MOLECULAR APPROACHES TO SYSTEMATIC PROBLEMS IN PARASITIC NEMATODES: RIBOSOMAL DNA VARIATION WITHIN *CYSTIDICOLA* SPP. (NEMATODA: HABRONEMATOIDEA) AND THE SUPERFAMILY DRACUNCULOIDEA

by

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HBSc., Lakehead University, Thunder Bay, 1996

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Department of Zoology)

We accept this thesis as conforming to the required standard.

THE UNIVERSITY OF BRITISH COLUMBIA

September 2000

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Date Oct. 12, 2000

DE-6 (2/88)

ABSTRACT

Morphological characters are traditionally used in nematode systematics, however, morphological convergence and marginal differences between close relatives can obscure species diversity and confound taxonomic studies. This thesis applies molecular approaches to systematic problems in two groups of parasitic nematodes where morphological data is ambiguous. Ribosomal DNA (rDNA) variable regions such as the first and second internal transcribed spacers (ITS-1 and ITS-2), and the D3 expansion loop of the large subunit have consistently distinguished nematode species and provided a limited basis for phylogenetic inference between close relatives. I assess rDNA variation within *Cystidicola* spp. (Nematoda; Habronematoidea) and the superfamily Dracunculoidea to examine species diversity in both groups, and phylogenetic relationships in the Dracunculoidea.

Phenotypic variation in *Cystidicola* spp. suggests unresolved variation within the genus. Distinct life histories, host ranges, reproductive strategies, and adult and egg morphologies define the two recognized *Cystidicola* spp. Variable host specificity and egg morphology in *Cystidicola farionis* is difficult to interpret and could reflect genetic species-level variation. I sequenced four rDNA regions (ITS-1, ITS-2, 5.8S, D3) from *Cystidicola* spp. isolates from a total of seven host species and nine locations in Ontario (ONT), British Columbia (BC) and Finland (FIN). The ITS-1, 5.8S, and D3 regions displayed no inter or intraspecific variation. Two ITS-2 types were identified which differed at four nucleotide positions: the ITS-2 from *C. farionis* (BC) and *C. stigmatura* was identical and 365bp long; the ITS-2 from ONT and FIN *C. farionis* was identical and 368bp long. No relationship between egg morphology and genetic variation was apparent. ITS-2 differences between morphologically distinct *C. farionis* (ONT and FIN) and *C. stigmatura* were expected but comparison of this region among *C. farionis*

isolates produced a surprising result. The ITS-2 distinguishes *C. farionis* (BC) from *C. farionis* (ONT and FIN) and suggests a closer relationship between *C. farionis* (BC) and *C. stigmatura*.

Morphological resemblance among close relatives and a lack of phylogenetically informative characters in the superfamily Dracunculoidea reiterates this need for more precise taxonomic markers. I examined the D3 and ITS-2 regions from a total of nine dracunculoid species to distinguish cryptic species (e.g. *Philonema* spp.), place unidentified nematodes within the current classification system, and infer phylogenetic relationships within dracunculoid families (e.g. the Philometridae and Guyanemidae). I sequenced the D3 of two dracunculoid species, *Philometroides huronensis* and an unidentified nematode from *Eopsetta exilis*, adding these to an existing D3 data set of seven dracunculoids and sequenced the ITS-2 from all nine species. These regions varied in their ability to distinguish close relatives. The D3 region distinguishes *Philonema agubernaculum* and *P. oncorhynchi* but not *Cystidicola* spp. whereas the ITS-2 is identical in the former taxa and distinct in the latter. Both ITS-2 and D3 data supported previous suggestions that the family Philometridae may be artificial, and that members of the Guyanemidae are affiliated with some philometrids (e.g. *Philonema* spp.).

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ACKNOWLEDGEMENTS

I wish to thank my advisor, Martin Adamson, for advice, encouragement and supervision throughout my studies. I am grateful to my supervisory committee, Al Lewis and Rick Taylor, who provided valuable discussions and comments on drafts of this thesis.

I am indebted to those who provided nematode samples for this project: Murray Lankester and Mark Klitch (Thunder Bay, Ontario); Ernest Keeley (British Columbia); Hans-Peter Fagerholm (Finland); David Cone (Nova Scotia); Amanda Brown (British Columbia worms and DNA samples). Many thanks go to Murray Lankester for providing lab space and resources during my 1997 collection trip in Thunder Bay. When the fish didn't bite, he wouldn't let me go home empty handed. I appreciate the advice of Neil Chilton (Melbourne, Australia), who suggested appropriate nematode ITS-2 primers.

I wish to thank members of the Adamson lab for providing a supportive and stimulating work environment. Thank you Shannon Bennett and Stephen Connor for sharing your knowledge and enthusiasm about parasitology (and life in general). Thank you Amanda Brown and Tammy Laberge for teaching me about molecular biology; your patient guidance in the lab, helpful discussions about my project, and comments on drafts of this thesis were invaluable. I am grateful for your support and friendship.

I sincerely appreciate the support of my friends and family throughout my studies. I wish to thank my parents, Patricia Miscampbell and Les Miscampbell, for believing in me through it all. Thank you Mom for countless phone calls and emails sharing your strength, love, and encouragement and for all the care packages (and coffee $$). Thank you Dad for all your advice and words of encouragement. I wish to thank my husband, Scott Morgan, for his strength, patience, and support which means more to me than I can say.

CHAPTER 1: GENERAL INTRODUCTION

Issues in nematode taxonomy

Nematodes are widespread with diverse life histories and habitats, yet their anatomy is highly conserved. There are approximately 16 000 described nematode species, several thousand of which are parasites including many economically or medically important species (Anderson 1992). Most estimates suggest even higher diversity; including undescribed nematodes more than 42 000 species may exist (Anderson 1992). Placing unidentified taxa within the current classification system (i.e. identification/species diagnosis), and establishing classifications that reflect natural, or evolutionary, relationships among taxa (i.e. phylogenetic systematics) are continual challenges for nematode taxonomists.

Morphological characters are the classical data of nematologists, however, morphological convergence and marginal differences between close relatives may obscure species diversity and confound taxonomic studies (Ferris 1983, 1994; Baldwin and Powers 1987). Advances in available technologies for investigating biodiversity, including scanning electron microscopy (SEM) and transmission electron microscopy for fine structure examination, and biochemical techniques such as protein electrophoresis, serology, and nucleic acid analysis, have improved nematode diagnostics and phylogenetic investigations (Baldwin and Powers 1987; Ferris and Ferris 1987; Ferris 1994). The search for congruence among a range of data sets will continue to advance nematode taxonomy (Hyman and Powers 1991; Ferris 1994).

Integration of these alternate data sets becomes increasingly important when considering species concepts. Most biologists reject the idea that all species can be recognized using only morphological criteria (Mayr 1963; Scudder 1974; Mallet 1995), yet

as detailed biological background for many nematode taxa is lacking many species remain defined by morphology alone. Deciding species boundaries by applying the biological species concept, where species are groups of actually or potentially interbreeding natural populations reproductively isolated from other groups (Mayr 1963), is difficult with nematodes as the necessary cross-breeding experiments are often impractical. Many other species definitions exist (see Scudder 1974 and Mallet 1995 for reviews). The genotypic cluster species concept recently proposed by Mallet (1995) considers species as groups that remain recognisable in sympatry because of morphological and genotypic gaps between them. This definition incorporates both genetic information and phenotypic characteristics and is practical for nematode taxonomy and systematics, however, it still defines a species by comparison to a given type (morphological or genetic). While the species problem has yet to be resolved, it is clear that information from multiple markers is desirable especially for recently diverged taxa (e.g. sibling species) where there may be no single diagnostic marker to differentiate between groups (Avise 1994, Anderson et al. 1998).

Reliable identification of a nematode parasite, irrespective of life cycle stage, is central to studying its life cycle, population biology, and epidemiology. This is particularly important when considering nematodes of medical and economic importance, such as human or agricultural parasites. Every organism bears a unique genetic tag, in the form of its DNA sequence, which can be applied to a range of taxonomic issues, from identification of individuals in a population to relationships among phyla (Litvaitis et al. 1994). Analysis of molecular data, such as DNA sequences, has become increasingly popular for nematode diagnostics (reviewed in the following section and Chapter 2) and phylogenetic inference (Vanfleteren et al. 1994; Blaxter et al. 1998; Nadler and Hudspeth 1998).

Ribosomal DNA as a taxonomic marker for nematodes

Molecular analysis of nematodes can be applied to a range of taxonomic and systematic problems such as diagnosis of economically important species, phylogenetic inference, and biodiversity issues. Ribosomal RNA (rRNA) genes and their associated spacer regions, collectively called rDNA, have been applied to studies ranging from the origin of life to more recent evolutionary events (Hillis and Dixon 1991), and can be a particularly powerful tool for nematode species-level diagnosis and phylogenetic studies (Gasser et al. 1993; Ferris et al. 1993; Powers et al. 1997). I refer the reader to Hillis et al. (1996) for a detailed review of molecular systematic techniques and terminology used in this and upcoming sections.

Ribosomes, the organelles that direct protein synthesis from messenger RNA in the cells of all living organisms, are comprised of several distinct rRNAs and ribosomal proteins. The rDNA array of a eukaryote nuclear genome typically includes hundreds of tandemly repeated copies of the transcription unit with a nontranscribed spacer (NTS) separating adjacent repeats (Figure 1.1). The transcription unit is subdivided into three coding regions and four noncoding spacers (including the NTS). Two internal transcribed spacers (ITS-1 and ITS-2) that separate the small subunit (SSU, 16-18S), 5.8S, and large subunit (LSU, 23-28S) genes, and the external transcribed spacer (ETS), located upstream from the SSU, are transcribed with the coding regions to form a single precursor RNA molecule (pre-RNA) (Hillis and Dixon 1991). Ordered cleavage and removal of the spacers occurs to produce mature rRNA (Gerbi 1985).

Several structural characteristics of rDNA contribute to its wide applicability, and facilitate nematode rDNA studies in particular. The rDNA repeat unit is divided into

domains that evolve at different rates (Gerbi 1985) so it can be applied to diagnostic and evolutionary problems at different taxonomic levels, allowing a range of phylogenetic questions to be addressed. Copies of the repeat unit within an interbreeding population or species tend to be identical because of concerted evolution (Arnheim 1983) so interspecific variation is greater than intraspecific variation. Polymerase chain reaction (PCR) amplification from small amounts of tissue (e.g. larval nematodes and eggs, Gasser et al. 1993) is also facilitated by the abundant and uniform nature of rDNA and the presence of highly conserved regions within most rDNA genes, which permit construction of "universal" primers and allow sequences from many different species to be targeted for comparison (Hillis and Dixon 1991).

Nuclear small subunit rDNA (16-18S) possesses one of the most slowly evolving sequences and has been useful for examining ancient evolutionary events among eukaryote taxa (Hillis and Dixon 1991) including nematodes (Nadler 1992; Fitch et al. 1995; Blaxter et al. 1998; Nadler and Hudspeth 1998). While nematode relationships at the family level may be resolved using the SSU, intrageneric relationships (e.g. within *Caenorhabditis* spp.) are not estimated sufficiently by this region because it evolves so slowly (Fitch et al. 1995). The 5.8S is similarly conserved, but its short length (~160 bp) limits its phylogenetic effectiveness across large time scales (Hillis and Dixon 1991).

The large subunit rDNA (23-28S) of eukaryotes consists of conserved "core" regions interspersed with ten to twelve variable regions, termed "expansion segments", which are absent in prokaryotes (Clark et al. 1984). These divergent segments, or D-domains (D1-D12), are under relatively reduced evolutionary constraint and length and sequence may vary considerably among taxa (Hancock and Dover 1988). Expansion segments D2, D3, D8 and

D10 are among those structurally characterised and appear to exhibit conserved secondary structure among eukaryotes (Michot and Bachellerie 1987; Michot et al. 1990; Ruiz Linares et al. 1991; Nunn 1992, 1996). The D3 region has proven to be a good marker for closely related species (Litvaitis et al. 1994; Nunn 1996) including nematodes (Nunn 1992; Deprés et al. 1995; Brown 1996; Baldwin et al. 1996; Al-Banna et al. 1997) because it exhibits interspecific, but not intraspecific, variation.

The ITS-1 and ITS-2 regions are noncoding and have no function in mature rRNA, so they are under fewer evolutionary constraints than rDNA genes (Michot et al. 1983; Hillis and Dixon 1991). The assumption that spacer sequences are completely free to diverge is questionable. Recent studies indicate a fundamental role for these spacers in processing pre-RNA which is directly related to their secondary structure (Musters et al. 1990; van der Sande 1992; van Nues 1994, 1995). Evolutionarily conserved portions of yeast ITS-2 have been shown to be the most important in post-transcriptional processing (van Nues 1995). While some of the spacer sequence is functionally constrained, the remainder may evolve at a rate close to neutral (Schlötterer et al. 1994). Since secondary structure plays an important role in ITS function, restrictions to maintain specific structural motifs would be expected, making it possible to compare taxa that have reduced primary sequence similarity. Ribosomal DNA secondary structure integrity can be maintained despite changes in primary sequence if mutations are non-disruptive; that is if compensatory nucleotide changes occurring on both strands of a stem base-pair, and/or insertions which add to stem length or occur in existing loops or bulges, do not alter the overall folding of the region. Secondary structures have been predicted for the ITS regions of plants (Coleman and Mai 1997; Mai and Coleman 1997), fungi (Musters et al. 1990; van der Sande 1992; van Nues 1994, 1995;

Lott et al. 1998; Shinohara 1999), insects (Wesson et al. 1991; Kwon and Ishikawa 1992; Schlötterer et al. 1994), mammals (Michot et al. 1983), trematodes (Michot et al. 1993; Morgan and Blair 1998), and nematodes (Chilton et al. 1998). While there is little conservation among the secondary structures of these taxa, common structures, usually supported by the existence of compensatory base-pair changes, can be found within each group despite high levels of sequence variation (Wesson et al. 1992; Schlötterer et al. 1994; Mai and Coleman 1997; Chilton et al. 1998; Morgan and Blair 1998; Shinohara 1999).

Nematode spacer sequences serve as valuable diagnostic tools, allowing researchers to identify and differentiate economically important species (Ferris et al. 1993; Campbell et al. 1995; Hoste et al. 1995; Stevenson et al. 1995, 1996; Hung et al. 1997, 1999; Leignel et al. 1997; Powers et al. 1997; Newton et al. 1998a, b). The spacers have also provided, in a more limited way, a basis for phylogenetic inference between closely related nematode species (Chilton et al. 1997; Hoste et al. 1998; Newton et al. 1998; Zarlenga et al. 1998). Given their increasing popularity as diagnostic and phylogenetic tools, and the growing number of taxa for which ITS sequences are available, the taxonomic level at which these regions are phylogenetically informative, or have phylogenetic signal, must be ascertained. Hypervariability of ITS sequences has generally excluded their application to higher level phylogenetic analyses (e.g. above the family level), however, since rRNA secondary structure conservation exceeds that of its nucleotides, secondary structures may more accurately guide assignment of homologous positions, in the form of partial sequence alignment, among taxa (Kjer 1995). Greater potential exists for examining higher level phylogenetic relationships using ITS sequences if their secondary structures are used to guide alignment across a greater taxonomic range. This method has been applied, with the effect of

resolving phylogenies, to *Drosophila* spp. (Schlötterer et al. 1994), Chlorophyta (Coleman and Mai 1997), and trematodes (Morgan and Blair 1998).

Application of rDNA to problems at different taxonomic levels

When considering close relatives, especially morphologically cryptic species, distributional, ecological, or behavioural differences may first indicate that more than one species is involved (Sturhan 1981). Phenotypic variation in *Cystidicola* spp. (Nematoda; Spirurida), a common parasite of salmonid fishes, suggests unresolved variation within the genus. Biological differences, including distinct life histories, host ranges, reproductive strategies, and adult and egg morphologies define the two recognized *Cystidicola* spp. Variable host specificity and egg morphology in *Cystidicola farionis*, however, is difficult to interpret; this variation could reflect host-induced developmental flexibility or indicate a fixed morphological polymorphism. The latter possibility would suggest genetic species-level variation within *C. farionis*. The current status of the genus is based solely on morphological information. Analysis of molecular level differences within *Cystidicola* spp. may resolve the above biological and morphological discrepancies and allow the validity of the current species designations to be assessed.

The use of morphological characters to establish phylogenetic relationships and distinguish closely related species within the nematode superfamily Dracunculoidea, which includes medically and economically important vertebrate parasites, has likewise proven difficult. The current classification may be based on primitive, phylogenetically uninformative, characters (Adamson et al. 1987). In addition, morphological similarity among close relatives (e.g. *Philonema* spp.) suggests that more precise taxonomic markers are required to reliably assess species diversity within this superfamily. Therefore, several

issues within the Dracunculoidea can be addressed with the help of appropriate molecular markers (i.e. rDNA), ranging from resolving species diversity within *Philonema* spp. and classifying unidentified dracunculoid species, to assessing the validity of the current dracunculoid classification based on morphological characteristics.

In this study I examine species-level variation within and between *Cystidicola* spp. (Nematoda; Habronematoidea) by assessing genetic divergence of four rDNA regions (ITS-1, ITS-2, 5.8S, D3) relative to morphological variation (egg type and appearance of the oral opening). I also compare ITS-2 and D3 variation among nine members of the Dracunculoidea (Nematoda; Spirurida) to evaluate species diversity and phylogenetic relationships within this superfamily. In addition to resolving taxonomic issues within these groups of nematodes, results from the rDNA variable regions can be compared to assess their overall taxonomic usefulness.

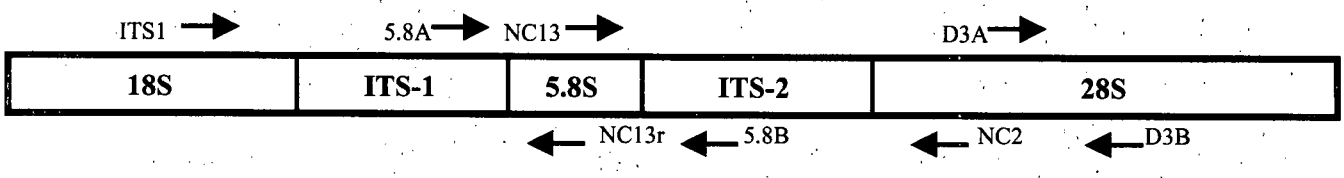


Figure 1.1 The rDNA tandem repeat is composed of 3 coding regions (18S, 5.8S, and 28S) and 4 non-coding spacers (ITS-1 and ITS-2 shown here). In this study, primer sets (indicated with arrows) amplified 4 regions: ITS-1 (ITS1 and NC13r), 5.8S (5.8A and 5.8B), ITS-2 (NC13 and NC2), and D3 (D3A and D3B).

CHAPTER 2: MOLECULAR AND MORPHOLOGICAL VARIATION WITHIN *CYSTIDICOLA* SPP.

Introduction

Nematodes of the genus *Cystidicola*, Fischer 1798 (Spirurida; Cystidicolidae) are common parasites in the swim bladder of physostomous fishes. Two species are recognized: *Cystidicola farionis* Fischer, 1798 infects fishes from Salmonidae and Osmeridae in Europe, Asia, and North America, and *C. stigmatura* (Leidy, 1886) Ward and Magath, 1917 occurs in *Salvelinus* spp. exclusively in North America. The two species are morphologically well-defined and can be distinguished based on the appearance of the egg and oral opening. *Cystidicola farionis* has filamented eggs and the anterolateral wall of the buccal cavity lacks a lip-like projection, whereas *C. stigmatura* has eggs with lateral lobes and a lip-like projection fused to the medial aspect of the pseudolabium (Ko and Anderson 1969; Black 1983a; Dextrase 1987).

The biology of these parasites is well documented (Smith and Lankester 1979; Black and Lankester 1980, 1981; Lankester and Smith 1980; Black 1983a, b, 1984, 1985). The amphipods *Gammarus fasciatus*, *Hyalella azteca* and *Pontoporeia affinis* are suitable intermediate hosts for *C. farionis*, while *C. stigmatura* develops in the opossum shrimp, *Mysis relicta*, before transmission to the final host (Smith and Lankester 1979; Black and Lankester 1980). *Cystidicola farionis* occurs most commonly in whitefish (*Coregonus* and *Prosopium* spp.), trout (*Salmo* spp.), and char (*Salvelinus* spp.) (Black 1983a). Lake trout (*Salvelinus namaycush*), Arctic char (*S. alpinus*) and brook trout (*S. fontinalis*) host *C. stigmatura* (Black 1983a).

