, with consideration of parasite-host coevolution. Journal of Parasitology 85(4):638-648.

CARRENO, R.A. AND M.W. LANKESTER. 1994. A re-evaluation of the phylogeny of *Parelaphostrongylus* Boev and Schultz, 1950 (Nematoda: Protostrongylidae). Systematic Parasitology 28:145-151.

COOMBS, N.J., A.C. GOUGH, AND J.N. PRIMROSE 1999. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. Nucleic Acids Research 27(16): e12.

CRONIN, M.A. 1991. Mitochondrial-DNA phylogeny of deer (Cervidae). Journal of Mammology 72:553-566.

DALLAS, J.F., R.J. IRVINE, O. HALVORSEN, AND S.D. ALBON. 2000. Identification by polymerase chain reaction (PCR) of *Marshallagia marshalli* and *Ostertagia gruehneri* from Svalbard reindeer. International Journal for Parasitology 30:863-866.

DAO, A. 2000. The application of PCR in the differentiation of elaphostrongyline nematodes. HBSc. Thesis. Lakehead University, Thunder Bay, Ontario, Canada. 40 pp.

DIVINA, B.P., E. WILHELMSSON, J.G. MATTSSON, P. WALLER, AND J. HÖGLUND. 2000. Identification of *Dictyocaulus* spp. in ruminants by morphological and molecular analyses. Parasitology 121:193-201.

DOUGHERTY, E.C. 1945. The nematode lungworms (sub-order *Strongylina*) of North American deer of the genus *Odocoileus*. Parasitology 36:199-208.

EDWARDS, W.H. 1995. *Parelaphostrongylus andersoni* (Nematoda: Protostrongylidae) in white-tailed deer (*Odocoileus virginianus*) of northeastern Wyoming. M.Sc Thesis, University of Wyoming, Laramie, Wyoming, 83 pp.

ELLIS, R.E., J.E. SULSTON, AND A.R. COULSON. 1986. The rDNA of *C. elegans*: sequence and structure. Nucleic Acids Research 14(5):2345-2364.

EPE, C., G.V. SAMSON-HIMMELSTJERNA, AND T. SCHNIDER. 1997. Differences in a ribosomal DNA sequence of lungworm species (Nematoda:Dictyocaulidae) from fallow deer, cattle, sheep and donkeys. Research in Veterinary Science 62(1):17-21.

FAGERHOLM, H.P.1979. Nematode length and preservatives, with a method for determining the length of live specimens. Journal of Parasitology 65(2):334-335.

FORRESTER, D.J. 1992. Parasites and diseases of wild mammals in Florida. Gainsville: University Press of Florida, Gainsville, 459 pp.

FORRESTER, S.G., AND M.W LANKESTER. 1997. Over-winter survival of first-stage larvae of *Parelaphostrongylus tenuis* (Nematoda: Protostrongylidae). Canadian Journal of Zoology 76:704-710.

GAJADHAR, A., T. STEEVES-GURNSEY, J. KENDALL, M.W. LANKESTER, AND M. STEEN. 2000. Differentiation of dorsal-spined Elaphostrongyline larvae by polymerase chain reaction amplification of ITS-2 of rDNA. Journal of Wildlife Diseases 36(4):713-722.

GAJADHAR, A., S.V. TESSARO, AND W.D.G. YATES. 1994. Diagnosis of *Elaphostrongylus cervi* infection in New Zealand Red Deer (*Cervus elaphus*) quarantined in Canada, and experimental determination of a new extended prepatent period. Canadian Veterinary Journal 35:433-437.

GAJADHAR, A., AND S.V. TESSARO. 1995. Susceptability of mule deer (*Odocoileus hemonius*) and two species of North American molluscs to *Elaphostrongylus cervi* (Nematoda: Metastrongyloidea). The Journal of Parasitology 81:593-569.

GALL, K., J. PAVELIĆ, D. JADRO-ŠANTEL, M. POLJAK, AND K. PAVELIĆ. 1993. DNA amplification by polymerase chain reaction from brain tissues embedded in paraffin. International Journal of Experimental Pathology 74:333-337.

GASSER, R.B., AND J.R. MONTI. 1997. Identification of parasitic nematodes by PCR-SSCP of ITS-2 rDNA. Molecular and Cellular Probes 11:201-209.

GASSER, R.B. 2001. Identification of parasitic nematodes and study of genetic variability using PCR approaches. In parasitic nematodes: Molecular biology, biochemistry, and immunology. Ed. M.W. Kennedy, and W. Harnett. CABI Publishing, New York, NY. pp 53-71.