Aside from having distinct morphologies and life cycles, *C. farionis* and *C.*

stigmatura exhibit striking differences in their reproductive strategies. Seasonal fluctuations of *C. farionis* numbers in many fish hosts (Lankester and Smith 1980) suggests that it is relatively short-lived, perhaps a year or less, while *C. stigmatura* may live for more than ten years (Black and Lankester 1981). The number of eggs per female worm (absolute fecundity) is 10 to 20 times greater in *C. farionis* than in *C. stigmatura* (Dextrase 1987). The low fecundity of *C. stigmatura* may be related to its long lifespan. Populations of *C. stigmatura* are relatively constant, exhibit density dependent regulatory mechanisms, and do not have seasonal changes in abundance or maturity (Black and Lankester 1981). Prevalence and intensity of *C. farionis* populations tend to fluctuate seasonally and there is a lack of density dependent regulatory mechanisms (Lankester and Smith 1980).

Considerable confusion surrounds the taxonomy of *Cystidicola* spp. At one time 21 species were assigned to the genus, but Ko and Anderson (1969) recognized only 3 valid species: *C. farionis, C. stigmatura* and *C. cristivomeri* White, 1941. The species known as *C. cristivomeri* had eggs bearing lateral lobes and was restricted to Salmoninae in North America, while the species with filamented eggs that infected both Coregoninae and Salmoninae was called *C. stigmatura*. The third species with filamented eggs from European Salmonidae and Osmeridae was named *C. farionis*. *Cystidicola stigmatura* was often considered a synonym for *C. farionis* (Campana-Rouget 1955; Arthur et al. 1976; Ko and Anderson 1969; Moravec and Ergens 1970; Margolis and Arthur 1979) since both species appeared morphologically identical. This taxonomic muddle is attributed to the fact that Leidy (1886) did not describe the egg morphology of *C. stigmatura*, and later workers (e.g. Skinker 1930, 1931) assumed that, like those of *C. farionis,* eggs of *C. stigmatura* were

filamented. Black (1983a) examined Leidy's specimens, and noted eggs bearing a pair of lateral lobes. As a result he proposed *C. cristivomeri* as a synonym of *C. stigmatura* (Leidy, 1886) Ward and Magath, 1917 which has eggs with lateral lobes, whereas *C. farionis* (syn *C. stigmatura* of Skinker 1931; Ko and Anderson 1969) has filamented eggs. Currently all *Cystidicola* spp. with filamented eggs from Europe, Asia, and North America, are recognized as *C. farionis*.

Further questions about the taxonomic status of *Cystidicola* spp. have recently been investigated, with equivocal results. Lankester and Smith (1980) speculated that the worm maturing in lake whitefish (*Coregonus clupeaformis*) may represent a new species of *Cystidicola.* They found few mature *C. farionis* in lake whitefish from lakes Superior and Nipigon, but up to 50% of worms were mature in lake whitefish from Surprise Lake, Black Sturgeon Lake and Lake Huron. Although adult worms from lake whitefish are otherwise morphologically indistinguishable from those in other hosts (Dextrase 1987), their eggs have mostly lateral filaments while eggs in other hosts have mainly polar filaments (Smith 1978; Dextrase 1987). Dextrase (1987) found immature worms that accumulated in the swimbladder of L. Nipigon lake whitefish developed toward sexual maturity in hatchery-reared lake whitefish from L. Simcoe. He concluded that development of *C. farionis* is dependent on host strain, with variation in egg morphology being either host-induced or a result of genetic differences of the worms involved.

The extent of variation implied by the above biological and morphological discrepancies has yet to be fully assessed. An approach which combines molecular and morphological markers may resolve taxonomic issues within *Cystidicola* spp. Evidence of

molecular variation could further support the hypothesis that the worm maturing in lake whitefish is a distinct species. In this study I investigate variation among *Cystidicola* isolates from seven host species and nine locations in Ontario, British Columbia, and Finland; I sequence the spacer regions (ITS-1 and ITS-2), D3 expansion loop of the large subunit (28S), and 5.8S rDNA and examine morphological features (e.g. egg type and appearance of the oral opening) to assess species level diversity within this genus.

Materials and methods

Collection

Nematodes were collected during 1997-1998 from various host species and localities across Canada and Europe (Table 2.1). Samples were obtained from fish caught by commercial fisheries on L. Superior and Arrow L., or from swim bladders collected by anglers in British Columbia (BC) and Ontario (Table 2.1). Worms collected from individual host species from each location were pooled, except those from BC rainbow trout, and Ontario lake whitefish from Arrow L. and L. Superior. Unpooled worms were counted and identified as male, female, or juvenile. Mature worms were identified according to Lankester and Smith (1980). All worms were fixed and stored in 95% ethanol.

Examination of Eggs

I determined the egg type of at least two worms representing each *Cystidicola* isolate before I extracted DNA. To do this, I placed mature female worms on clean microscope slides and moistened them with proteinase K buffer (0.1M TRIS, 0.05M EDTA, 1% SDS, 0.2M NaCl). Using a sterile scalpel blade, I removed the worm's midsection (≅ 2mm) to a

new slide with a drop of glycerine. The rest of the worm was set aside for DNA extraction. Eggs were forced out of the uterus when I placed a cover slip on the slide. Using a light microscope I determined whether the eggs had lateral filaments and/or polar filaments, or lateral lobes.

Scanning Electron Microscopy (SEM)

For SEM examination, nematodes fixed in 95% EtOH were further dehydrated with 3 washes of 100% EtOH. Dehydrated worms were critical point dried with CO_2 in a Balzers Union CPD 020 critical point dryer, mounted on aluminum stubs, and gold-coated in a Nanotech SEM Prep II sputter coater. Worms were examined with a Cambridge 250 scanning electron microscope at an accelerating voltage of 10-20kV.

DNA Extraction

Individual female worms were prepared for DNA extraction as follows: each worm was cut in 2-3 pieces and soaked in proteinase K buffer for 45 min. Samples were placed in 1.7ml microcentrifuge tubes with 0.25 ml proteinase K buffer + 0.1 ml proteinase K enzyme (10mg/ml) and incubated at either 65°C for 10 hours or 55°C for 5 hours to digest histones and other proteins associated with DNA. Most extraction replicates represent separate individual host infections. Worms were taken from different host individuals from BC rainbow trout, Arrow L. and L. Superior lake whitefish (e.g. unpooled samples). Worms chosen from the remaining (pooled) samples may be from different infected hosts.

DNA was extracted twice with phenol followed by one phenol-chloroform-isoamyl alcohol (25:24:1) and one chloroform-isoamyl-alcohol (24:1) extraction. DNA was precipitated overnight in 95% ethanol at -20°C. DNA precipitate was centrifuged and the

resulting pellet washed twice with 70% ethanol and dried in a vacuum centrifuge. Pellets were resuspended in 30µl Milli-pore water + 1.5µl RNAse (0.02mg/ml).

PCR and Sequencing

Primers that targeted the ITS-1, ITS-2, 5.8S, and D3 expansion loop of the 28S rDNA were used for PCR amplification of *Cystidicola* spp. DNA (Figure 1.1).

ITS-2: Primers derived from 5.8S (NC13 5'-ATCGATGAAGAACGCAGC-3' Chilton et al. 1997) and 28S (NC2 5'-TTAGTTTCTTTTCCTCCGCT-3' Gasser et al. 1993) rDNA sequences of *C. elegans* (Ellis et al. 1986) amplified the ITS-2 rDNA region of *Cystidicola* spp.

D3: Primers (D3A 5'-GACCCGTCTTGAAACACGGA-3' and D3B 5'-TCGGAAGGAACCAGCTACTA-3' Nunn 1992) based on *C. elegans* 28S rDNA sequences (Ellis et al. 1986) amplified a portion of conserved core DNA as well as the D3 expansion loop of *Cystidicola* spp.

ITS-1: Primers based on *C.elegans* 18S rDNA (Ellis et al. 1986) (ITS1 5'-CCCTTTGTACACACCGCC-3') and 5.8S rDNA (NC13r 5'-GCTGCGTTCTTCATCGAT-3'complementary of NC13 from Chilton et al. 1997) amplified the ITS-1 of *Cystidicola* spp.

5.8S: Primers based on sequences obtained from *Cystidicola* spp. ITS-1 (5.8A 5'-TTGTTCGAGCTATCGCCCTT-3') and ITS-2 (5.8B 5'-TTGCAACATTGCTCGACG-3') amplified the 5.8S region of *Cystidicola* spp.

PCR amplification was performed in 25µl reactions containing 0.2mM each dNTP, 2.5mM $MgCl_2$, 1x reaction buffer (10x buffer = 200mM $(NH_4)_2SO_4$, 750mM Tris-HCl (pH 8.8) and 0.1% Tween 20), 0.6µM each primer and 1.25U Taq polymerase. PCR conditions

for ITS-1, D3 and 5.8S consisted of one cycle of 95°C for 2 min (denaturation), followed by 40 cycles of 95°C for 50s (denaturing), 55°C for 25s (annealing), 72°C for 1 min (extension) and one cycle of 72°C for 7 min (extension). Identical conditions were used for ITS-2 except the annealing phase was 54.5°C for 30s. I viewed PCR products by running 4µl PCR product and 3µl loading buffer (40% sucrose, 0.25% xylene cyanol FF, and ethidium bromide 0.20µg/µl) on 2% TBE (44.5mM Tris, 44.5mM boric acid, 1mM EDTA) agarose gels.

Sequencing reactions were done using fluorescently-labelled chain-terminating (dideoxy) nucleotides which interrupt DNA synthesis to produce a distinct fragment for each sequence position. Sequences were generated directly from a gel using the ABI Prism 377 automated DNA sequencer. Both the 5' and 3' strands were sequenced for three regions (ITS1, ITS2, D3) for at least two worms of each isolate, except the D3 sequence of only one worm from Arrow L. lake whitefish was obtained (Table 2.2). Sequencing in both directions was done for the 5.8S region of one worm from L. Superior ONT, Squeers L. ONT, Baltic Sea Finland, and Chaunigan L. BC.

Using the Clustal W version 1.7 program (Thompson et al. 1994), I aligned *Cystidicola* spp. sequences with rDNA of *C. elegans* (Ellis et al. 1986) to determine the ends of the four rDNA regions obtained. Sequence length in base pairs (bp) and base composition (guanine and cytosine, G + C, content) of each rDNA region is reported. *Cystidicola* spp. sequences from each direction were compared and overlapping regions aligned manually using the ESEE version 3.2s program (Cabot 1998). Secondary structure of rRNA is often more conserved than nucleotide sequence (see Chapter 1), therefore I determined the secondary structure of the D3 and ITS-2 of *Cystidicola* spp. for comparison to other

nematodes (Brown 1996; Chilton 1998). Secondary structure for the D3 region was based on the conserved eukaryote model (Michot et al. 1990) and drawn using loopDloop version 1.2 (Gilbert 1992). The mfold program (Mathews et al. 1999; Zucker et al. 1999), based on an energy minimisation approach, was used to predict the secondary structure of the ITS-2. Mfold generates the most energy efficient, or optimal, folding for a given nucleotide sequence, as well as other foldings that are termed suboptimal because they have a higher free energy (ΔG) (default setting is $\leq 5\%$ of the optimal folding's ΔG). I compared all predicted secondary structures for *Cystidicola* spp. ITS-2 sequences for evidence of compensatory changes which would suggest ITS-2 secondary structure is maintained within this genus despite nucleotide changes.

Results

All eggs examined from *C. farionis* had polar filaments except those from Chaunigan L, which had no filaments (Table 2.2). *Cystidicola farionis* eggs from lake whitefish had both polar and lateral filaments. Eggs of *C. stigmatura* from lake trout had lateral lobes and no filaments (Table 2.2). Four rDNA regions in *Cystidicola* spp. (Figure 2.1) were compared (Table 2.2) for variation between isolates. No intraspecific variation was found at any of the rDNA regions examined. No interspecific variation was detected within the ITS-1, 5.8S, or D3 sequences from any *Cystidicola* isolates. *Cystidicola farionis* from Ontario (ONT) and Finland (FIN) were identical at all rDNA regions sequenced regardless of whether their eggs had both polar and lateral filaments (ONT lake whitefish) or only polar filaments. The ITS-2 was the only rDNA region examined that varied between *Cystidicola* isolates, but nucleotide differences in this region did not correspond with egg morphology (Table 2.3). British

Columbia isolates with polar filamented eggs differed in ITS-2 type from isolates from Ontario and Finland with lateral and/or polar filaments but were identical in ITS-2 type to isolates with lobed eggs.

SEM examination (Figure 2.2) revealed that *C. farionis* from Ontario were the only isolates lacking a lip-like projection on the anterolateral wall of the buccal cavity. While this feature distinguished them from *C. stigmatura* as suggested by Black and Lankester (1980) and Dextrase (1987), it also differentiated them from *C. farionis* BC and FIN and represented another morphological character shared by *C. farionis* BC and *C. stigmatura*.

ITS-1, 5.8S and D3 rDNA sequences were invariant among all samples analysed. The ITS-1 was 605bp in length with a G + C content of 33.05%. The 5.8S was 158bp in length and G + C content was 49.37%. The D3 was 171bp in length and G+C content was 41.86%. The D3 secondary structure for *Cystidicola* spp. (Figure 2.3), which consisted of the universally conserved H14 stem and 4 subdomains, conformed to the general eukaryote model proposed by Michot et al. (1990), and adopted by Nunn (1992), Deprés et al. (1995), and Brown (1996).

Two ITS-2 types were identified (Table 2.3): ITS-2 type 1 from *C. farionis* in Ontario and Finland hosts was 368bp in length and its G + C content was 26.63%; ITS-2 type 2 from *C. stigmatura*, and *C. farionis* from BC rainbow trout was 365bp in length with a G + C content of 26.85%. The ITS-2 types differed at 4 nucleotide positions (1.1%) which included 3 transversions (G/C, T/A, G/T), and a 3 bp insertion/deletion (AAA) (Figure 2.1).

Mfold produced 12 and 11 possible foldings for the ITS-2 secondary stucture of *Cystidicola* spp. type 1 and 2 respectively, all of which included a conserved stem (I) formed

by the complementary pairing of the 3' end of the 5.8S gene with the 5' end of the 28S gene. Optimal structures (ΔG = -106.01 type 1; -106.57 type 2) were not identical, but comparison of foldings within 5% suboptimality revealed common structures for the ITS-2 types. Two possible configurations, comprised of seven helices in addition to the conserved stem I, are shown in Figure 2.4. Differences between the sequences did not represent compensatory changes.

New distribution data found in this study for *C. farionis* in British Columbia and Ontario are summarized in Table 2.4. The range of mature worms (0-77.2%) found in BC rainbow trout from five BC lakes highlights the importance of broad sampling to assess host suitability. Lake Superior lake whitefish had a mean intensity of 3.8 worms, 38% of which were mature females (number of mature males not recorded), in contrast to previous studies where mature *C. farionis* were rarely found in lake whitefish from Lake Superior (Lankester and Smith 1980; Dextrase 1987).

Discussion

The rDNA of *Cystidicola* spp.

Limited sequence data is available for the rDNA spacer regions of nematodes in the Order Spirurida. This study represents the first report of sequences from the D3 expansion loop and spanning the rDNA ITS regions of cystidicolid nematodes. The conserved nature of rDNA genes flanking the ITS regions allowed comparison with other nematode sequences to reliably determine the spacer regions and D3 boundaries for *Cystidicola* spp. Comparison of *Cystidicola* spp. ITS and D3 characteristics with those of other nematode species is important

because sequence length and G + C content are often diagnostic of group affiliation (Powers et al. 1997). Vertebrate rDNA sequences are usually longer and have higher G + C content compared to invertebrates (Michot et al. 1983; Hassouna et al. 1984; Michot and Bachellerie 1987; Hancock and Dover 1988) so these characteristics can serve to rule out contamination with host DNA, and confirm amplification of the target sequence. The ITS-1 for *Cystidicola* spp. (605bp) is much longer than the same region in strongylids (372-404bp, Hoste et al. 1998; Hung et al. 1999; Zarlenga 1998) and *C. elegans* (464bp, Ellis et al. 1986), but similar to *Heterorhabditis* spp. (550bp, Adams et al.1998). The ITS-2 for *Cystidicola* spp. (365 and 368bp) is slightly longer than that reported for some nematodes (217-337bp, for strongylids Campbell et al. 1995; Chilton et al. 1995; Newton et al. 1998; Romstad et al. 1998; Zhu et al. 1998b, 1999) but similar to that of *C. elegans* (383bp, Ellis et al. 1986). Hoste et al. (1998) noted that the ITS-1 is usually longer than the ITS-2. The ITS-1 of *Cystidicola* spp. is ~235bp longer than the ITS-2, a much greater difference than that reported for *Trichostrongylus* spp. (150bp, Hoste et al. 1998), *Cylicocyclus* spp. (~50bp, Hung et al. 1997) and *C. elegans* (120bp, Ellis et al. 1986). The 5.8S (158bp) is similar in length to those of *C. elegans* (158bp, Ellis et al. 1986), ascarids (157bp, Zhu et al. 1998a) and strongylids (153bp, Chilton et al. 1997). The 5.8S is highly conserved among nematodes: *Cystidicola* spp. and *C. elegans* differ by only 29%. The D3 of *Cystidicola* spp. (171bp) is similar to *C. elegans* (165bp, Ellis et al. 1986) and *Philonema* spp. (169-172bp, Deprés et al. 1995; Brown 1996). In comparison, some vertebrate ITS regions are much longer; in the mouse, the ITS-1 is 999bp and ITS-2 is 1089bp long (Michot et al. 1984). The ITS of fish is similar to some nematodes; the ITS-1 of *Coregonus* spp. is 590-725bp (Sajdak and Phillips 1997) and the

ITS-2 of *Salvelinus namaycush* is 370bp (Phillips et al. 1994). The high G + C content of these species' ITS regions distinguishes them from nematode ITS sequences.

Overall, the G + C content of *Cystidicola* spp. rDNA was similar to other nematodes. ITS-1 G + C content for *Cystidicola* spp (33.05%) was lower than *Trichostrongylus* spp. (40-42%, Hoste et al. 1998) but higher than its ITS-2 G + C content (~27%) as is the case for other nematodes (e.g. 40% and 30% respectively for *Trichostrongylus* spp., Hoste et al. 1998). G + C content for the 5.8S (49.37%) was similar to strongylids (47.7-49.7%, Chilton et al. 1997). D3 G + C content was 41.86%, similar to *Philonema* spp. (49%, Brown 1996). In comparison rDNA G + C content for vertebrates is much higher, ranging from 70-74.3% in mouse ITS regions (Michot et al. 1983) and 80-86% in vertebrate LSU expansion segments (Hassouna et al. 1984; Michot and Bachellerie 1987; Hancock and Dover 1988).

Genetic variation in *Cystidicola* spp.

Evaluating the significance of sequence variation and conservation in *Cystidicola* spp. requires comparison of rDNA variability within other groups of nematodes. Lack of variation in the 5.8S of *Cystidicola* spp. is not surprising given the conserved nature of this gene in nematodes (Ferris et al. 1994; Chilton et al. 1997; Zhu et al. 1998a, b, 1999) and other eukaryote taxa (Hillis and Dixon 1991). This result confirmed the limitations of 5.8S sequences for discriminating between closely related species, but characterisation of *Cystidicola* spp. 5.8S may prove useful for future phylogenetic studies to assess relationships among nematode orders.

Analysis of the ITS-1 and D3 did not distinguish between *C. farionis* and *C. stigmatura* which are biologically and morphologically well-defined. These regions have

discriminated between other closely related nematode species (Nunn 1992; Deprés et al. 1995; Hung et al. 1997; Hoste et al. 1998; Zhu et al. 1999), however, their relative ability to distinguish between species may vary. Studies comparing variation in the ITS-1 and ITS-2 among morphologically distinct strongylid species have shown that the ITS-2 reveals more interspecific variation than the ITS-1 (Hung et al. 1997; Hoste et al. 1998; Zhu et al. 1998b, 1999). For example, between *Cylicostephanus goldi* and *C. longibursatus* the ITS-1 differs by 0.8% compared to a 3.8% ITS-2 difference (Hung et al. 1997). In contrast, the D3 region distinguishes between *Philonema sibirica*, *P. agubernaculum* and *P. oncorhynchi* (0.6-1.3%, Deprés et al. 1995; Brown 1996; Chapter 3 this study) but the ITS-2 is identical in *P. agubernaculum* and *P. oncorhynchi* while that of *P.sibirica* differs by 5.1% (Chapter 3 this study).

The ITS-2 of strongyloid nematodes has been studied extensively, providing valuable insight into the degree of variation within this region in relation to other traits, which can be applied to an interpretation of molecular and morphological variation within *Cystidicola* spp. Studies fall into 3 categories: morphologically distinct species with distinct ITS-2 sequences; morphologically distinct taxa with identical ITS-2 sequences (dimorphic species, or recent divergences); and morphologically identical taxa having distinct ITS-2 sequences (cryptic species). The first category includes nine studies using ITS-2 sequences to develop diagnostic tools for differentiating between nematodes of veterinary importance (Hoste et al. 1995; Campbell et al. 1995; Stevenson et al. 1995, 1996; Leignel et al. 1997; Hung et al. 1997, 1999; Newton et al. 1998a, b). Interspecific ITS-2 variation in these reports ranges from 1.3-29%. In the second category, morphologically distinct taxa with identical ITS-2

sequences are viewed as dimorphic species if only morphological features support their distinction (Stevenson 1996; Newton et al. 1998a). The absence of ITS-2 variation may suggest a relatively recent divergence, if other evidence differentiates the taxa. For example, in light of RAPD and morphological evidence, Leignel et al. (1997) held that *Metastrongylus confusus* and *M. salmi* are distinct species even though no differences were found in their ITS-2 sequences. Similarly, biochemical, immunological, morphological (Kurimoto 1974) and ITS-1 (1.3%, Zhu et al. 1999) differences suggest that lack of ITS-2 variation between *Ascaris lumbricoides* and *A. suum* reflects a recent speciation event within this genus. The third category includes evidence of cryptic species, where morphologically identical taxa exhibit much greater ITS-2 sequence differences between operational taxonomic units (OTUs) than between morphologically recognizable species (Chilton et al. 1995; Romstad et al. 1998; Hung et al. 1999). For example, 3 *Hypodontus macropi* OTUs have ITS-2 differences ranging from 25-28.3% (Chilton et al. 1995), and 2 *Cylicostephanus minutus* OTUs differ by 7.4% (Hung et al. 1999).

Given the preceeding interpretations of ITS-2 variation, it can be concluded that the molecular and morphological variation exhibited by *Cystidicola* spp. places representatives in all three categories: *C. farionis* ONT and *C. stigmatura* are distinct species, *C. farionis* FIN and *C. farionis* ONT are dimorphic species, *C. stigmatura* and *C. farionis* BC are recent divergences and *C. farionis* FIN and *C. farionis* BC are cryptic species. I further discuss the basis and implications of this interpretation in the following sections on morphological variation.

Egg morphology

The number and arrangement of egg filaments is variable within *Cystidicola* spp. This study found no relationship between egg morphology and genetic variation within the genus *Cystidicola*. The lack of variation in the ITS-1, 5.8S and D3 of *Cystidicola* spp. was uninformative in answering the question of whether variable egg filament placement constitutes species-level differences, because these regions failed to discriminate between the morphologically well-defined *C. farionis* ONT and *C. stigmatura*. More informative was the existence of isolates with different ITS-2 types but the same egg type (e.g. polar filamented eggs of *C. farionis* from BC and ONT/ FIN) and identical ITS-2 sequences from worms possessing different egg types (e.g. *C. farionis* with lateral and/or polar filaments ONT and FIN; BC *C. farionis* and *C. stigmatura* with lobed eggs). This result supports Ko and Anderson's (1969) assertion that filament arrangement is not a good taxonomic character, a conclusion based on observed variability in the number and distribution of egg filaments among worms from the same host. The possibility remains that these taxa represent a recent divergence, and a more appropriate marker (e.g mitochondrial DNA) could differentiate between *C. farionis* populations with different egg types.

The cause of variation in filament placement remains unclear. Egg morphology may be influenced by environmental or host cues and therefore reflect host-induced developmental flexibility, or indicate a fixed morphological polymorphism. The latter possibility would suggest genetic species-level variation between worms from different hosts. Evidence supporting both possibilities exists. Dextrase (1987) found that eggs from worms in lake whitefish have more lateral filaments and a greater ratio of lateral to polar filaments

than eggs from worms in other salmonids. Ninety-six percent of *C. farionis* eggs from lake whitefish had lateral filaments, and 87% had polar filaments. Only 21% of *C. farionis* eggs from rainbow trout and lake herring had lateral filaments, while all eggs had polar filaments. Dextrase (1987) noted similar polar filament counts for eggs from worms in lake whitefish, lake herring and rainbow trout. While eggs from lake whitefish consistently have both polar and lateral filaments (Ko and Anderson 1969; Dextrase 1987; this study) other fish species have been found to host worms with the same egg type. For example, *C. farionis* eggs with polar and lateral filaments have been obtained from brown trout in England (Ko and Anderson 1969), and lake herring, brook trout, and lake trout from Ontario lakes (Ko and Anderson 1969; Smith and Lankester 1980; Dextrase 1987). Ninety percent of eggs from Pettit L. lake trout had lateral filaments and appeared similar to those in lake whitefish (100% laterally filamented) from the same lake (Dextrase 1987). By comparison, worms from lake trout from northwestern Canada have only polar filaments (Ko and Anderson 1969).

Differences between the egg morphology of *C. farionis* and *C. stigmatura* seem to facilitate transmission to their respective intermediate hosts. Filaments occur on the eggs of various nematodes in the Family Cystidicolidae, many of which use crustaceans, usually amphipods, as intermediate hosts (Appy 1981; Moravec and Kazuya 1999). Attachment of eggs to vegetation or to feeding appendages (e.g. gnathopods) of amphipods may increase the probability of being eaten by this host (Smith 1978; Dextrase 1987). Dextrase (1987) observed amphipods using their gnathopods to clean entangled *C. farionis* eggs from their setae and antennae. Similarly, Dextrase (1987) suggested that lobes on the eggs of *C. stigmatura* may increase their chances of being ingested by the intermediate host *Mysis*

relicta. *Mysis relicta* feeds on planktonic crustacea, especially cladocercans (Cooper and Goldman 1980) as well as detritus, cysts, and benthic diatoms (Kinsten and Olsen 1981). Lobed eggs of *C. stigmatura* are 40 to 45 μm long (Ko and Anderson 1969), similar in size and shape to many diatoms, raising the possiblility that mysids become infected by eating eggs directly or through eating cladocercans which have already ingested eggs. Whether the differences in filament placement between *C. farionis* isolates present an additional advantage for intermediate host transmission is unknown, but this could account for patterns observed in this (Table 2.2) and other studies (Smith 1978; Lankester and Smith 1980; Dextrase 1987).

Morphology of the oral opening

Previous studies indicated that *Cystidicola* spp. can be distinguished based on differences in the appearance of their oral opening (Black and Lankester 1980; Dextrase 1987), however SEM examination of an expanded range of *Cystidicola* isolates indicates this feature alone does not distinguish *C. farionis* across Canada and Europe from *C. stigmatura*. Rejecting the taxonomic value of this character because of this finding would be unwise given the considerable overlap of other traits within *Cystidicola* spp., and the likely significance of any morphological variation between these sister species. Morphology of the oral opening is a conserved trait among other nematode sister species. The cystidicolids *Salvelinema walkeri* and *S. salmonicola* show no substantial interspecific differences in their cephalic morphology (Moravec and Nagasawa 1999) so the importance of the variable nature of this trait within *Cystidicola* spp. should not be dismissed. While the presence or absence of lips on the buccal cavity wall alone may not be species-defining, it remains a useful

diagnostic character when taken in conjunction with other morphological and molecular data, especially since it can distinguish these sister species in some locations (e.g. Ontario). *Cystidicola farionis* BC shares the same ITS-2 and mouth type as *C. stigmatura*, but exhibits vastly different egg morphology (filamented vs. lobed), life history traits (e.g. intermediate and final hosts), and distribution, therefore I suggest it is a species distinct from both *C. stigmatura* and *C. farionis* ONT/ FIN. *Cystidicola farionis* FIN and *C. farionis* ONT have different mouth types, but share other diagnostic traits (polar egg filaments, ITS-2 type 1) and therefore represent the same species.

Glacial history and distribution of *Cystidicola* spp.

Analysis of the ITS-2 revealed expected differences between the morphologically distinct *C. farionis* and *C. stigmatura*, but comparison of this region among *C. farionis* isolates produced a surprising result: *Cystidicola farionis* from northwestern Canada (BC rainbow trout) have a different ITS-2 sequence from other *C. farionis*, but the same ITS-2 type as *C. stigmatura* ONT. The ITS-2 distinguishes *C. farionis* (BC) from *C. farionis* (ONT and FIN) and implies a closer relationship between *C. farionis* (BC) and *C. stigmatura.* This result suggests that the *C. farionis* population diverged before *C. stigmatura* arose as a species, likely before the most recent (Wisconsin) glaciation. Glaciations during the Pleistocene era significantly shaped the evolution and distribution of aquatic organisms through temporal and geographic isolation in separate refugia while advancing ice sheets covered much of Canada and parts of the United States (McPhail and Lindsey 1970). Details of glacial events previous to the last glacial period (the Wisconsin, from 50 000 to 10 000

years ago) are uncertain since each advancing ice sheet tended to cover traces of its predecessor (McPhail and Lindsey 1970). While it is not possible to trace the ITS-2 divergence to a particular isolating event, the distribution of ITS-2 types across Canada and Europe, in conjunction with the probable glacial history of *Cystidicola* spp. and its hosts, suggests that ITS-2 type 1 (ONT and FIN) preceded type 2 (BC and *C. stigmatura*) since it is shared by isolates that would have been separated long before the last glaciation.

The distribution of *Cystidicola* spp. in some fish hosts has been attributed to their isolation in separate drainages during the height of glaciation (Black 1983b, c). Both ITS-2 data and distribution patterns support the survival of *C. farionis* in more than one glacial refugia during the Wisconsin. *Cystidicola farionis* matures in a variety of fish species throughout North America, but when Black (1983b) examined lake trout from across the continent he noted that only lake trout from the northwest consistently hosted mature *C. farionis*. He proposed the existence of two *C. farionis* strains to explain this distribution pattern, suggesting one strain survived with fishes in a Bering refugium during the Wisconsin glaciation. Upon glacial retreat, parasite and host had access to isolated watersheds to achieve their current northwestern distribution. *Cystidicola farionis* found in hosts other than lake trout in eastern North America represent a population (strain) which survived glaciation in a southern refugium (e.g. Upper Mississippi River). The existence of different ITS-2 types from *C. farionis* from hosts of Bering (BC rainbow trout) and Mississippi (ONT lake whitefish and lake herring) origin supports the proposed glacial history of parasite and host.

The present distribution of *C. stigmatura* is also attributed to its survival in an Upper Mississippi River refugium during Wisconsin glaciation. Black (1983c) found *C. stigmatura*

in lake trout and Arctic char from the Great Lakes and St. Lawrence drainage basins, from lakes extending from northwestern Ontario to Great Bear Lake, and from the Canadian Arctic Archipelago, but not elsewhere. This distribution pattern suggests *C. stigmatura* dispersed with lake trout and *M. relicta* from the Upper Mississippi refugium, following the retreating glacier in a northwesterly direction. Black (1984) found the morphology of *C. stigmatura* varied randomly among populations from different geographic locations, which is consistent with its survival in a single glacial refugium. Furthermore, the apparent restricted preglacial distribution of *C. stigmatura* and recent history of its intermediate host (*M. relicta*) in freshwater suggests that *C. stigmatura* arose as a species during the last glaciation (Black 1983c).

Data from the ITS-2 region supports the possibility that the Wisconsin glaciation provided conditions for morphological divergence within the genus *Cystidicola*. *Cystidicola farionis* and *C. stigmatura* from the same refugia have distinct ITS-2, egg, and mouth types which suggests an absence of gene flow between the diverging species. *Cystidicola* isolates from BC and *C. stigmatura,* which represent different glacial refugia, share ITS-2 type 2, and *C. farionis* ONT and FIN share type 1, therefore rather than acting as the isolating mechanism responsible for the divergent ITS-2 sequences, the unique ITS-2 types were apparently maintained during the last glaciation.

Development and Host Specificity

Results of this study confirm that *C. farionis* has readily transferred from native hosts to introduced salmonids such as chinook and pink salmon, and rainbow trout, (Lankester and Smith 1980; Table 2.2 and 2.3) in Lake Superior, indicating this parasite is

not always restricted to fish with which it has had an historic association, as suggested by Black (1983b, c). If a physostomous fish inhabits a lake with *Cystidicola* spp. several variables could influence its infection and the nematode's subsequent development; host diet is the first factor determining infection. Once a host ingests an infected amphipod, other aspects of the host's biology could affect the ability of larval *C. farionis* to develop to sexual maturity. For example, host habitat (e.g. depth in the lake) will determine variables such as temperature, and swimbladder gas pressure and composition, which may in turn affect cues the parasite receives for moulting/maturation.

The best available index of host species suitability for *Cystidicola* spp. is the proportion of mature worms found infecting fishes in a given locality (Lankester and Smith 1980). To assess this, it is necessary to distinguish between the proportion of hosts infected with only immature worms, and the complete absence of *Cystidicola* spp. in a given host population. A host group consistently infected with only immature worms is viewed as unsuitable, whereas the absence of any infection suggests differences in host diet. The variable distribution and development of *C. farionis* in lake herring and lake whitefish from across Canada serves to illustrate this point.

Dextrase (1987) identified three Ontario lakes where lake whitefish, but not lake herring, were infected with *C. farionis*. While this may imply lake herring is unsuitable for *Cystidicola* spp. maturing in lake whitefish, it is also possible that lake herring in these lakes do not feed on amphipods. Watson and Dick (1979), for example, noted mature worms in lake whitefish, but attributed the absence of worms in lake herring from Southern Indian Lake, Manitoba to the low frequency of amphipods in their diet (<3%). Sampling Lake

Superior, Lankester and Smith (1980) found lake herring were commonly infected with mature worms, whereas all worms from lake whitefish sampled in the spring, fall, and winter were immature. *Cystidicola farionis* larvae that accumulated in large numbers in L. Nipigon lake whitefish developed to sexual maturity in experimentally infected rainbow trout (Black and Lankester 1980). Not surprisingly, these observations along with its distinctive, laterally filamented eggs, led researchers to speculate that the worm maturing in lake whitefish is different from *C. farionis* maturing in other salmonids (Black and Lankester 1980; Lankester and Smith 1980). Given the extensive sampling these researchers performed over a number of years and seasons (spring, winter, fall for 2-3 years) it is unlikely that their findings are a result of sampling error. Dextrase (1987), however, later demonstrated that larval *C. farionis* from L. Nipigon whitefish would develop towards maturity in hatchery-reared lake whitefish of Lake Simcoe stock. Recent sampling efforts also raise questions about the perceived host specificity of *C. farionis*.

Distribution data obtained in this study does not support previous observations that certain *C. farionis* populations may be highly host specific (Black and Lankester 1980; Lankester and Smith 1980). Although my collection method did not permit prevalence and intensity calculations for *C. farionis* in Lake Superior lake whitefish (i.e. commercial fishermen pooled worms from infected fish), many mature specimens were obtained (38% mature females), despite previous reports suggesting that maturation in this host type is a rare occurrence (Black and Lankester 1980; Lankester and Smith 1980). Population levels of this parasite seem to fluctuate greatly so rigorous sampling is necessary to determine host type suitability. This concept is reinforced by the range of mature worms (0-77.2%) obtained

from BC rainbow trout (Table 2.4) during one season. Also, in the fall of 1988, M.W. Lankester collected mature *C. farionis* from L. Nipigon lake whitefish for the first time. Attempts to extract DNA from these specimens were unsuccessful, likely because of glycerine in the fixative, so whether they differed genetically from other *C. farionis* isolates is unknown. Given the complete lack of variation between isolates from lake whitefish and lake herring at all four gene regions sequenced, I predict these worms would be identical to other *C. farionis* from L. Superior and inland Ontario lakes. These specimens further support the idea that only one type of *C. farionis* exists in Ontario, where lake whitefish apparently have variable resistance to this parasite.

The suitability of other species hosting *Cystidicola* spp. is thought to vary with locality. For example, mature *C. farionis* are found in smelt (*Osmerus eperlanus*), but not in whitefish (*Coregonus laveratus*) in Bothnian Bay, Finland (Fagerholm 1982). Rainbow smelt (*Osmerus mordax*) and lake whitefish from Lake Superior are close relatives of these European fishes, and only lake whitefish appear suitable for *C. farionis* (Lankester and Smith 1980). Brown trout (*Salmo trutta*) is infected with *C. farionis* in several British rivers, and has transferred the worm to rainbow trout introduced into River Itchen, Wales (Poynton 1985). In contrast, *C. farionis* matures in rainbow trout from Lake Superior, but not brown trout (Lankester and Smith 1980). These examples, along with the distribution of *Cystidicola* spp. in North American lake trout, may indicate the host specificity of different *Cystidicola* strains. Alternatively, variable host suitability may indicate more about differences in the biology of fish hosts throughout their range. The lack of rDNA variation in *C. farionis* isolates from Ontario and Finland (Table 2.3) supports the latter hypothesis.

Cystidicola farionis exhibits seasonal fluctuations in prevalence and intensity in a number of host species (Lankester and Smith 1980). The perceived unsuitability of brown trout in Lake Superior is based on samples taken during one season from only 26 fish. Prevalence and intensity was low in this host (12% and 1% respectively) so it is possible that further sampling would reveal mature worms. Although Black (1983b) observed *C. farionis* to be specific to a Bering strain of lake trout, there is one instance where *C. farionis* was collected from lake trout outside the dispersal range of this host strain. Mature *C. farionis* were found in lake trout from Lake Nipigon, and immature *C. farionis* have also been collected from Lake Superior and Lake Huron char (Lankester and Smith 1980). The perceived host specificity of *C. farionis* may result from sampling bias, but only rigorous sampling in lakes Nipigon and Superior each season for a number of years could test this possibility. Whereas the presence of *C. stigmatura* in a particular host population can be determined by examining a few hosts in a lake and prevalence is often near 100% (Black and Lankester 1981), assessing infection with *C. farionis* is apparently more difficult. Prevalence of *C. farionis* in certain host populations may be low, so extensive sampling must be done to confirm suitability.

Table 2.1. *Cystidicola* spp. collected from Canada and Europe.

Location	Date Collected*	Host
Cystidicola farionis		
Ontario		
Arrow Lake	November 1997 (AEM)	*Coregonus clupeaformis* (lake whitefish[c])
Nipigon River	May 1999 (MWL)	*Oncorhynchus tshawytscha* (chinook salmon[p])
Lake Superior	November 1997 (AEM and MK)	*C. clupeaformis* (lake whitefish[c])
Lake Superior	November 1997 (AEM)	*C. artedii* (lake herring[cp])
Lake Superior	September 1997 (MWL and MK)	*O. gorbuscha* (pink salmon[p])
Lake Superior	April 1999 (MWL)	*O. mykiss* (rainbow trout[p])
British Columbia		
Chaunigan Lake	July 1998 (EK)	*O. mykiss* (rainbow trout)
Ealue Lake	July 1998 (EK)	*O. mykiss* (rainbow trout)
Fish Lake	July 1998 (EK)	*O. mykiss* (rainbow trout)
Tatlatui Lake	June 1998 (EK)	*O. mykiss* (rainbow trout)
Finland		
Baltic Sea	April 1998 (HPF)	*Osmerus eperlanus* (boreal smelt[cp])
C. stigmatura		
Squeers Lake, ONT	March 1998 (MWL)	*Salvelinus namaycush* (lake trout[p])

*Date followed by collectors intials in brackets. AEM=A.E. Miscampbell, MK= M. Klitch, MWL= M.W. Lankester, EK= E. Keeley, HPF= H.P. Fagerholm.
[c]Hosts obtained by commercial fisheries, all other taken by anglers.
[p]*Cystidicola* isolates from numerous hosts pooled, all others unpooled.

Table 2.2. Summary of sequences obtained from 4 regions of *Cystidicola* spp. rDNA.

Origin	Host	Individual Females Sequenced*			
		ITS-1	5.8S	ITS-2	D3 (26S)
Cystidicola farionis					
Arrow L, ONT	lake whitefish[a]	2		3	1
Lake Superior, ONT	lake whitefish[a]	4		3	3
Lake Superior, ONT	lake herring[b]	3	1	3	3
Lake Superior, ONT	chinook salmon[b]	2		2	
Lake Superior, ONT	rainbow trout[b]	3		3	
Nipigon R, ONT	pink salmon[b]	3		3	2
Baltic Sea, FIN	smelt[b]	3	1	3	3
Chaunigan L, BC	rainbow trout[c]	2	1	3	
Ealue L, BC	rainbow trout[b]			3	
Fish L, BC	rainbow trout[b]	2		3	
Tatlatui L, BC	rainbow trout[b]	2		2	3
C. stigmatura					
Squeers L, ONT	lake trout[d]	3	1	3	3

[a]eggs with both polar and lateral filaments
[b]eggs with predominantly polar filaments
[c]eggs with no filaments
[d]eggs with lateral lobes
*worms from numerous hosts pooled ∴ each replicate likely represents infections from more that one fish.