GASSER, R.B., N.B. CHILTON, H. HOSTE, AND I. BEVERIDGE. 1993. Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. Nucleic Acids Research 21(10):2525-2526.

GIBBONS, L.M., O. HALVORSEN, AND G. STUVE. 1991. Revision of the genus *Elaphostrongylus* (Nematoda: Metastrongyloidea) with particular reference to species of the genus occurring in Norwegian cervids. Zoologica Scripta 20(1):15-26.

GRAY, J.B, AND W. M. SAMUEL. 1986. *Parelaphostrongylus odocoilei* (Nematoda: Protostrongylidae) and a protostrongylid nematode in woodland caribou (*Rangifer tarandus caribou*) of Alberta, Canada. Journal of Wildlife Diseases 22:48-50.

GREER, C.E., S.L. PETERSON, N.B. KIVAIT, AND M.M. MANOS. 1991. PCR amplification from parafin-embedded tissues: effects of fixative and fixation time. American Journal of Clinical Pathology 95(2):117-124.

HANDELAND, K. 1994. Experimental studies of *Elaphostrongylus rangiferi* in reindeer (*Rangifer tarandus tarandus*): life cycle, pathogenesis, and pathology. Journal of Veterinary Medicine 41:351-365.

HANDELAND, K. AND A. SKORPING. 1992. The early migration of *Elaphostrongylus rangiferi* in goats. Journal of Veterinary Medicine B 39:263-272.

HASEGAWA, M., H KISHINO, AND T. YANO. 1985. Dating the human-ape split by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22:160-174.

HEISE, M., C. EPE, AND T. SCNHIEDER. 1999. Differences in the second internal transcribed spacer (ITS-2) of eight species of gastrointestinal ruminants. Journal of Parasitology 85(3):431-435.

HEMMINGSEN, W., O. H, A. HALVORSEN, AND A. SKORPING. 1993. Migration of adult *Elaphostrongylus rangiferi* (Nematoda: Protostrongylidae) from the spinal epidural space to the muscles of reindeer (*Rangifer tarandus*). Journal of Parasitology 79:728-732.

HENEGARIU, O., N.A. HEEREMA, S.R. DLOUHU, G.H. VANCE, AND P.H. VOGT. 1997. Multiplex PCR: Critical parameters and step by step protocol. BioTechniques 23:504-511.

HOBMAIER, A. AND M. HOBMAIER. 1934. *Elaphostrongylus odocoilei* n.sp., a new lung worm in black-tailed deer (*Odocoileus columbianus*): description and life history. Proceedings of the Society for Experimental Biology and Medicine 31:509-514.

HÖGLUND, J., E. WILHWLMSON, D. CHRISTENSSON, T. MÖRNER, P. WALLER, AND J.G. MATTSSON. 1999. ITS-2 sequences of *Dictyocaulus* species from cattle, roe deer and moose in Sweden: molecular evidence for a new species. International Journal for Parasitology 29:607-611.

HOSTE, H., N.B. CHILTON, R.B. GASSER, AND I. BEVERIDGE. 1995. Differences in the second internal transcribed spacer (ribosomal DNA) between five species of *Trichostrongylus* (Nematoda: Trichostrongylidae). International Journal for Parasitology 25(1):75-80.

HUNG, G.C., N.B. CHILTON, I. BEVERIDGE, A. McDONNELL, J.R. LICHTENFELS, AND R. B. GASSER. 1997. Molecular Delineation of *Cylicocaulus nassatus* and *C. ashworthi* (Nematoda: Strongylidae). International Journal for Parasitology. 27(5):601-605.

ISOLA, J., S. DeVRIES, L. CHU, S. GHAZVINI, AND F. WALDMAN 1994. Analysis of changes in DNA sequence copy number by comparative genomic hybridization in archival paraffin-embedded tumor samples. American Journal of Pathology 145(6):1301-1308.

JANNASCH, H.W., C.O. WIRSEN, S.J. MOLYNEAUX, AND T.A. LANGWORTHY. 1992. Comparative physiological studies on hyperthermophilic archaea isolated from deep-sea hot vents with emphasis on *Pyrococcus* strain GB-D. Applied and Environmental Microbiology 58(11):3472-3481.

KUTZ, S.J., A.M. VIETCH, E.P. HOBERG, B.T. ELKIN, E.J. JENKINS, AND L. POLLEY. 2001. New host and geographic records for two protostrongylids in Dall's sheep. Journal of Wildlife Diseases 37(4):761-774.