Table 2.3. Egg and ITS-2 types of *Cystidicola* spp. from hosts in Canada and Europe.

Species and Host Locality	Egg Type	ITS-2 Type
C. farionis – lake whitefish (**ONT**)	**A** = eggs with both polar & lateral filaments	**1**
C. farionis - lake herring, pink & chinook salmon, rainbow trout (**ONT**); boreal smelt (**FIN**)	**B** = eggs with predominantly polar filaments	**1**
C. farionis – rainbow trout (**BC**)		**2**
C. farionis – rainbow trout (**BC**)	**C** = eggs with no filaments	**2**
C. stigmatura – lake trout (**ONT**)	**D** = eggs with lateral lobes	**2**

Table 2.4. New distribution records of *Cystidicola farionis*.

Location	Host	Mean intensity	% Mature worms	N	W
British Columbia					
Chaunigan Lake	Rainbow trout	27.4 (4-78)*	56.3	51°30.9'	123°50.5'
Ealue Lake	Rainbow trout	5.2 (1-9)	50	57°45.3'	129°50.0'
English Lake	Rainbow trout	7.7 (1-15)	31.5	51°30.8'	120°35.8'
Fish Lake	Rainbow trout	3.8 (1-14)	77.2	51°25.4'	123°35.3'
Tatlatui Lake	Rainbow trout	9.3 (2-36)	67.8	56°55.8'	127°15.3'
Thutade Lake	Rainbow trout	1.6 (1-3)	0	56°50.8'	126°50.6'
Ontario					
Arrow Lake	Lake whitefish	6 (1-13)	46.7	48°09'	90°16'

* range is indicated in brackets

18S>
Type 1 - *GGGACTGAGCCGTTTCGAGAAACGTGGGGACTGCTGTTTTTCCGCCAATTTTGGTTGGAAATTCTTTAGTGGAAACCATCTTAATCGCA*
Type 2 - . . . . . .

ITS-1>
*GTGGCTTGAACCGGGCAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATC*GAACCAAGACAAAAAAAAAGTCT
. . . . . .

CAAACTTGTGCATACGACCATTTGCGCAATAATGAGCATTATAGCACAAGCAAAATTGTGTTTTTGTGATTACAATAAAAATTTTATTG
. . . . . .

TTGCTGTTGCTCTGTTAATGTTTATAAAATGAATATTAGCTAAATATTCTAATTTTTTTTTTTATAACAATAATAACAGCAGTTTGAGC
. . . . . .

AAAAACAGTTGTTGGCGGACAAGGCTAACAATAACTATTAACAATTGCTGGGGTTTTTATTGATGAGAGTAAATAAGTGGCACAATAAG
. . . . . .

CGTGTTAAATTGTGTCAGCGCAAGAAAATGCTTGTTTACTCGTTAGTTGTATCAACGGCGTCCATGTTGGCGTCTATTTCTTGTTCGAG
. . . . . .

CTATCGCCCTTGGCCGTCGGTAGCAATGAAAGATGAGAAATTTAGCTGTTATTTTTTTTTCTTACAGGTGAAAATTTAATAGCAGTAGA
. . . . . .

CTTAATGAGCACTAGCTTGTAGTGCCGCCAACCCACACACACACACAAACACACAAGTTATATGCATTGTAAAAAATGTGTATAATTTT
. . . . . .

5.8S>
GTTAAAATATATATATATATTGTTGTTTAAATTTATTTGTGTTGTTTTTATAAATCAGCATGAAAAAAATTTACAAATAAAAAAAAT
. . . . . .

ACTCTTAGCGGTGGATCACTCGGCTCGTGGATCGATGAAGAACGCAGCTAGCTGCGATAATTAGTGCGAATTGCAGACACATTGAGCAC
. . . . . .

ITS-2>
*AAAAATTTCGAACGCAAATTGCGCCATCGGGTTTATTCCCCTTGGCACGTCTGGCTGAGGGTCGGTT*TTGTAGAAAATGTAGTTATTGT
. . . . . .

TGTTATTGCTGAATAACGATGGCTAATAATTTCGTCGAGCAATGTTGCAATATATATTTAGTTTGCACCTTACCTCGATAAGCATTATA
. . . . . .

TTGTTGCGTTTTTTTAACGTACCAGTTAGAATTTTATCAAAC**G**TAT**TG**TATGTTTGATTTATTAGCAAGTAAAATGTTATTTTGTTATA
. . . . . C . . **AT** . . . . . .

ATTTGTTGCTATTTATTTGTTGTATTTTTGTGTGCACAAA**AAA**TAAATGGTTTTCATTGTGTGTGTATGTGTTTCTAATAGTGTTTTAG
. . . . . .

28S>
CGCACATTTGTGTGTTGACTTATTTAGAACATATGACAACAAAATTAAAACAATTAAAGCATTAATTTGTATTTTATAT*TGAGACCTCA*
. . . . . .

D3 >
GCTCAGTCGTGATTACCCGCTGAATTTAAGCATATTACTAA√*CTCTTGAAACACGGACCAAGGAG*TCTAGCATATGCGCAAGTTATTTG
. . . . . √ . . . . . .

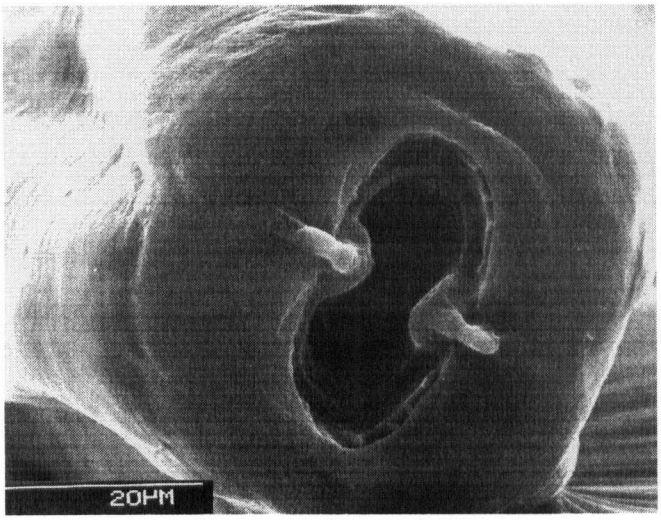
GTGGTAAACCATAAAAACGTAATGAAAGTAAACGTCGTCTTGATACGATGAAATATGTGAGCTGTGTGTTGATTAATATGCGCACAGTG
. . . . . .

CAACATAGCCCTGTTCTCGTTGCTTGCAATAGAACGGAGGTAGAGCGCATACGCTGGGA*CCCGAAAGATGGTGAACTATGCCTGAGCAG*
. . . . . .

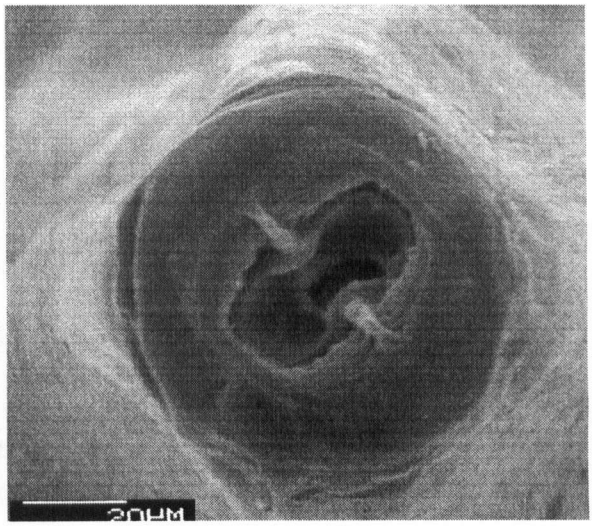
GATGAAGCCAGAGGAAACTCTGGTGGAGGTCCGAAGCGATTCTGACGTGCAAATCGATCGTCTGACTTGGGTATAGGGGCGAAAGACTA
. . . . . .

ATCGAACTATCTAGTATCTGGTTCC
. . . . . .

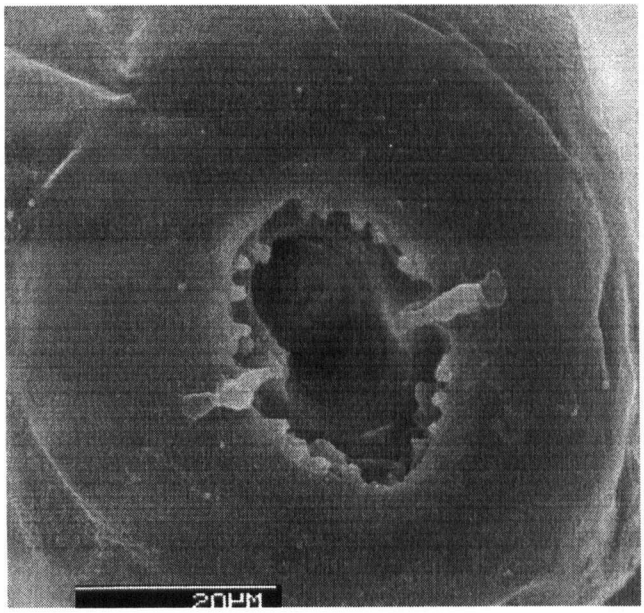
Figure 2.1. Sequences spanning the ITS regions of rDNA (18S 3' end, ITS-1, 5.8S, ITS-2, 28S 5' end) and the D3 expansion segment (28S) from *Cystidicola* isolates. √ indicates an unsequenced portion of 28S upstream of the D3 region. No variation was found in the ITS-1, 5.8S, or D3 but 4 variable positions (indicated in **bold**) were identified in the ITS-2 from Type 1 (*C. farionis* ONT/FIN) and Type 2 (*C. stigmatura & C. farionis* BC).



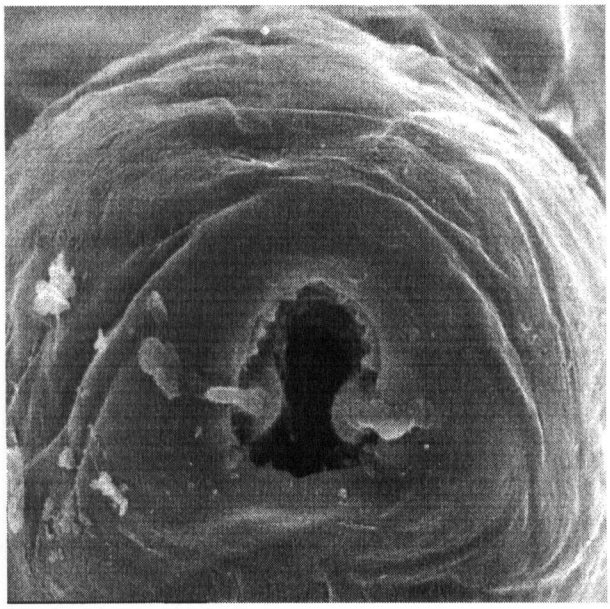
C. stigmatura from lake trout, Squeers L. ONT



C. farionis from rainbow trout, Tatlatui L. BC



C. farionis from lake whitefish, Lake Superior ONT



C. farionis from *Osmerus eperlanus*, Baltic Sea Finland

Figure 2.2. SEM photos showing the oral opening of *Cystidicola* spp. from Ontario, British Columbia and Finland.

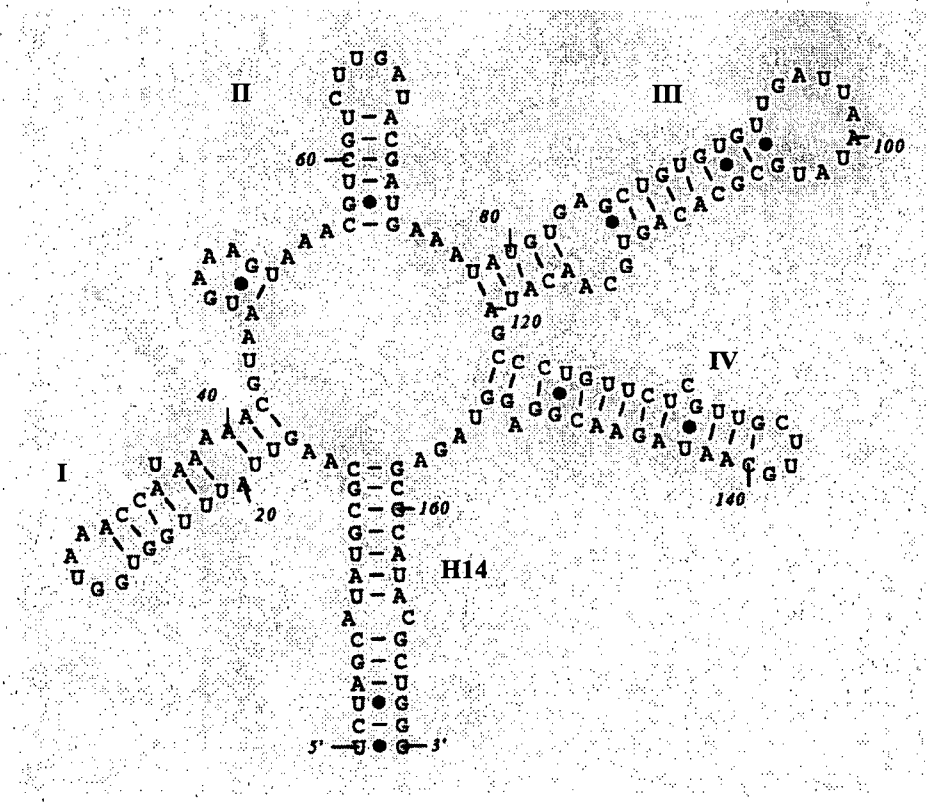


Figure 2.3. Proposed secondary structure of the D3 expansion segment of *Cystidicola* spp., based on the conserved eukaryote model (Michot et al. 1990) and drawn using loopDloop (Gilbert et al. 1992). This rRNA region was identical for all *Cystidicola* isolates examined.

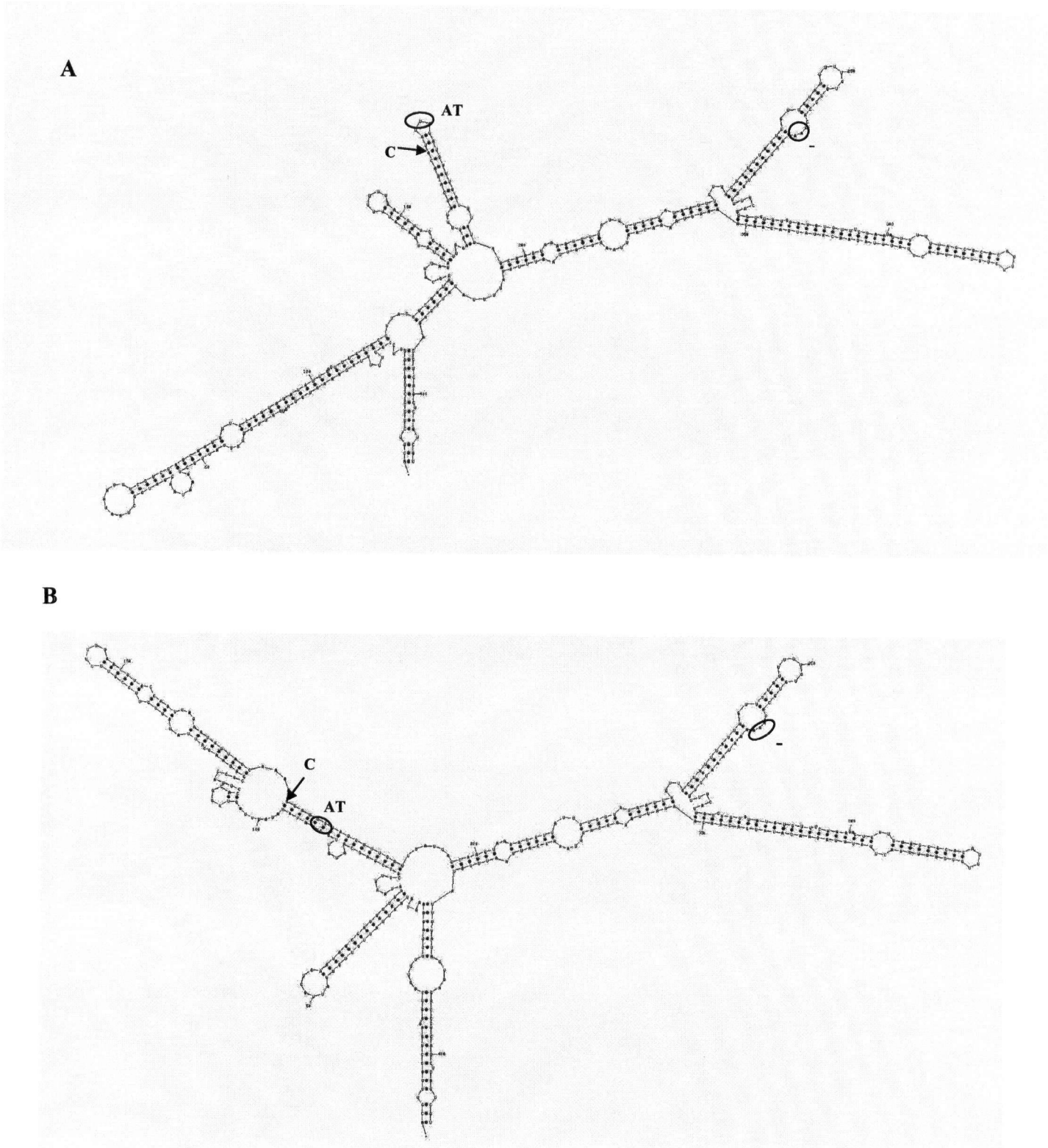


Figure 2.4. Predicted secondary structures of the ITS-2 for *Cystidicola* spp. Type 1 and 2. A is the most energy efficient folding for Type 1 (dG = -106.01) and the second suboptimal folding for Type 2 (dG = -106.31). B is the most energy efficient folding for Type 2 (dG = -106.57) and third suboptimal folding for Type 1 (dG = -102.9). Differences between ITS-2 Type 1 sequence (shown here) and Type 2 are indicated by circles and arrows and do not represent compensatory changes.

CHAPTER 3: RIBOSOMAL DNA VARIATION WITHIN THE DRACUNCULOIDEA

Introduction

Nematodes of the superfamily Dracunculoidea occur in extraintestinal tissues of fish, reptiles, birds, and mammals (Chabaud 1975). Moravec and Køie (1987) revised Chabaud's (1975) five family classification based on structure of the buccal capsule, esophagus, reproductive system in females, and caudal extremity in males, and further divided the superfamily among 7 families: Anguillicolidae, Daniconematidae, Dracunculidae, Guyanemidae, Micropleuridae, Philometridae, and Skrjabillanidae. The use of morphological characters to establish phylogenetic relationships and distinguish closely related species within the Dracunculoidea, however, has proven difficult. The current classification may be based on primitive, phylogenetically uninformative, characters (Adamson et al. 1987). In addition, morphological similarity among close relatives suggests that more precise taxonomic markers are required to reliably assess species diversity within this superfamily.

Recent research has focussed on using molecular techniques to address phylogenetic and species diagnostic problems within the Dracunculoidea (Adamson et al. 1992; Deprés et al. 1995; Brown 1996). The D3 expansion segment of 28S rDNA has been particularly useful for discriminating members of the holarctic genus *Philonema* (Dracunculoidea; Philometridae), which are parasites in the body cavity of salmonids. The 9 nominal *Philonema* species were previously defined only in terms of host or zoogeographical differences and considerable morphologicial overlap (Platzer 1964) suggested the existence of only 2 or 3 valid species. Molecular characterisation of four species, *P. agubernaculum, P. oncorhynchi, P. sibirica* and *P. tenuicauda,* established species-level differences in the D3 region (Deprés et al. 1995; Brown

1996). Although morphologically cryptic, *Philonema agubernaculum* and *P. oncorhynchi* represent distinct genetic stocks (Adamson et al. 1992; Deprés et al. 1995; Brown 1996). Similarly, while morphology and measurements of *P. sibirica* and *P. agubernaculum* suggest they may be synonymous (Moravec 1994), the D3 region distinguished these two species (Brown 1996).

The Guyanemidae, which includes *Guyanema* Petter, 1975 and *Pseudodelphis* Adamson and Roth, 1990 presents another taxonomic challenge. This family consists of species that occur in distantly related hosts inhabiting diffuse zoogeographic regions and different habitats (marine and freshwater), suggesting the family is artificial, or that known guyanemids represent only a small fraction of those that exist (Adamson and Roth 1990). An undescribed nematode obtained from slender sole, *Eopsetta exilis*, appears to be closely related to *Pseudodelphis oligocotti* because of shared guyanemid characteristics (e.g. anterior position of the vulva, pseudodelphic condition) and may represent a new guyanemid species. Phylogenetic analysis using the D3 region placed *P. oligocotti* within the Philometridae (Brown 1996), and suggested *P. oligocotti* was closer to *Philonema* spp. than *Philonema* spp. are to other philometrids (e.g. *Clavinema mariae*) but *Guyanema* spp. could not be included in the analysis. Further molecular analysis of the Dracunculoidea including other guyanemid (e.g. the slender sole worm) and philometrid species (e.g. *Philometroides huronensis*), may resolve this questionable affiliation.

In this study I investigate genetic variation within the Dracunculoidea; I characterise the D3 and ITS-2 of two and nine dracunculoid species respectively to evaluate species diversity and phylogenetic relationships within this superfamily. Dracunculoid relationships are assessed using sequences from the D3 region of two species, the slender sole worm and *P. huronensis*,

added to an existing D3 data set of seven dracunculoid species (Brown 1996), and ITS-2 sequences from all nine species. The relative ability of these regions to distinguish cryptic species (e.g. *Philonema* spp.), place unidentified nematodes within the current classification system, and infer phylogenetic relationships (e.g. within the Philometridae and Guyanemidae) is compared. I also explore ITS-2 secondary structure characteristics to determine if there is a discernable common structure which could guide sequence alignment and thus expand the phylogenetic utility of this region.

Materials and methods

Two nematode species were collected in May 1999: *Philometroides huronensis* from the fin of white sucker (*Catastomus commersoni*) from Timber L. drainage, Nova Scotia, and an undescribed nematode species from slender sole (*Eopsetta exilis*) caught during a research trawl south of Barkley Sound BC (Table 3.1). The undescribed nematodes were found encysted in a mass of 20-25 worms in the body cavity of slender sole. M.L. Adamson identified the species as a close relative to *Pseudodelphis oligocotti* because of shared morphological features characteristic of the Guyanemidae (e.g. anterior position of the vulva; pseudodelphic condition). DNA extraction of these samples followed the protocol outlined for *Cystidicola* spp. (Chapter 2 this study). A.M.V. Brown provided DNA extracted from 7 other nematode species representing 4 families within the superfamily Dracunculoidea (Table 3.1).

Two rDNA regions were targeted for PCR: primers NC13 and NC2 amplified the ITS-2 while D3A and D3B amplified the D3 region of 9 and 2 dracunculoids respectively (Table 3.1). Primers, PCR cycles, and sequencing methods followed those for *Cystidicola* spp. (Chapter 2

this study). When possible both the 5'and 3' directions of each region were sequenced for 2 worms per species.

The ITS-2 and D3 boundaries were determined using the Clustal W version 1.7 program (Thompson et al. 1994) to align these regions with those of *C. elegans* (Ellis et al. 1986). ITS-2 sequences were aligned with multiple alignment parameters set at gap opening penalty = 10 (on a scale of 1-100) and gap extension penalty = 4 (on a scale of 1-8) to allow the conserved ends of the flanking 5.8S and 28S genes to align since large size differences exist in the enclosed ITS-2 sequences. Secondary structures for the D3 region of *P. huronensis* and the undescribed guyanemid worm from slender sole (SS1) were modelled after those of *C. elegans* (Ellis et al. 1986; Nunn 1992) and *Philonema* spp. (Brown 1996; Deprés et al. 1995) and drawn using loopDloop version 1.2 (Gilbert 1992). D3 sequences from *P. huronensis,* SS1, *Cystidicola* spp., and 7 dracunculoids (Brown 1996) were aligned manually in ESEE version 3.2s (Cabot 1998) using secondary structure as a guide, and automatically in Clustal W (Thompson et al. 1994).

Sequence data was analysed using the Unweighted Pair Group Method using Arithmetics averages (UPGMA) included in the PAUP * 4.0 package (Swofford et al. 1998). UPGMA calculates pairwise comparisons based on sequence dissimilarity which is equal to the number of alignment positions containing non-identical bases divided by the total number of sequence positions compared (Swofford et al. 1996). Gaps were treated as missing data. Bootstrap values (n=100) calculated according to 50% majority rule (Swofford et al. 1998). Phenograms generated by UPGMA were drawn and viewed using TreeView 1.6 (Page 1996).

The mfold program (Mathews et al. 1999; Zucker et al. 1999), based on an energy minimization approach, was used to generate ITS-2 secondary structures for all 9 dracunculoid

species and to confirm D3 secondary structure. Aligned ITS-2 sequences were examined to identify close species pairs with potentially common structures. Optimal and suboptimal (≤ 5%) foldings were compared within and between species pairs to find conserved stems. When secondary structures differed, but sequence alignment strongly indicated a close relationship between species, a common stem was forced. Forced structures within 10% suboptimality were considered acceptable. Stems were then compared between species pairs to find any conserved domains, and to determine an overall ITS-2 structure for dracunculoids.

Results

ITS-2 sequence comparisons

The ITS-2 of 9 dracunculoid species was 299-523 bp in length, and G + C content ranged from 28.8-55.3% (Table 3.2). The ITS-2 distinguished all species examined, except *Philonema agubernaculum* and *P. oncorhynchi* which had identical sequences. Percent difference between dracunculoid species' ITS-2 sequences ranged from 0-58% (Table 3.4). *Avioserpens* sp. had the most distinct ITS-2 compared to the other 8 taxa (52.8-58% difference), while *P. agubernaculum/P. oncorhynchi* and *P. sibirica* were least distinct (5.1%). Their sequences differed at 49 nucleotide positions including 22 substitutions and 27 insertions/deletions. Of the 22 substitutions, 5 were transitions between purines (e.g. A and G), 7 were transitions between pyrimidines (e.g. C and T), and 10 were transversions (e.g. substitutions between purines and pyrimidines). Sequence alignment and consequently intergeneric ITS-2 comparisons were complicated by a number of factors such as sequence hypervariability resulting from point mutations/substitutions, and repeat motif

insertion/deletions which produced large size differences and questionable sequence homology between taxa.

Predicted ITS-2 secondary structures representing the most energy efficient shape for each species (ΔG values of -144.6 FIN, -132.71 PAG&PON, -142.1 PSE, -151.1 SS1, -132.15 PHU, -174.84 CLA, -99.37 ANG, and -154.93 AVI) were composed of 6-8 distinct domains (not shown). All structures included a central helix (I) formed by complementary base pairing of the 5.8S 3' end with the 28S 5' end (approximately 20 bps long). Attempts to predict a universally conserved dracunculoid structure were confounded by the high degree of sequence variation between species, and the large number of competing structures generated by Mfold. Analysis of optimal and suboptimal foldings indicated structures with a similar overall structural morphology could be achieved, composed of helix I plus an additional 5-6 distinct domains (Figure 3.1). This usually involved altering at least one stem per species to resemble presumed homologous stems. All configurations maintained helices from their most energy efficient folding and were within 5% suboptimality, except FIN (11% suboptimal), and PHU and AVI which maintained the most energy efficient folding in its entirety (ΔG values of -128.44 FIN, -129.76 PAG&PON, -138.66 PSE, -143.04 SS1, -132.15 PHU, -168.96 CLA, -101.35 ANG, -154.93 AVI).

Predicting common ITS-2 secondary structures did not facilitate ITS-2 sequence alignment. Determining homologous stems even among close relatives was problematic. For example, *P. oligocotti* and SS1 (25.3% different) shared many regions of primary sequence conservation which occurred in stems of questionable homology. Forcing conserved nucleotide regions to form presumed common stems in one area would often disrupt the best primary

sequence alignment of subsequent conserved regions. On the other hand, some of the most variable and virtually unalignable sequences folded into similar structures with minimal interference (e.g. *P. huronensis* and *P. sibirica* 48% difference).

Optimal structures for *P. agubernaculum/P. oncorhynchi* and *P. sibirica* were similar, but not identical, despite minimal sequence variation (5.1%). Comparison of consensus structures (Figure 3.2) showed 7 sequence differences in helix loops or bulges that did not affect base pairing in the stem structures. Remaining substitutions and indels occurred within stems and did not maintain base pairing (i.e. were disruptive or non-compensatory). The most striking secondary structure difference between all foldings for these taxa resulted from 10 and 8 nucleotide indels on each sides of stem IV. These caused an additional 8 bp stem to form near the terminal loop of stem IV in *P. sibirica*'s optimal folding. Incorporating these nucleotides into stem IV increased the free energy of the resulting structure to 11% suboptimality (Figure 3.1a) for the 6 domain model and 3% suboptimality for the 7 domain consensus structure (Figure 3.2).

D3 expansion segment

The 28S rDNA region sequenced for *P. huronensis* and SS1 included 138 bp conserved core flanking the D3 expansion segment of 240 bp and 179 bp respectively (Figure 3.3). G + C content was 46% and 50.3% respectively. No intraspecific variation was detected. Secondary structures based on the universal eukaryote model (Michot et al. 1990) matched those generated by Mfold (Mathews et al. 1999; Zucker et al. 1999) in all but stem II. Comparison over an alignment length of 398 bp indicated a 17.7% difference between these species. The D3 expansion segment of *P. huronensis* and SS1 consisted of 4 variable subdomains and the

universally conserved H14 stem as in Michot et al. (1990). One partially compensatory transversion (T to G) and 2 compensatory transitions existed in stem H14 of *P. huronensis* and SS1. There were four substitutions in stem I, which was the most conserved subdomain of the 2 species. Stems II, III, and IV exhibited large size and sequence variation between the species, making substitutions/indels difficult to discern. Comparison of the core region in these 2 species revealed 2 transitions over 138 bp representing a 1.5% difference. In contrast, differences in the core region between *Cystidicola* spp. and these dracunculoids was 2.17-2.90%.

Pairwise comparisons of percent differences among D3 sequences (plus 138bp conserved core region) from this study and Brown (1996) are shown in Table 3.3. Interspecific differences between dracunculoid species aligned based on secondary structure were usually greater than those obtained using the Clustal W alignment (0.6%-26.2% vs 0.6%-26.8% respectively). *Avioserpens* sp. was most genetically distinct (18.8-26.8%) from the other 8 species, while *P. oncorhynchi* and *P. sibirica* were the most genetically similar (0.6% difference).

Pairwise D3 comparisons revealed SS1 was most genetically similar to *P. oligocotti* (Brown 1996) (5.0% difference) while *P. huronensis* was most similar to *Clavinema mariae* (Brown 1996) (11% difference). SS1 and *P. oligocotti* had identical core regions, and their D3 differed at 31 nucleotide positions, consisting of 11 substitutions and 20 indels over all 4 subdomains, with III and IV being the most variable (Figure 4b). There were 6 transitions (5 between pyrimidines, 1 between purines), and 5 transversions. Substitutions occurring in stem regions were partially compensatory (e.g. a substitution on one side of a helix to maintain base

pairing, 5 in total) or completely compensatory (substitutions/indels on both sides of a helix to maintain base pairing, 8 in total) with the remainder being non-compensatory. Twelve substitutions/indels occurred in loops and thus did not affect stem base-pairing.

No differences existed between core regions of *P. huronensis* and *C. mariae*. Comparison of their predicted D3 secondary structures revealed a transversion in the terminal loop of subdomain I and highly variable subdomains II, III, and IV. These stems exhibited large mutational differences and were too variable to differentiate substitutions/indels between the 2 species. While the base of each subdomain was conserved, considerable variation occurred at the terminal ends in both stem and loop regions. *P. huronensis* maintained the first 3 bp of stem II and 8 bp of stem III, but the rest of these stems were elongated by repeat insertions. Most of subdomain IV was conserved, with 5 substitutions (2 transitions, 3 transversions) and 3 indels in the first 21 bp; the terminal portions were too divergent for comparison.

Figure 8 shows phenograms depicting genetic similarity of 9 dracunculoid species, as well as *C. elegans* and *Cystidicola* spp. type 1, based on their ITS-2 and D3 sequences. Phenograms based on Clustal W or secondary structure alignments of the D3 region differed in their branch lengths, however, except for the placement of *Avioserpens* sp. and *Anguillicola crassus,* taxa clustered identically. ITS-2 and D3 phenograms both split members of the family Philometridae, and placed *Philonema* spp. with *P. oligocotti* (Guyanemidae) and SS1 rather than with the other philometrids, *P. huronensis* and *Clavinema mariae*. The D3 showed more similarity among these 7 species (Philometridae/Guyanemidae) than other dracunculoids *A. crassus* and *Avioserpens* sp., but the ITS-2 did not. The ITS-2 showed the Philometridae as

highly diverged and *Philonema* spp. and the Guyanemidae as more similar to *A. crassus* and *C. elegans* than to other philometrids.

Discussion

D3 and ITS-2 sequences can resolve systematic and taxonomic issues within the Dracunculoidea, but the extent of their usefulness depends on the taxonomic level to which they are applied. Important issues include the appropriateness of ITS-2 and D3 data for determining the validity of the current dracunculoid classification and whether the dracunculoids represent a coherent taxonomic group. Before such questions can be addressed, it is necessary to assess the extent of D3 and ITS-2 variation, and hence the phylogenetic utility, of these variable regions. Although the phylogenetic value of the ITS-2 across the 4 dracunculoid families analysed in this study was limited by the small number of taxa I was able to examine, comparison of D3 and ITS-2 variation within the Dracunculoidea allowed general observations about the evolution of rDNA expansion and spacer regions. I will first discuss characteristics of dracunculoid ITS-2 and D3 regions before extending an interpretation of their diagnostic value.

ITS-2 characteristics

Dracunculoid ITS-2 sequences ranged from 299-521 bp (Table 3.2), consistent with ITS-2 sequence lengths of other nematodes (strongylids 217-337 bp, *C. elegans* 383 bp, *Cystidicola* spp. 365-368 bp, as discussed in Chapter 2 this study), but the ITS-2 of *Avioserpens* sp. at 521 bp was exceptionally long. G + C content (28.8-55.3%) was higher than for other nematodes (e.g. *Cystidicola* spp. G + C = 27% and Trichostrongylids G + C ≈30%). Further comparison of

ITS-2 sequence characteristics, except within *Philonema* spp., was hindered by the high degree of variability in length and nucleotide composition within this group. Phylogenetic analysis using dracunculoid ITS-2 sequence data was not performed because the ITS-2 was so divergent among the species analysed that the alignment was questionable and a robust phylogeny unlikely. Establishing a common secondary structure might have allowed more reliable identification of homologous regions and guided ITS-2 alignment across this broad assemblage. This technique has been successfully applied to plants (Mai and Coleman 1997), trematodes (Morgan and Blair 1997), and *Drosophila* spp. (Schlötter et al. 1994), yielding phylogenies which agree with those derived using morphological data.

Conserved ITS-2 secondary structures have been predicted for several groups (Wesson et al. 1992; Schlötterer et al. 1994; Mai and Coleman 1997; Chilton et al. 1998; Morgan and Blair 1998; Shinohara 1999), however, the only ITS-2 structure unambiguously resolved is that of *Saccharomyces cerevisiae*. Determining this structure involved an approach which combined enzymatic and chemical probing, minimum free energy calculations, and mutation and insertion/deletion experiments (Yeh and Lee 1990; van der Sande et al. 1992; van Nues et al. 1995). Computer modelling is now the technique most commonly used to predict secondary structures. This involves generating models that rely on algorithms that search for the state of minimum energy based on stacking and destabilizing energies, however, since some molecules may not adopt the minimum energy structure, additional algorithms allow suboptimal structures to also be generated (Zucker et al. 1999). The increased rRNA database has facilitated a combined comparative and energetic approach resulting in improved secondary structure estimates since those based solely on computer-generated energetic calculations are poor

predictors of rRNA secondary structure because they ignore the contributions of ribosomal proteins (Kjer 1995). Results from this study confirm the limitations of a strictly energetic approach as Mfold could not form D3 structures that should conform to a universally conserved model and are easily produced manually.

A common secondary structure for trichostrongylid nematodes was predicted despite distinct interspecific sequence differences (1.3-26%, Chilton et al. 1998). This common structure represented the most energy efficient shape for 6 of 7 species analysed. In light of these results it is surprising that the most energy efficient foldings for *P. agubernaculum/P. oncorhynchi* and *P. sibirica* were not identical, since their sequence differences were relatively minor (5.1%). Arriving at a common ITS-2 secondary structure for a given group can be complicated by either a high rate of sequence divergence among distantly related organisms or a level of conservation too great to be structurally revealing within a group of close relatives (Mai and Coleman 1997). A greater range of taxa, spanning more appropriate levels of conservation (e.g. more *Philonema* spp. and philometrids), should be examined in order to estimate a common dracunculoid structure which could then be applied to phylogenetic analysis.

D3 characteristics

The D3 expansion segment of 28S rDNA has been studied extensively from a variety of organisms, including crustaceans (Nunn 1996), free-living nematodes (Nunn 1992), plant parasitic nematodes (Al-Banna et al. 1997) and dracunculoids (Adamson et al. 1992; Deprés et al. 1995; Brown 1996). Characterisation of the D3 regions from *P. huronensis* and the slender sole worm provides further insight into dracunculoid molecular diversity and evolutionary relationships. In addition, detailed examination and comparison of proposed D3 structures for

P. huronensis and SS1 with those of other organisms (Nunn 1992, 1996), particularly dracunculoid nematodes (Brown 1996), supports current views on patterns of divergence and possible evolutionary mechanisms within rDNA expansion segments. In the following section I first examine variation in the D3 regions of *P. huronensis,* SS1, and other dracunculoids, before focussing on more general evolutionary patterns within the D3 region.

The length and G + C content of the D3 region from SS1 (179 bp and 50.3%) and *P. huronensis* (240 bp and 46%) are comparable to those obtained from other dracunculoids (160-232 bp in length, 31-55.1% G + C content, Brown 1996), *C. elegans* (Ellis et al. 1986) and *Cystidicola* spp. (172 bp and 41.86%). The absence of intraspecific D3 variation noted in this study is consistent with findings from other nematodes (Adamson et al. 1992; Nunn 1992; Deprés et al. 1995; Brown 1996; Al-Barra 1997).

Several patterns of divergence observed in this study reflect apparent general characteristics of the D3 expansion segment, including conserved secondary structure despite considerable size and sequence variation (Michot et al. 1990; Nunn 1992; Deprés et al. 1995; Brown 1996); highly conserved single-stranded regions between subdomains (Michot et al. 1990; Nunn 1992); and subdomains II-IV which sustain considerable variation, often exhibiting repeat insertion motifs perhaps generated by replication slippage (Hancock and Dover 1988; Nunn 1996). The first trend is apparent when comparing the D3 of distant nematode species; for example, despite the high level of D3 sequence divergence between *P. huronensis* and *C. elegans* (26.9%), a common overall secondary structure is maintained which resembles models proposed for other nematodes (Nunn 1992; Brown 1996) and conforms to the general eukaryote structural model (Michot et al. 1990).

The pattern of conserved and variable regions I observed within the D3 expansion segment corresponds with previous findings (Michot et al. 1990; Nunn 1992; Brown 1996). The most conserved areas of D3 primary sequence were the single stranded regions between stem-loops. Nucleotides in stem I were the next most conserved between taxa compared in this study (*P. huronensis*/*C. mariae*; *P. huronensis*/SS1; and SS1/*P. oligocotti*), a characteristic shared by other dracunculoids (Brown 1996), *Caenorhabditis* spp., and *Heterorhabditis* spp. (Nunn 1992). Stems II-IV are considerably more variable. Stem II may exhibit large variation in primary sequence, but is usually structurally conserved. Internal bulges are absent in this region of *Caenorhabditis* spp. and *Heterorhabditis* spp. (Nunn 1992), *Philonema* spp., *C. mariae*, *P. oligocotti* (Brown 1996), *P. huronensis* and SS1, but are present in other dracunculoids, including *Dracunculus insignis*, *A. crassus*, and *Avioserpens* sp. (Brown 1996). Stem III is highly variable in size and structure, but remains extensively base-paired. Although in some nematodes (*Caenorhabditis* spp., and *Heterorhabditis* spp.) insertion events are mainly single nucleotides inserted in terminal loops (Nunn 1992), I found evidence of base-pair inserts looking motif-like (e.g. TG x 6 repeat in stem II and GTT x 5 repeat in stem III of *P. huronensis*), which is also characteristic of stems in other organisms (e.g. isopods, Nunn 1996).