LANKESTER, M.W. 2001. Extrapulmonary lungworms of cervids. In Parasitic diseases of wild mammals. W.M. Samuel, M. J. Pybus, and A.A. Kocan ed pp. 228-278.

LANKESTER, M.W., AND R.C. ANDERSON. 1968. Gastropods as intermediate hosts of *Pneumostrongylus tenuis* Doughtery of white-tailed deer. Canadian Journal of Zoology 46:373-383.

LANKESTER, M.W., AND D. FONG. 1989. Distribution of Elaphostrongyline nematodes (Metastrongyloidea: Protostrongylidae) in Cervidae and possible effects of moving *Rangifer spp.* into and within North America. Alces 25:133-145.

LANKESTER, M.W., AND D. FONG. 1998. Protostrongylid nematodes in caribou (*Rangifer tarandus caribou*) and moose (*Alces alces*) of Newfoundland. Rangifer, Special Issue No. 10, 73-83.

LANKESTER, M.W., AND P.L. HAUTA. 1989. *Parelaphostrongylus andersoni* (Nematoda: Protostrongylidae) in caribou (*Rangifer tarandus*) of northern and central Canada. Canadian Journal of Zoology, 67:1966-1975.

LANKESTER, M.W., AND T. H. NORTHCOTT. 1979. *Elaphostrongylus cervi* Cameron 1931 (Nematoda: Metastrongyloidea) in caribou (*Rangifer tarandus caribou*) of Newfoundland. Canadian Journal of Zoology 57:1384-1392.

LANKESTER, M.W., AND W. J. PETERSON. 1996. The possible importance of deer wintering yards in the transmission of *Parelaphostrongylus tenuis* to white-tailed deer and moose. Journal of Wildlife Diseases 32:31-38.

MACHIELS, B.M., T. RUERS, M. LINDHOUT, K. HARDY, T. HLAVATY, D.D. BANG, V.A.M.C. SOMERS, C. BAETEN, M. von MAYENFELDT, AND F.B.J.M. THUNNISSEN. 2000. New extraction protocol for DNA extraction of stool. BioTechniques 28:286-290.

McMANUS, D.P., M. KNIGHT, AND A.J. SIMPSON. 1985. Isolation and characterisation of nucleic acids from the hydatid organisms, *Echinococcus* spp. (Cestoda). Molecular and Biochemical Parasitology 16(3):251-66.

MITSKEVITCH, V.Y., 1958. On the interpretation of the developmental cycle of the nematode *Elaphostrongylus rangiferi* n. sp. from a reindeer. Doklady Akademii Nauk SSR 119:621-624. (In Russian).

MITSKEVITCH, V.Y., 1964. Life cycle of *Elaphostrongylus rangiferi* Mitskevitch 1958. In Parasites of farm animals in Kazakhastan. S.N. Boev ed. Izdatel'stvo Akademii Nauk SSR Alma-Ata 3: 49-60. (In Russian).

MONKEY, T., M. TROLL, M. ERMAN, AND N. SIX. 2002. Thermocycling and exponential growth of hindquarters: effects of increasing doses of glucose and fatty lipids. Journal of Degenerate Wildlife 1: 1-100.

NEWTON, L.A., N.B. CHILTON, I. BEVERIDGE, AND R.B. GASSER. 1998a. Differences in the second internal transcribed spacer of four species of *Nematodirus* (Nematoda:Molinedae). International Journal for Parasitology 28:337-341.

NEWTON, L.A., N.B. CHILTON, I. BEVERIDGE, AND R.B. GASSER. 1998b. Genetic evidence indicating that *Cooperia surnabada* and *Cooperia oncophora* are one species. International Journal for Parasitology 28:331-336.

OLSSON, I.M., R. BERGSTRÖM, M. STÉEN, AND S. SANDGREN. 1995. A study of *Elaphostrongylus alces* in an island moose population with low calf body weights. Alces 31:61-75.

OLSSON, I.M., M.W. LANKESTER, A.A. GAJADHAR, AND M. STÉEN. 1998. Tissue migration of *Elaphostrongylus spp.* in guinea pigs (*Cavia porcellus*) Journal of Parasitology 84:968-975.

PLATT, T.R., 1984. Evolution of the Elaphostrongylinae (Nematoda: Metastrongyloidea: Protostrongylidae) parasites of cervids (Mammalia). Proceedings of the Helminthological Society Washington 51:196-204.

POSDA, D. AND K.A. CRANDALL. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14(9): 817-818.