Diagnostic value of rDNA variable regions: D3 and ITS-2 sequences

Comparison of dracunculoid D3 and ITS-2 sequence data allowed the diagnostic value of these rDNA regions to be assessed. Since they are non-coding, spacer regions probably experience fewer evolutionary constraints, and thus display greater variation than rDNA gene expansion segments. If ITS-2 sequences within a given lineage change at a uniformly higher rate than the D3 expansion segment then we would expect that ITS-2 differences within the

group will always be higher than D3 differences. The degree of interspecific/intrageneric D3 and ITS-2 variation, however, was found here to be inconsistent. Within *Philonema* spp. the range of variation between D3 (0.6-1.3%) and ITS-2 (0-5.1%) sequences did not reveal the predicted pattern. The D3 region discriminated between all *Philonema* spp. examined, indicating that *P. sibirica* and *P. oncorhynchi* were as closely related as *P. oncorhynchi* and *P. agubernaculum* (0.6% difference between each pair), while *P. agubernaculum* and *P. sibirica* (1.3% difference) were more diverged (Brown 1996). The ITS-2 gave an alternative view of variation within *Philonema* spp. This spacer distinguished all dracunculoids examined, with one exception: ITS-2 sequences from *P. agubernaculum* and *P. oncorhynchi* were identical. On the other hand, ITS-2 variation between these species and *P. sibirica* was substantially greater (5.1% difference) than the D3 (1.3%).

Ribosomal DNA variation within *Cystidicola* spp. (Figure 1, Chapter 2 this study) also illustrated D3 and ITS-2 disagreement. This genus demonstrated greater conservation in its D3 than in its ITS-2; *Cystidicola* isolates had identical D3 but variable ITS-2 regions. Examination of a broader range of *Cystidicola* isolates as well as other members of the Cystidicolidae may provide a more consistent pattern of D3 and ITS-2 divergence rates. At this point the discrepancy between D3 and ITS-2 divergence within *Philonema* spp. and *Cystidicola* spp. suggests that these markers vary in their ability to discriminate interspecific relationships, depending on the taxa, so examining both regions for species identification is advisable.

The ITS-1 in nematodes displays a similarly variable diagnostic value. For example, *Ascaris lumbricoides* and *Ascaris suum* had identical ITS-2 sequences, but their ITS-1 sequences differed by 1.3% (Zhu et al. 1999), whereas in other species the ITS-1 is less variable

than the ITS-2 (see discussion Chapter 2). Phenograms depicting genetic similarity among *Trichostrongylus* spp. differed depending on whether they were derived using ITS-1 or ITS-2 sequence data (Hoste et al. 1998).

ITS-2 and D3 data reveal comparable intergeneric affiliations. For example, these regions ally *P. huronensis* with *C. mariae* and SS1 with *P. oligocotti*, their morphologically closest relatives for which rDNA sequence data is available. The D3 and ITS-2 regions also agree in their placement of *Pseudodelphis oligocotti* and SS1 within the Philometridae, as their sequences are more similar to *Philonema* spp. (an average difference of 7.65% and 37.88% respectively) than *Philonema* spp. are to other philometrids (an average difference of 15.65% and 49.38% respectively). Phylogenetic analysis of the Dracunculoidea using the D3 also supported *Pseudodelphis oligocotti*'s affiliation with *Philonema* spp. (Brown 1996).

In comparison to D3 divergence, the ITS-2 demonstrated an accelerated rate of change between dracunculoid families. Within the Dracunculoidea D3 differences ranged from 0.6-26.8% compared to 0-58% for ITS-2 sequences. ITS-2 differences among 5 species within the family Philometridae alone were 0-50.3%. This increased ITS-2 divergence rate is consistent with that found in other nematode superfamilies; within the Strongyloidea ITS-2 differences ranged from 8.9-57.5%, and intrafamilial differences were as follows: Family Cloacinidae 8.9-42.8%, Family Strongylidae 11.3-50.1%, Family Chabertiidae 29.2% (Chilton et al. 1997). Variation among 3 genera within the subfamily Anisakinae (Ascaridoidea:Anisakidae) (49.9-53.4%) was also considerable (Zhu et al. 1998).

The diagnostic value of the ITS-2 becomes far less reliable above the family level. This is most apparent when comparing dracunculoid sequence differences with the outgroup species

C. elegans and *Cystidicola* spp. High ITS-2 variablity causes some dracunculoids (e.g. *Avioserpens* sp.) to appear closer to *Cystidicola* spp. than to other superfamily members. This unlikely represention is presumably an artifact of sequence alignment. Homologous sites become increasingly difficult to identify, and alignments less robust, among highly divergent sequences. When paired sequences differ by more than 30% in a given region the probability of homoplasy (parallelism, convergences and reversals) increases and short areas of sequence similarity are more likely to match at random (Hillis and Dixon 1991). This problem may be partially related to the method of analysis (UPGMA vs parsimony) as Chilton et al. (1997) found strongyloid families were not upheld when percent nucleotide similarity was the criteria for analysis, but parsimony analysis supported recognized family affiliations.

Table 3.1. Dracunculoid nematode sequences used to compare utility of ITS-2 (this study) and D3 regions (Brown 1996, except x,y) for species identification and genetic similarity.

Family and Species	Location	Host	Individuals sequenced	
			ITS-2	D3
Anguillicolidae:				
Anguillicola crassus	Bracciano L. Italy	*Anguilla anguilla* (European eel)	1	Brown 1996
Dracunculidae:				
Avioserpens sp.	Vancouver BC	*Ardea herodias* (great blue heron)	1	Brown 1996
Guyanemidae:				
Pseudodelphis oligocotti	Stanley Park BC	*Apodichthys flavidus* (penpoint gunnel)	2	Brown 1996
Undescribed species[x]	Barkley Sound BC	*Eopsetta exilis* (slender sole)	2	2
	Stanley Park BC	*Platichthys stellatus* (starry flounder)	1	Brown 1996
Philometridae:				
Clavinema mariae	Timber L, NS	*Catostomus commersoni* (white sucker)	2	2
Philometroides huronensis[y]				
Philonema agubernaculum	Fulton R, BC	*Oncorhynchus mykiss* (steelhead trout)	1	Brown 1996
P. agubernaculum	O'Connor L, BC	*Oncorhynchus mykiss* (rainbow trout)	1	Brown 1996
P. oncorhynchi	Lake Kuril, Kamchatka Russia	*Oncorhynchus nerka* (sockeye salmon)	2	Brown 1996
P. sibirica	Lake Yli-Katka Finland	*Coregonus albula* (vendace)	1	Brown 1996

Table 3.2. ITS-2 length and G+C content of 9 dracunculoid nematodes. Species abbreviations are indicated in parentheses.

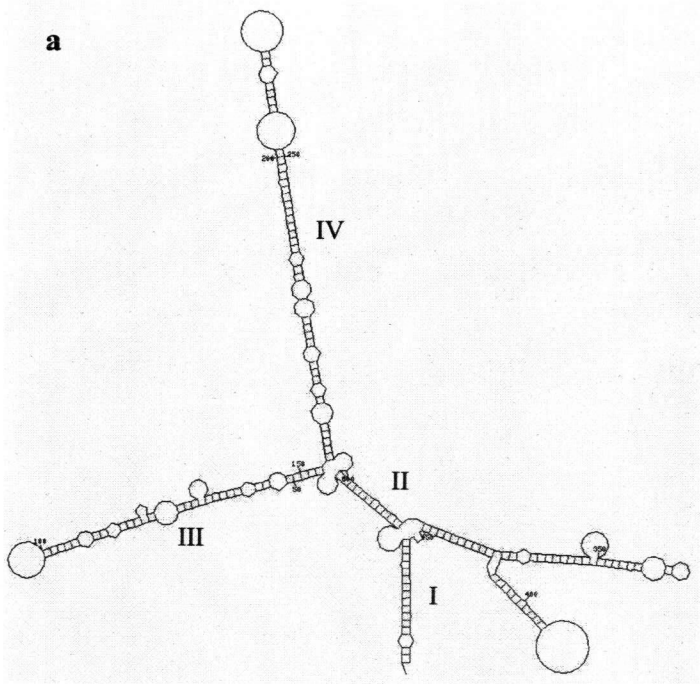
Species	ITS-2 length	G+C content
Anguillicola crassus (ANG)	299 bp	49.8%
Pseudodelphis oligocotti (PSE)	338 bp	55.3%
Philometroides huronensis (PHU)	406 bp	38.4%
Slender sole worm (SS1)	411 bp	48.2%
Philonema agubernaculum & *P. oncorhynchi* (PAG &PON)	416 bp	43.3%
P. sibirica (FIN)	435 bp	42.3%
Clavinema mariae (CLA)	448 bp	40.2%
Avioserpens sp. (AVI)	521bp	28.8%

Table 3.3. Pairwise comparisons of % differences in D3 sequences. Secondary structure alignment (upper diagonal) and Clustal W alignment (lower diagonal). Dracunculoid species abbreviations are given in Table 3.2, with CEL=*C. elegans* and CY1=*Cystidicola* spp. type 1.

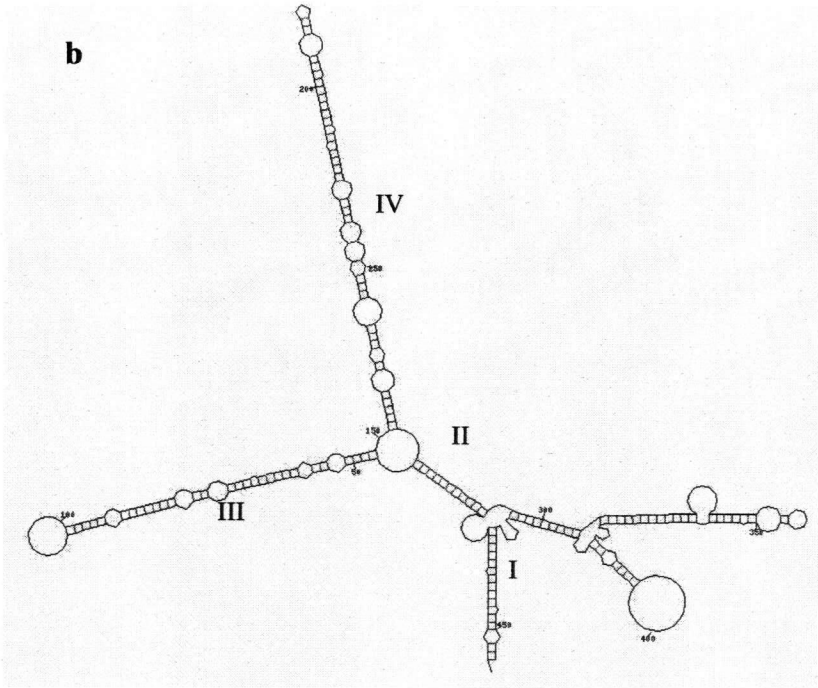
	PHU	CLA	PAG	PON	FIN	SS1	PSE	ANG	CEL	AVI	CY1
PHU	-	16.0	20.4	20.8	21.3	21.7	18.5	23.8	32.1	26.2	23.1
CLA	11.0	-	16.9	16.7	17.2	17.1	13.4	22.8	28.8	19.7	22.3
PAG	16.3	14.8	-	0.6	1.3	7.2	5.4	20.0	29.8	18.9	21.8
PON	15.8	14.9	0.6	-	0.6	7.2	5.8	20.5	29.4	19.3	21.8
FIN	16.4	15.7	1.3	0.6	-	8.1	6.4	21.3	29.2	19.7	22.1
SS1	17.7	16.5	8.7	8.6	8.8	-	5.1	20.6	30.1	21.4	21.1
PSE	15.0	12.1	6.4	6.7	6.7	5.0	-	18.7	29.9	18.8	20.4
ANG	21.0	18.3	20.4	20.7	20.7	21.5	18.4	-	30.4	23.0	20.4
CEL	26.9	27.2	27.1	26.9	26.9	28.9	26.5	24.4	-	31.2	32.2
AVI	26.8	21.4	18.8	19.2	19.7	22.0	19.7	23.6	28.9	-	22.5
CY1	22.5	19.7	17.5	17.7	17.8	18.7	18.2	17.8	26.5	16.5	-

Table 3.4. Pairwise comparisons of % differences in D3 sequences (upper diagonal) and ITS-2 sequences clustal (lower diagonal).

	PHU	CLA	PAG	PON	FIN	SS1	PSE	ANG	CEL	AVI	CY1
PHU	-	11.0	16.3	15.8	16.4	17.7	15.0	21.0	26.9	26.8	22.5
CLA	41.4	-	14.8	14.9	15.7	16.5	12.1	18.3	27.2	21.4	19.7
PAG	48.8	50.0	-	0.6	1.3	8.7	6.4	20.4	27.1	18.8	17.5
PON	48.8	50.0	0	-	0.6	8.6	6.7	20.7	26.9	19.2	17.7
FIN	48.4	50.3	5.1	5.1	-	8.8	6.7	20.7	26.9	19.7	17.8
SS1	50.9	57.2	37.0	37.0	37.8	-	5.0	21.5	28.9	22.0	18.7
PSE	52.0	57.8	38.1	38.1	39.3	25.3	-	18.4	26.5	19.7	18.2
ANG	53.6	55.5	47.5	47.5	47.3	54.2	54.1	-	24.4	23.6	17.8
CEL	50.6	54.2	45.1	45.1	46.6	54.1	55.0	55.6	-	28.9	26.5
AVI	53.9	57.2	53.2	53.2	52.8	56.6	58.0	53.3	58.5	-	16.5
CY1	50.4	50.7	45.8	45.8	45.2	54.8	57.0	50.6	51.7	46.8	-

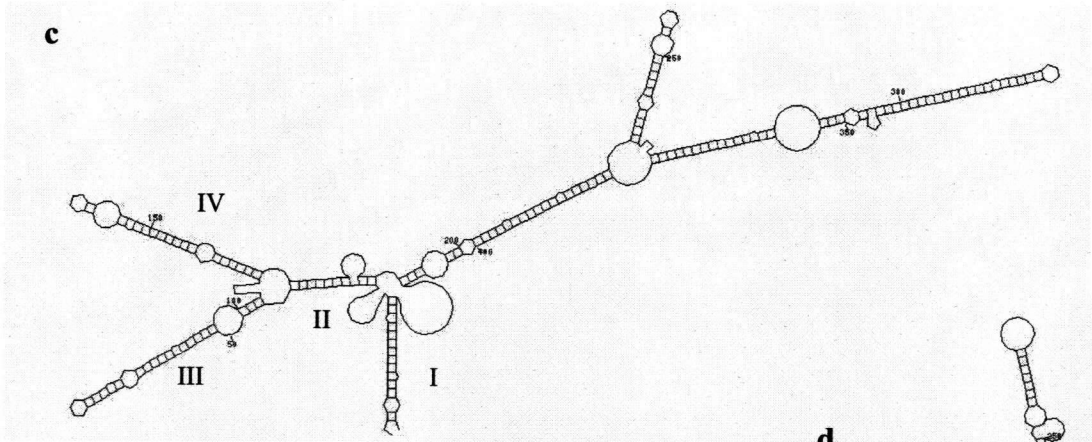


P. sibirica ΔG =-128.44

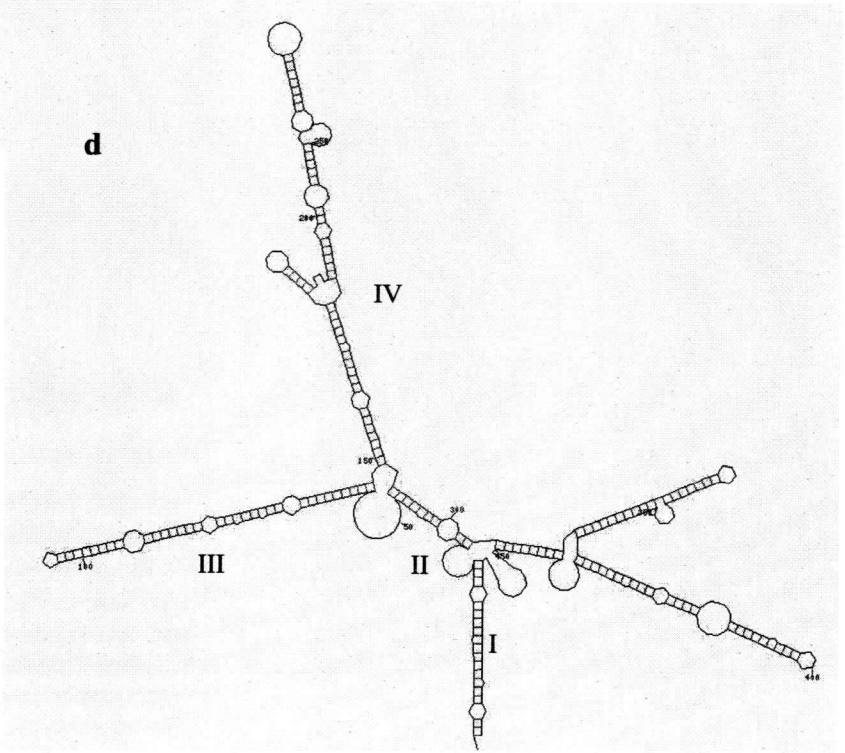


P. agubernaculum and *P. oncorhynchi* ΔG = -129.76

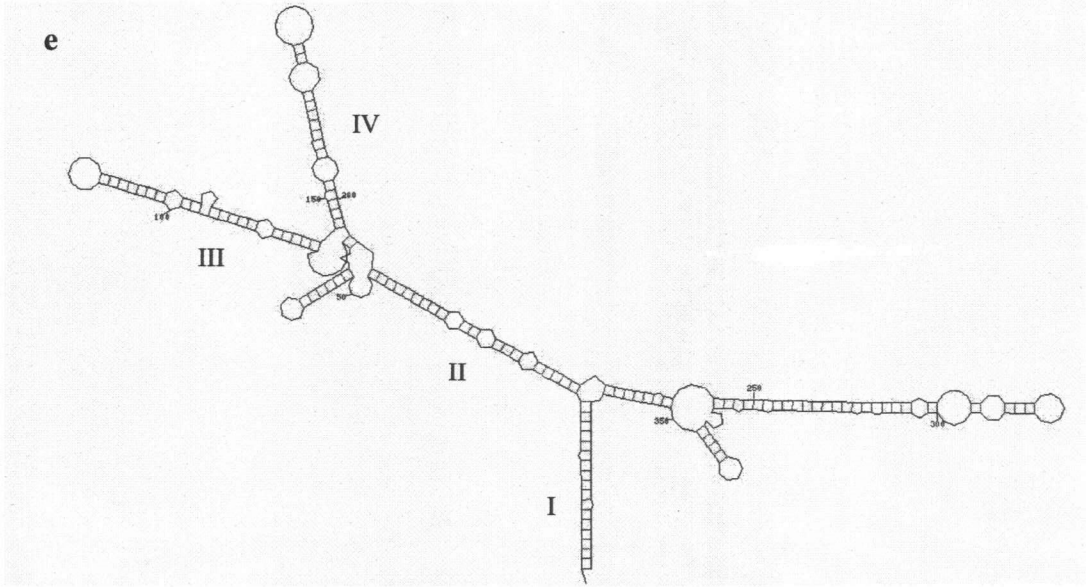
Figure 3.1. Predicted ITS-2 secondary structures for 7 dracunculoid species depicting a similar overall structural morphology consisting of 5-6 distinct domains in addition to coding stem I. Helices were retained from each species' optimal folding and were within 5% suboptimality [except *P. sibirica* (11%) and *P. huronensis* and *Avioserpens* sp. (most energy efficient structures)]. Homologous stems (I-IV) are indicated and though the 3' end of each sequence consistently formed 3 domains, their homology was uncertain.



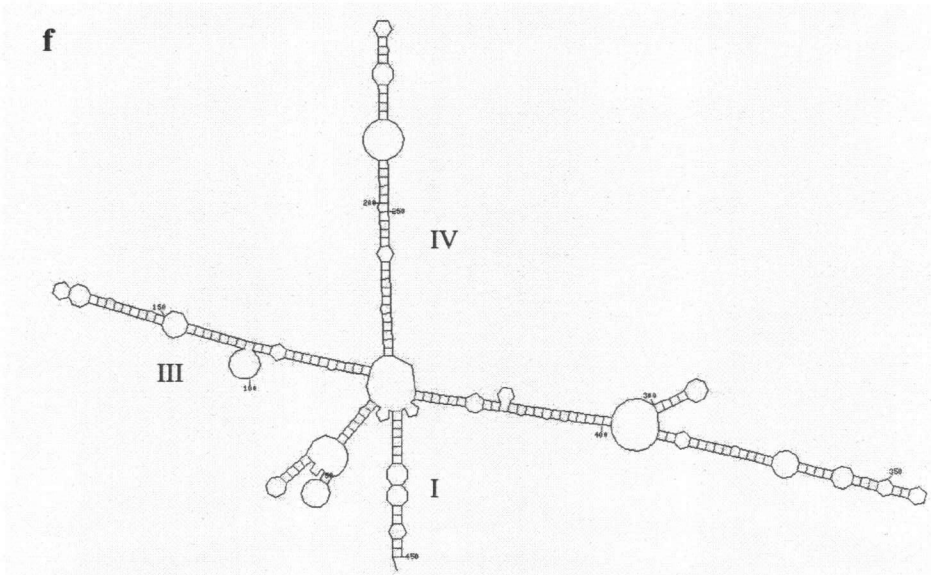
Philometroides huronensis ΔG = -132.15



Clavinema mariae ΔG = -168.96



Pseudodelphis oligocotti ΔG = -138.66



Slender sole worm ΔG = -143.04

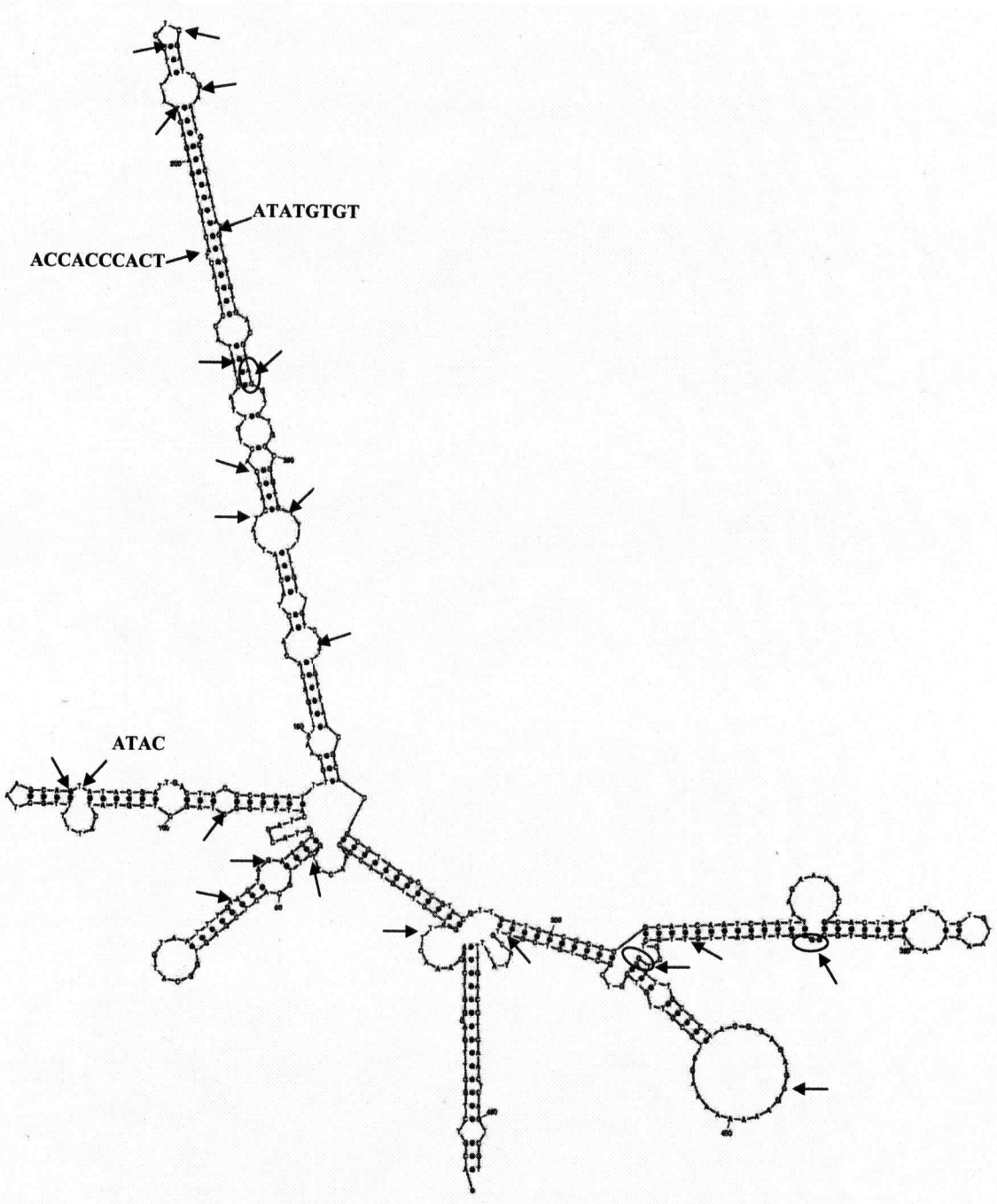


Figure 3.2. Consensus ITS-2 secondary structure for *Philonema* spp. Substitutions between the ITS-2 sequence of *P. agubernaculum/P. oncorhynchi* (shown here) and *P. sibirica* are indicated, but do not represent compensatory changes.

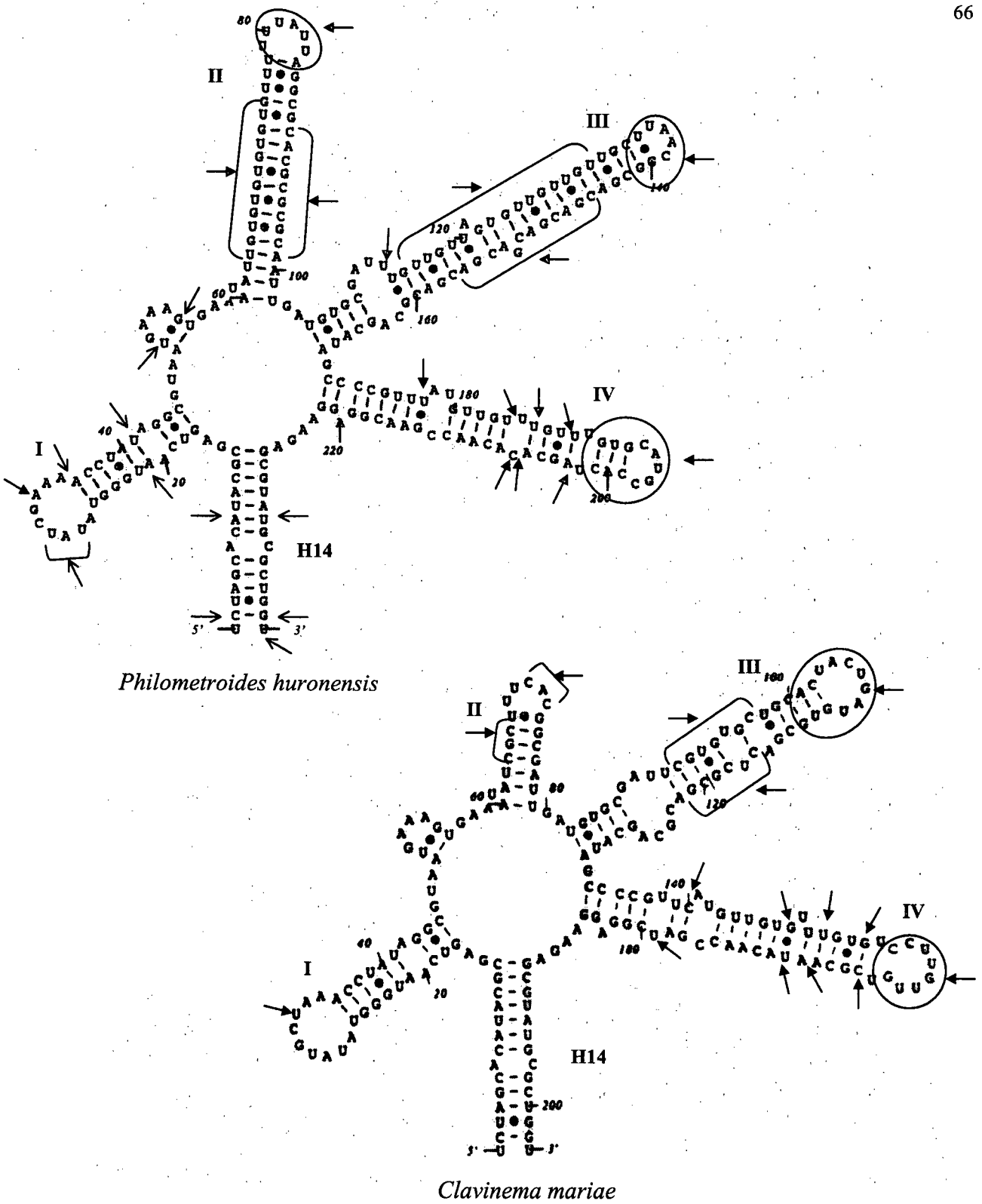
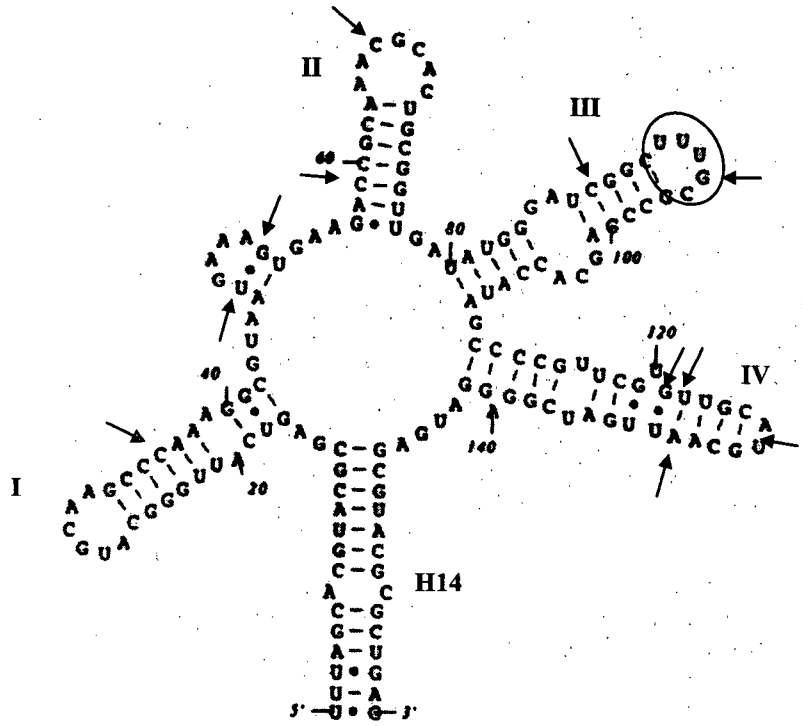
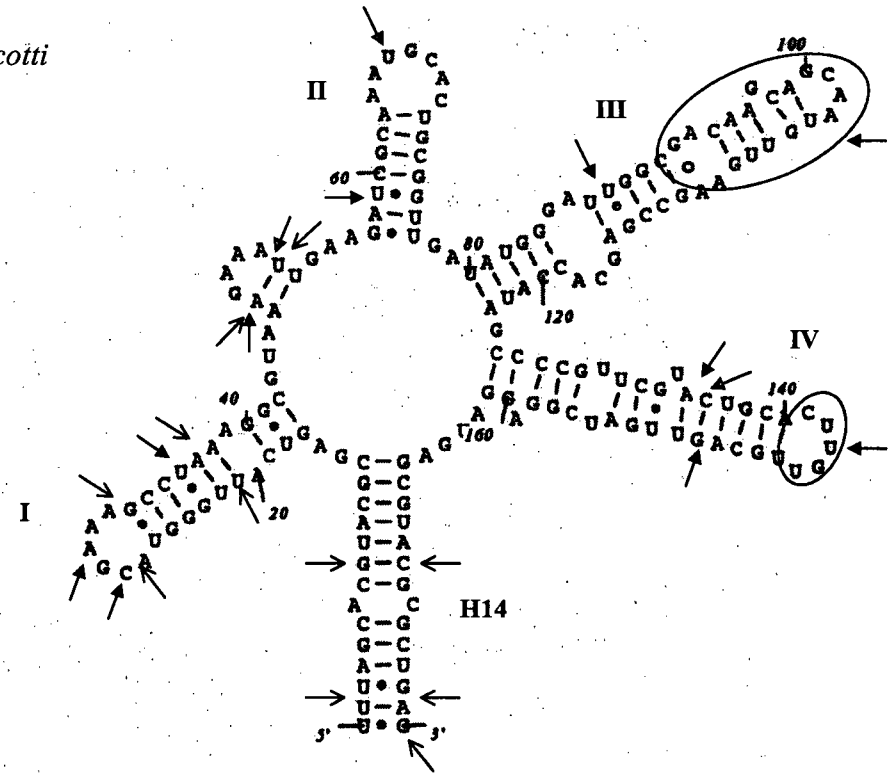


Figure 3.3. Proposed secondary structures for the D3 expansion segment of *Philometroides huronensis* and the slender sole worm, in comparison to those determined for their relatives, *Clavinema mariae* and *Pseudodelphis oligocotti* (Brown 1996). Differences between *P. huronensis* and slender sole worm are indicated by open arrows, and between *P. huronensis* and *C. mariae*, and SS1 and *P. oligocotti* with closed arrows.



Pseudodelphis oligocotti



Slender sole worm

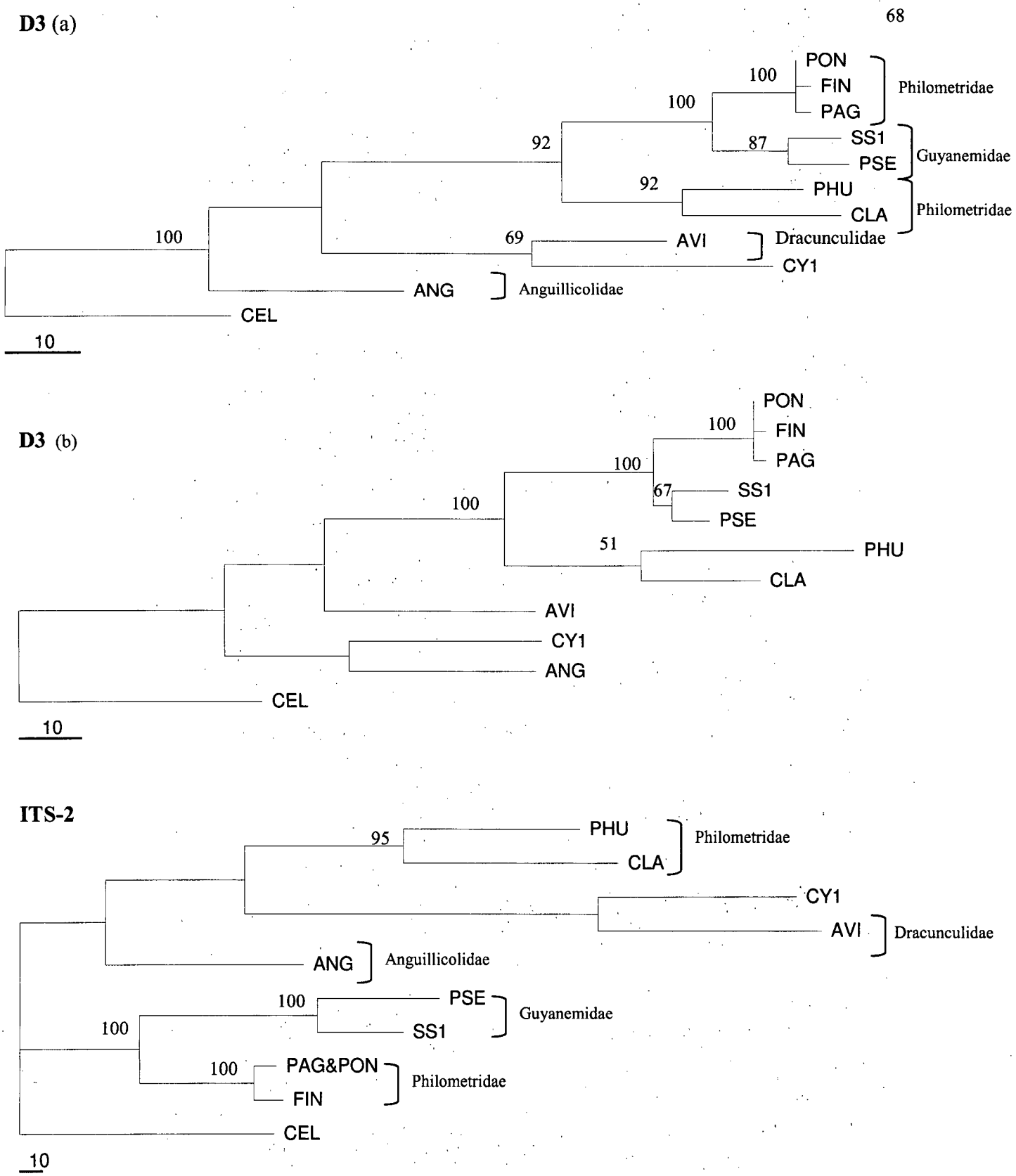


Figure 3.4. Phenograms depicting the percent genetic differences between 9 dracunculoid species based on the D3 (a = clustal and b = secondary structure alignment) or ITS-2 regions. Bootstrap values are indicated on branches (n=100), values under 50% not shown.

CHAPTER 4: GENERAL CONCLUSIONS

The considerable morphological differences within species and overlap between species within the genus *Cystidicola* are difficult to interpret. *Cystidicola* spp. appear to exhibit a continuum of variation where species are defined by a set of shared traits which if considered individually are not diagnostic. Egg morphology alone is not a good taxonomic indicator since *Cystidicola* spp. with morphologically distinctive eggs can be identical in rDNA sequence, and those with morphologically identical eggs can be genetically distinct. Morphology of the oral opening is also a dubious taxonomic indicator if viewed in isolation. The range of variation observed within *Cystidicola* spp. suggests that unless it is assumed that none of the characteristics examined here are informative, a suite of traits must be considered before determining the significance of differences in a given trait between *Cystidicola* isolates. Information from multiple markers is desirable especially for recently diverged taxa (e.g. sibling species) where there may be no single diagnostic marker to differentiate between groups (Avise 1994, Anderson et al. 1998). Based on the morphological and genetic differences observed in this study, I conclude that: *C. farionis* with laterally filamented eggs from lake whitefish is the same species that infects other Ontario salmonids; *C. stigmatura* and *C. farionis* ON/FIN are separate species; *C. farionis* FIN and BC are cryptic species; *C. farionis* BC is sufficiently different from *C. farionis* ON/FIN to be considered a separate species; *C. farionis* BC and *C. stigmatura* represent a recent divergence and thus share the same ITS-2 type, but since other biological traits distinguish these species (intermediate and definitive hosts, distribution, egg morphology) I suggest they be viewed as separate species. Therefore there may be 3 species belonging to the genus *Cystidicola*: *C. farionis*, *C. stigmatura* and

Cystidicola sp. from BC. The biology and morphology of *Cystidicola* sp. from BC deserves further study, and may support re-naming this species.

Further molecular analysis of *Cystidicola* isolates, including a broader range of isolates from across Canada (e.g. Manitoba, Yukon) and Europe, using a more rapidly evolving marker such as mitochondrial DNA may resolve the pattern of molecular and morphological variation/conservation observed in this study. Characterisation of the D3 and ITS-2 regions from other cystidicolids, especially other sister species, may more clearly indicate the connection between morphological and molecular variation and thus confirm the taxonomic value of these rDNA regions for the Cystidicolidae. Assessing the degree of variation within these regions in *Salvelinema walkeri* and *S. salmonicola* would be particularly interesting, since these cystidicolids are morphologically very similar, and are distinguished only by their egg morphology (e.g. placement and number of egg filaments).

D3 and ITS-2 sequences can resolve systematic and taxonomic problems within the Dracunculoidea, but their diagnostic value depends on the taxonomic level being examined. These rDNA regions vary in their ability to differentiate close relatives (e.g. *Cystidicola* spp. and *Philonema* spp.), so characterising both sequences is advisable for species diagnosis. ITS-2 and D3 data revealed comparable intergeneric dracunculoid relationships, and supported previous suggestions that the family Philometridae may be artificial, and that *P. oligocotti* and SS1 (currently within Guyanemidae) are affiliated with others in this family (e.g. *Philonema* spp.). The diagnostic value of dracunculoid ITS-2 sequences was poor above the family level. At this level sequences were too divergent to reliably assess homology without using a more conserved feature, such as secondary structure, for alignment. Attempts to predict a common

dracunculoid secondary structure were confounded by high levels of variation in ITS-2 sequence length and nucleotide composition, and the large number of competing structures generated by mfold. Attempts to predict a common structure which could be applied to phylogenetic analysis would be improved by examining ITS-2 sequences from a greater range of taxa, especially more genera within a particular family (e.g. more philometrids). Overall, the relatively reduced sequence variability exhibited by the D3 region produced more reliable alignments than the ITS-2, so phylogenetic relationships would be expected to be inferred with greater confidence. As a result, the D3 appears to be a more precise taxonomic marker for the dracunculoids, particularly above the family level, and is better suited for phylogenetic studies within this group.