POTTS, W.K. 1996. PCR Based cloning across large taxonomic distances and polymorphism detection: MHC as a case study. In Molecular Zoology Advances, Strategies, and Protocols J.D. Ferraris and S.R. Palumbi ed.

PRESIDENTE, P.J.A. 1986a. First report of the tissue worm in Australia. The Federal Deerbreeder 5:13-17.

PRESIDENTE, P.J.A. 1986b. Tissue worm: Implications for live deer exports. In deer farming into the nineties. P. Owen ed. Based on the proceedings of the Australian Deer 86 conference. Brisbane: Owen Art and Publishing, pp. 192-202.

PRESTWOOD, A.K. 1972. *Parelaphostrongylus andersoni* n. sp. (Metastrongyloidea: Protostrongylidae) from the muscle of white-tailed deer (*Odocoileus virginianus*). Journal of Parasitology 58:897-902.

PRESTWOOD, A.K., AND V.F. NETTLES. 1977. Repeated low level infection of white-tailed deer with *Parelaphostrongylus andersoni*. Journal of Parasitology 63:974-978.

PRESTWOOD, A.K., V.F. NETTLES, AND F.E. KELLOGG. 1974. Distribution of muscle worm (*Parelaphostrongylus andersoni*) among white-tailed deer of the southeastern United States. Journal of Wildlife Diseases 10:404-409.

PYBUS, M. J. 1983. *Parelaphostrongylus andersoni* Prestwood 1972 and *P. odocoilei* (Hobmaier and Hobmaier 1934) in two cervid definitive hosts. Ph.D. Dissertation, University of Alberta, Edmonton, 185 pp.

PYBUS, M. J., AND W.M. SAMUEL. 1981. Nematode muscle worm from white-tailed deer of Southeastern British Columbia. Journal of Wildlife Management 45(2):537-542.

PYBUS, M.J., AND W.M. SAMUEL. 1984. *Parelaphostrongylus andersoni* (Nematoda: Protostrongylidae) and *P. odocoilei* in two cervid definitive hosts. Journal of Parasitology 70(4):507-515.

PYBUS, M. J., W.M. SAMUEL, D.A. WELCH, AND C.J. WILKE. 1990. *Parelaphostrongylus andersoni* (Nematoda: Protostrongylidae) and *P. odocoilei* in white-tailed deer from Michigan. Journal of Wildlife Diseases 26(4): 535-537.

QIAamp® Tissue Kit and QIAamp DNA Blood Mini Kit Handbook. QIAGEN, January 2000.

SAMUEL, W.M., T.R. PLATT, AND S.M. KNISPEL-KRAUSE. 1985a. Gastropod intermediate hosts and transmission of *Parelaphostrongylus odocoilei*, a muscle-inhabiting nematode of mule deer, *Odocoileus h. hemonius* in Jasper National Park, Alberta. Canadian Journal of Zoology 63:928-932.

SAMUEL, W.M., A.W. SHOSTAK, AND M.J. PYBUS. 1985b. *Varestrongylus alpenae* (Nematoda Metastrongyloidea) in white-tailed deer (*Odocoileus virginianus*) of Saskatchewan. Canadian Journal of Zoology 63:1499-1454.

SIMPSON, A. J., A. SHER, AND T.F. McCUTCHAN. 1982. The genome of *Schistosoma mansoni*: isolation of DNA, its size, bases and repetitive sequences. Molecular and Biochemical Parasitology 6(2):125-37.

STEEN, M. 1991. Elaphostrongylosis: a clinical, pathological, and taxonomical study with special emphasis on the infection in moose. Ph.D. Dissertation. Swedish University of Agricultural Sciences. Uppsala, Sweden.

STEEN, M., A.G. CHABAUD, AND C. REHBINDER. 1989. Species of the genus *Elaphostrongylus* parasite of Swedish cervidae: a description of *E. alces* n. sp. Annals de Parasitologie Humaine et Comparee 64:134-142.

STEEN, M., W.E. FABER, AND A. OKSANEN. 1998. Disease and genetical investigations of Fennoscandian cervids: a review. Alces 34:287-310.

SWOFFORD, D. 1998. PAUP 4.0: Phylogenetic analysis using parsimony and other methods. Sinauer, Sunderland, MA.

TAMARIN, R.H., 1996. Principles of Genetics, 5th Edition. Wm. C. Publishers Dubuque Iowa. 683 pp.

YANG, D.Y., B. ENG, J.S. WAYE, J.C. DUDAR, AND S.R. SAUNDERS. 1998. Technical note: Improved DNA extraction from ancient bones using silica-based spin columns. American Journal of Physical Anthropology 105:539-543.