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```
PSE             ACGTCTGGTTGAGGGTCGAC--------CATTGCAGTCGTTGTACGTGCGC-----GCGA
SS1             ACGTCTGGTTGAGGGTCGAC--------AATTGCAGTCGTTGTACGCAAGCA-CACGCAA
PAG&PON         ACGTCTGGTTGAGGGTCAAC--------AAACTTAGTCGTTGTACGCAACTTGCTTGCAC
FIN             ACGTCTGGTTGAGGGTCAAC--------AAACATAGTCGTTGTACGCAACTTGTTTGCAC
CEL             ACGTCTGGTTCAGGGTTGTT--------TAACTCAAT-GCCTTAGGCTTCTC-TTCGGAG
CY1             ACGTCTGGCTGAGGGTCGGTTTTGTAGAAAATGTAGTTATTGTTGTTATTGC---TGAAT
AVI             ACGTCTGGTTGAGGGTCTTCAT---A--TATTTCAATTATCATTCATATCA----TAGAT
ANG             ACTTCTGGCTGAGGGTCGA----------------TTGTCG-AGTCAGCA-----GCAG
PHU             ACGTCTGGTTGAGGGTCGTATTATAAAACAACTC-GT-GTACGTCGCCGCTCAGTTGGTG
CLA             ACGTCTGGTTGAGGGTCATAATACACAAAAACTC-GT-GTACTA--CATCTTCATTATCT
                ** ***** * *****                    *

PSE             TATATTTGAGCGGAAAGTTCAG-AGCAGCGTACTA---TCGTTCGGCTAGCTAGCTGACG
SS1             CCTATTCGAATAGAATGTTC-G-ACTAGCGTACGA---TCATTTGGCTAGCTAGCTAACT
PAG&PON         TCGAGTGGCATGTAATTTGAAGTAGAAGCGTACGA---TCATTTGGCTAGCTCG-TATC-
FIN             TCGAGTGGCATGTAATTTGA-GTACAAGCGTACGA---TCATTTGGTTAGCTCG-TATC-
CEL             AGTCTTCGGCTTGTCGGGCAAC-ATTAGTGAGCTG---AGATTCGCGT--CTCGGCATA-
CY1             AACGATGGCTAATAATTTCGTCGAGCAATGTTGCA---ATATATATTTAGTTTG-CACC-
AVI             AGAGAGAGAAAATATAGTTCTC-ACAAAATTTGAT---ATTTTTTGTGAGAAAAATATC-
ANG             CAGCACGTCGAGCACGGTTA-----AGCGAAGC---------TGTGAAGACTG------
PHU             TATGTGGTGACTGGCTGTAGTGCA--AACGTACGACTAACAATCGGCTA--TATCTAGC-
CLA             TCTACTATCACACACACACA--CAC-ACACAACCACTTAACTATATGTATGTTG-TATGT
                                        *

PSE             ----TGTCTC--AGCCAGTG----GGC--------------GCGTGGGCAC--G-----
SS1             CACATATTTT--TGCCACGGCAACGGCATACTATTGTACTGTGTGTGTGTGT--G-----
PAG&PON         -----ATTTT--TGCTAA-------GCA-------------GTATAC------GA----
FIN             -----ATTTT--TGCTAA-------GCA-------------CTATACATAC--GA----
CEL             --CTGATCTTG-TCCTAGTCCAGAAGCAT-CACAA-------GTCAAGACAGA-GC----
CY1             ---TTACCTC---GATAA-------GCAT-------------TATATT-----G-----
AVI             ----TATGTGA-AAATGAAAACACAACAAACGGGTATTATGTGTTTATTTTTTTGAAAAA
ANG             ----TATTTGA-CGGCAG-------GCA-----------------ACT-----G-----
PHU             ---ATATCTA----CTACT------GCA--C-----------TCTATATGTGTGTT---
CLA             ---ATATGTGGGTGGTGGTGGTGCA----G-TGTGTGATGTGTATGTATA-GATGTG
                      *                 *                          *

PSE             -TGGCTACCCGAATTTGCACTTTT------GAACCG--ACC-ATAGA------------
SS1             -TGACTACCCGAATTTGTATTTTT------GAACTA--ACC-ATCAACTAACCCGCATGT
PAG&PON         TTGACTACCCGAATTTGCAAATTG------ACACAACTACA-ATTATCACCACTGTTACT
FIN             TTGACTACCCGAATTTGCAAATTG------ACACAACTACA-ATTACCACTACTGTTACT
CEL             TTCACCGTTCTGTCTTGTAATGTG------ACGCGC-TACT-CTTATATCGGA-GG----
CY1             TTG------CGTTTTTTTAACGT--------ACCAGTTAGA-ATTTT------------
AVI             TAAAAAATACATGTTTGT-TTGTT------TTTTTGCTACATATGTTGTATGTATATATG
ANG             -AGAGT----GATGTTGTGTATTA------TGTCGGTAACC--TGCT------------
PHU             TTGA-TGCA-GTGGTTGTGGTATGC-----ATACGCGTATACCTGATTCGAGATTT----
CLA             TGGATTAT--GTTGTTGTGGTACGACTAGCAATCGGCTATATGTGATC---GCG-T----
                          **                        *    *

PSE             ----GCACATCAA-------------------------------AAAAAGTGGAA----
SS1             --AAGCGCACCAACGCGGCGACGAC-GACGGTG---------CACAAAATGCGGTGGTTC
PAG&PON         -ATACCACACTAC------------TGTTGAG---------AGTATATCCTCGATCGAA
FIN             TATACCACACTACCAC--CCACTAC-TGTTGAG---------AGCATATTCTTGATCTAA
CEL             ---AGCTGCCAAA------------TGGCGTC---------CGACTTGACTCGGCTGA-
CY1             ---ATCAAACGTA--------------TTG--------------TATGTTTGATTTA-
AVI             TATAAAAGAAAAAGATTACTAGCATGTGTTGTTTAAAACAAAAAAATATTCTCAAATACC
ANG             ----T-AGAGAGA----------------GAG---------AGAATAAGAGGTGAGTG-
PHU             ----TGACATGTACA-------------TGATT--------TGATTATTGTGCTGCTTG
CLA             ----GCACCAGCAAAT-----------TTGTTG----------CTGTTGGTGTGCTGT
```

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PSE          ---TGGTCTTTGAATGCG---GCGCGTG-CGTACG---CACGCCGATAGCG----TGGCG
SS1          --GTGGTTTTTAAATGCG---GCGCACG-CACACG---CCCGCCGATGACTACGTTGACG
PAG&PON      --TTGCTCAATAT--------GTGTGTGACGTAGG---TGTGGCGGTGTTTTTGTTGTCA
FIN          --TTGCACAATATATATGTGTGTGTGTGGTGTGTG---TGTGGCGGTGGTTTTGTTGTCT
CEL          ---TCATCAAGACGTGTTTAACTGCGTGTCTTATGA--TGTGAAGCTACTG----CATCT
CY1          ---TTAGCAAGTAAAATGTTATTTTGT----TATAA--TTTGTTGCTATTTATTTGTTGT
AVI          CGTTTTGAAATGAATTTAATAAAGAGTG--ATATGATGTATGATGATGGGATATAATAGT
ANG          --GTGCACAGTTTTTATCGAAGAGGAC----AACCG--TTTCAAGGTGGTG-----TTAT
PHU          --TTTGACAACAACAATGGAAATGTTTG-TTGACGA---ATGCATGTTGTG----TGTGT
CLA          ACCCGATCGAGATACCCGATTTTGATT---TGACATGTAATGCATATAGATG---TGTGT
                                                                *

PSE          AACGTA--GCA--ACCG------G--CAACGTAGC-ACCGTGTGACAGCAGACGA-----
SS1          ATCAT----CA--ACAG------C--GAACGTAGCTAACGT-----AACAAGCGC-----
PAG&PON      TTGTTAACGCA--CGCGTACGCAC--GTACGTTGATGACGCGCG-CAACAATCAATTAAG
FIN          TTGTTAACGCA--CGCGTACGCAC--GTACGTTGATGACGCGCG-CAACAATCAATTAAG
CEL          ATGTCA--GAA--TGTGT-----C--GT-CGCTCCTTTCGAATACTGGGATTCGTCTA--
CY1          ATTTT--------TGTGT--------GCACAAAAAATAAATG-----GTTTTCAT-T---
AVI          AAATTGGAGAAATTTTGCTACTACTACTACTACTACTACTACTACTACTACTACTAACCC
ANG          TGGTT---GCA--AAAG------C---CGCGATTATTGAAA------GCGTCCGT-----
PHU          GTGTGTGAGGG--GGTGTTG--ATTTATACATATATATAGTAT----GTATATATAT---
CLA          GCGATA-CGCGCGCGCGC----ATATATGCTGCTGTGAATTGTCATGGTATATATGT--G
                          *           *

PSE          ---GCGGCATTGAA-CGCCGAAT-GAAA-CTCTC-ATCA-GC---TG-----GTGC-TAC
SS1          ---ACCG---TGTA-ATATGTAT-ATAA-CTGTG-GT---GC---TGAGTGCGTGC-TGC
PAG&PON      AAAGCAGCACTGTGTATGTGTAA-CAAA-AACTA-GTGC-GGGGTTGATTG-TTGC-TGC
FIN          AAAGCAGCACTGTGTATGTGTAA-CAAA-AACTA-GTGC-GG--TTGATTG-CTGC-TGC
CEL          ---GTCTCG-TGTGTGTGTTGAT-ATCG-AATTA-ATTT-TCGATTGA-----TGC-GGC
CY1          ---GTG----TGTGTATGTGTTT-CTAA-TAGTG-TTTTAGCGCACATTTGTGTGT-TGA
AVI          CTACTACTGGTGGTTAGTAGTTTACTACCTACTA-GTAGGGGAGGAGTGGTAGTGTATGC
ANG          ---GGA-----GAG-AGGCGGTCGCTATAGAGAA-AAAAAGG---TG-----GTGG-TGT
PHU          ---ATA----TGTATGTATGTATGGTAT--GTGA-ACAACGACGACGA-----CGCACGC
CLA          TATGTGCGTTTGTGTGTGTGTATCGTTTGAATGACACAATAACAAGGTTGTTTTGTGTGA
                        *                                           *

PSE          GA--TTG----ACTGT---GGCCTA------TGCGC---------GTAG-CGTG------
SS1          GG--TTG----ATTTTTTTGTCATC------TGGAT---------GGAG-CGCGACGCG-
PAG&PON      TGGATTGCGCAACGGAGAGGGAAAAAAAAGATGCGTT--------GTCGTCGCGTGTCG-
FIN          GACATTGCGCAACGGAGAGAGAAAAAAAAGATGCGTT--------GTCGTCGCGTGTCG-
CEL          GC---TG------------AGAAGA-------GAGAC--------GGTG-CGTGTC----
CY1          CTTATTTAGAACATATGACAACAAAATTAAAACAATT--------AAAGCATTAATTTG-
AVI          GTGTGTATGCAAAAAA-AGAGAATTTTTATTTCCATTTTTACTATATATTCTCTATTCGT
ANG          AT----------A---GAGAGAGCA--------CCTTT-------G-AAACGGGTTGTGC
PHU          AC-AATG-----AAACGTATGCACA----TGATCATC--------ATGGACACCACAAAC
CLA          CG----------ACGACACACAACATACACTATAAACTGTG-TGTGTAGAGAAAAAAA--


PSE          -----TCGCACGTTTT-----------------TTGACCTCAACTCAGGCGT
SS1          -----TCGCACATTT-------------------TGACCTCAAGTCAGTCGT
PAG&PON      -----TTGCATATTT-------------------TGACCTCAACTCAGTCGT
FIN          -----T-GCATATTT-------------------TGACCTCAACTCAGTCGT
CEL          -----TTGCTAATCT-------------------CAACCTGAACTCAGTCGT
CY1          -----TATTTTATATT-----------------GAGACCTCAGCTCAGTCGT
AVI          CACTCTTTCGTATATGAATTTTAACGCAAACTTTTGACCTCAACTCAGTCGT
ANG          G----TGTGAGATGTTA---C------------TGACCTCAGCTCAGTAGA
PHU          AATTATTACTTTTTTT-----------------TTGACCTCAACTCAGTCGA
CLA          -----TTCATTATTT-------------------TGACCTCAACTCAGTCGT
                  *       * *                      **** *  ****  *
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Appendix 3.1. Alignment of the ITS-2 sequences for nine dracunculoid species, *C.elegans* (CEL) and *C. farionis* type 1 (CY1). Asterisks indicate nucleotide similarity among the eleven species.

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PHU         TCCAGGAGTCTAGCACATACGCGAGTCAATGGGTATA-TCGAAAACCT--ATAGGCGTAA
CLA         CCAAGGAGTCTAGCACATACGCGAGTCAATGGGTATA-TGCTAAACCT--ATAGGCGTAA
SS1         CCAAGGAGTTTAGCACGTACGCGAGTCATTGGGTA----CGAAAGCCT--AAAGGCGTAA
PSE         CCAAGGAGTTTAGCACGTACGCGAGTCATTGGGCA----TGCAAGCCC--AAAGGCGTAA
PAG         CCAAGGAGTTTAGCACGTACGCGAGTCATTGGGTTT----GAAAACCC--AAAGGCGTAA
PON         CCAAGGAGTTTAGCACGTACGCGAGTCATTGGGTTT----GAAAACCT--AAAGGCGTAA
FIN         CCAAGGAGTTTAGCACGTACGCGAGTCATTGGGTTT----GAAAACCT--AAAGGCGTAA
ANG         CCAAGGAGTCTGGCATGTGCGCAAGTCACTAGTTG-----GTAAATCGGGAGAGGCGAAA
CEL         --GCGGAGTGCTTGTCTACTGCGAGTCAAAGGGTGT----TAAAACCT--TGCGGCGAAA
CY1         CCAAGGACTCTAGCATATGCGCAAGTTATTTGGT----GGTAAACCAT--AAAAACGTAA
AVI         CCAAAGGGTTAAGCACATACGCAAGTCAATAGGTATTGGTTAAAACCT--ATCGGCATAA
                 *  *           ** *** *   *          **           * **

PHU         TGAAAGTGAAATA--TTGTGTGTGTG--TGTTTTTTATTAGGCGCACGCG------CGCA
CLA         TGAAAGTGAAATA--TCGC-----------------TTT----CACG---------GCG
SS1         AGAAATTGAAG-A--TCGC-------------------AAATGCACT---------GCG
PSE         TGAAAGTGAAG-A--CCGC-------------------AAACGCACT---------GCG
PAG         TGAAAGTGAAG-A--TCGC-------------------AAATCTAAT---------GCG
PON         TGAAAGTGAAG-A--TCGC-------------------AAATCTAAT---------GCG
FIN         TGAAAGTGAAG-A--TCGC-------------------AAATCAAAT---------GCG
ANG         TGAAAGTGAAA------GC----------------------C-GACTCG------ATCG
CEL         TGAAAGTAAAGGT--CAGTC-----------------T---CGAATT----------G
CY1         TGAAAGTAAACGTCGTCTTGA-------TACGATGAAATATGTGAGCTG----------
AVI         TGAAAGTAAATGTTGTAGTGAAATAGAATACGACAAAATATGTGATCTGCTAAAAAAACA
             **** * **

PHU         ATTGATGTGCGATTTGTTGTT-AGTGTTGTTGTTGCTTAACGGCGACGACGACGACGACG
CLA         ATTGATGTGCGATTCGT------GTGCTGCACTA-CTGATGTGCGACT----CG----CG
SS1         GTTGATATGGGATTGGCGA---CAAGCAGCAATG--TTGAAGCCGA--------------
PSE         GTTGATATGGGATCGGC-----------------TTTGCGCCGA--------------
PAG         GTTGATATGGGATCGTCGT----------AGTG-AGTGTCGTCGA--------------
PON         GTTGATATGGGATCGTCGTC-----GT---AGTG-AGTGTCGTCGA--------------
FIN         GTTGATATGGGATCGTCGTC-----GTCGTAGTG-AGTGTCGACGA--------------
ANG         GCCGCTATGGGATTCGTGCT---------TTATG-CTTGGGCGCGAC------------
CEL         GCCGACGTGGGATCTGTGTT--------------CTTCGGAGTGCAG------------
CY1         ---------------------------------TGTGTTGATTAAT-ATGCGCACAGTG
AVI         ATAGTAATAATAATAATAATAGTAAAATATTAATTGTTGTTATTATTTATGAAATTAGTG


PHU         ACGCATCATAGCCCCGTTTATGTTGTTTGTTTGTGCATGCCACTAGCACACAACCGAACG
CLA         ACGCAGCATAGCCCCGTTCATGTTGTGTTTGTGTCCTTGTTGTCGCAATACAACCGATCG
SS1         --GCACCATAGCCCCGTTCGTACTGCAC--TTGT-------------TGCAGTTGATCG
PSE         --GCACCATAGCCCCGTTCGTGTTGCA----T----------------GCAATTGATCG
PAG         --GCACCATAGTCCCGTTCGTATTGCGC--TCACG------------CGCAATTGATCG
PON         --GCACCATAGTCCCGTTCGTATTGCGC--TCGCG------------CGCAATTGATCG
FIN         --GCACCATAGTCCCGTTCGTATTGCGC--TCGCG------------CGCAATTGATCG
ANG         --GCACCATAGCCCCGTCTTTGTTGCTT--------------------GCAATAGGGCG
CEL         -CGCACCACGGCCCTGTGCGTGTCACTT--------------------GTGACTGTGCA
CY1         ---CAACATAGCCCTGTTCTCGTTGCTT--------------------GCAATAGAACG
AVI         GAGCAACATAGACCCGTTCGTGTTGCGTA------------------TGCAATTGATCG
               ** **  * ** **                                  *  *
```

```
PHU             GAGGAAGAGCGTATGCGCTGGTACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
CLA             GAGGAAGAGCGTATGCGCTGGTACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
SS1             GAGGATGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
PSE             GAGGATGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
PAG             GAGAATGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
PON             GAGAATGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
FIN             GAGAATGAGCGTACGCGCTGAGACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
ANG             GAGGTAGAGCGTACATGCCGGTACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
CEL             GAGGTTGAGCAGTTGGCAAACGACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
CY1             GAGGTAGAGCGCATACGCTGGGACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
AVI             GAGTTAGAGCGTATGCGCTGGTACCCGAAAGATGGTGAACTATGCCTGAGCAGGATGAAG
                ***   ****            ************************************

PHU             CCAGAGGAAACTCTGGTGGAAGTCCGAAGCGGTTCTGACGTGCAAATCGATCGTCTGACT
CLA             CCAGAGGAAACTCTGGTGGAAGTCCGAAGCGGTTCTGACGTGCAAATCGATCGTCTGACT
SS1             CCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGATCGCCTGACT
PSE             CCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGATCGCCTGACT
PAG             CCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGATCGCCTGACT
PON             CCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGATCGCCTGACT
FIN             CCAGAGGAAACTCTGGTGGAAGTCCGAAGCGATTCTGACGTGCAAATCGATCGCCTGACT
ANG             CCAGAGGAAACTCTGGTGGAAGCTCGAAGCGGTTCTGACGTGCAAATCGATCGTCTGACT
CEL             CCAGAGGAAACTCTGGTGGAAGTCCGTATCGGTTCTGACGTGCAAATCGATCGATAGACT
CY1             CCAGAGGAAACTCTGGTGGAGGTCCGAAGCGATTCTGACGTGCAAATCGATCGTCTGACT
AVI             CCAGAGGAAACTCTGGTGGAAGTCCGAAGCGGTTCTGACGTGCAAATCGATCGTCTGACT
                ******************** *  ** * ** **********************   ****

PHU             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
CLA             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
SS1             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
PSE             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
PAG             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
PON             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
FIN             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
ANG             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
CEL             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
CY1             TGGGTATAGGGGCGAAAGACTAATCGAACCATC
AVI             CGGGTATAGGGGCGAAAGACTAATCGAACCATC
                 *******************************
```

Appendix 3.2. Alignment of the D3 region for nine dracunculoid species, *C.elegans* (CEL) and *C. farionis* type 1 (CY1).