A molecular approach to the community ecology of parasites of freshwater fish

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Abstract

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Freshwater fish have been model hosts in the study of the community and evolutionary ecology of parasites for decades. Most studies have dealt only with adult parasites, although larval stages often dominate fish parasite communities. In addition, few studies include replicates of both host species and sampling localities. For this thesis, both larval and adult parasites were surveyed in six fish species (*Notemigonus crysoleucas*, *Pimephales notatus*, *Perca flavescens*, *Etheostoma nigrum*, *Lepomis gibbosus*, *Ambloplites rupestris*) collected from six localities in the St. Lawrence River and molecular techniques were used to distinguish species of strigeid metacercariae (Platyhelminthes: Trematoda). Novel primers were developed to sequence the barcode region of the cytochrome c oxidase I (COI) gene and sequences of internal transcribed spacer (ITS) regions of ribosomal DNA were also obtained. Both markers indicated unexpectedly high numbers of species in strigeid metacercariae, but resolution between species was clearer with COI than with ITS sequences. Strigeid species inhabiting the lens of the eye of fish were significantly less host specific than species inhabiting other tissues, possibly due to limited immune activity in the lens. Patterns of host specificity were consistent across the separate fish communities, which included fish species that are

ecologically distinctive but closely related. Together, these findings suggest that physiological incompatibility between host and parasite is a more important determinant of host specificity than the ecological availability of host species to parasites in strigeid metacercariae. The high host specificity of most strigeid species had important effects on the parasite communities as a whole. Closely related fish species showed a significant tendency to have similar parasite communities that was much stronger than the tendency of parasite communities in spatially proximate fish to be similar. Geographic distance was strongly associated with parasite community similarity only when comparisons were limited to fish of the same species. Host taxonomy explained much more structure in parasite communities than spatial categories, suggesting that host phylogeny is more important than habitat in determining parasite community composition and abundance.

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This thesis represents the work of more than one person. Many people contributed time, energy, expertise, equipment and samples. However, any shortcomings in this work are my responsibility alone.

Teresa Crease and Anna Moszczynska made a key methodological contribution which vastly improved the quality of the data (Chapter 1, see also Contributions of the Authors).

The input of Selvadurai Dayanandan was important to several key molecular aspects of this project. He also served on my committee, along with Emma Despland, Edward Maly, Paul Widden, Dylan Fraser, Barbara Woodside and Kym Jacobson, all of whom provided critical feedback that is gratefully acknowledged.

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Contributions of authors

I collected, processed and identified most of the parasites used in Chapter 1. Teresa Crease designed the primers. Anna Moszczynska performed most of the extraction and PCR of DNA; I performed DNA extraction and PCR for a small number of samples. I rewrote an early draft of the chapter by Teresa Crease and Anna Moszczynska, made subsequent revisions, and performed most of the data analysis.

I was responsible for the conception, sampling design, field work, specimen identification and processing and data analysis described in Chapters 2-4. I traveled to the Canadian Centre for DNA Barcoding in Guelph and assisted with the molecular processing (DNA extraction, PCR and DNA sequencing) of approximately 1100 out of 2000 specimens.

Selvadurai Dayanandan provided editorial input to Chapter 2. David Marcogliese and Daniel McLaughlin oversaw and contributed to all aspects of the research and provided editorial input to all chapters.

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TABLES AND FIGURES

General introduction

Parasitism is a common way of life in the animal kingdom, yet few generalizations can be made about how parasites are distributed in space and among host species (Poulin and Morand 2004; Poulin 2007a). This is true of even well-studied parasite communities such as those in freshwater fish (Kennedy 2009). Typically, the findings of any given study are not duplicated in subsequent studies. This may point to insufficient replication in sampling designs in the original studies. Most are based on samples of multiple populations of a single fish species (spatial replication) and a few are based on multiple host species sampled in a single locality (host species replication). Very few studies include both types of replication, and in most cases, only adult parasites are enumerated because larval stages cannot be identified to species. This thesis describes an attempt to avoid these problems in a survey of the parasites of six fish species sampled across six localities, using molecular data to distinguish species in a dominant group of larval parasites. A substantial portion of the thesis is concerned with the evaluation of molecular data and their implications for the parasite communities under study.

One of the main difficulties of studying fish parasites is species identification. Parasites are usually small, soft-bodied and have few stable morphological characters. Moreover, morphological traits used to identify parasite species can vary depending upon host species (Blankespoor 1974; Graczyk 1991; Pérez-Ponce de León 1995). Paradoxically, the identity of the host often has a crucial role in taxonomic keys to parasitic taxa (e.g., Gibson 1996; Hoffman 1999; Niewiadomska 2002). These problems

are particularly acute for larval parasites, which differ morphologically from adults and have fewer easily distinguishable morphological features.

Identification of species is notoriously difficult in larval strigeids (Platyhelminthes: Trematoda), which are common and pathogenic parasites of freshwater fish around the world (Szidat 1969; Lemly and Esch 1984; Chappell 1995; Sandland and Goater 2001; Niewadomska 2002; Overstreet and Curran 2004; see Figure 3.1 for the strigeid life cycle). Molecular data offer a solution to species-identification problems and constitute the only practical means of obtaining species-level resolution in a field survey of parasites that includes larval stages. In this study, we use DNA sequences to distinguish species of larval strigeids, i.e., metacercariae, which are common in fish from the St. Lawrence River (Marcogliese *et al.* 2006).

Most studies using DNA to distinguish trematode species have employed sequences of non-coding spacer regions between nuclear genes encoding ribosomal subunits (internal transcribed spacers, or ITS) (Nolan and Cribb, 2005). A smaller number have employed sequences of mitochondrial DNA (Olson and Tkach 2005). The use of a particular region of mitochondrial DNA to distinguish species is the goal of the Barcode of Life, a campaign to increase the rate and reliability of species identification and discovery. There is little dispute that mitochondrial sequences or other molecular markers can be used to distinguish species; what is original (and controversial) in barcoding is the designation of a single molecular target for distinguishing species in all taxa, including undescribed species. The region advocated is also the principal molecular target used in this thesis, namely a 648-base-pair region near the 5` end of the gene

encoding cytochrome c oxidase I (COI) commonly referred to as the "DNA barcode" (Hebert *et al.*, 2003a, b).

Sequences from the barcode region of the COI gene have been used to distinguish described species and to detect unknown species in diverse groups of animals (Hebert *et al.* 2003a, b, 2004; Smith *et al.* 2005, 2007; Ward *et al.* 2005; Cywinska *et al.* 2006; Hajibabaei *et al.* 2006; Saunders 2008). However, this approach has been applied to only a few parasitic helminths (Elsasser *et al.* 2009; Ferri *et al.* 2009) and, prior to the publication of Chapters 1 and 2 of this thesis, no trematodes (Moszczynska *et al.* 2009; Locke *et al.* 2009). This is partly attributable to lack of available primer sequences that amplify the barcode region in trematodes. Chapter 1 addresses this situation with the development and evaluation of primers that amplify the barcode region in diplostomatids (Trematoda: Strigeida) as well as other flatworms.

Delineating undescribed species on the basis of DNA sequences from a single molecular marker is controversial (Kunz 2002; Desalle *et al.* 2005; Hickerson *et al.* 2006; Frézal and Leblois 2008). The utility of COI sequences for species distinction can be compromised by the presence of imperfect copies of mitochondrial sequences in the nuclear genome or in intracellular symbionts (e.g. van Herwerden *et al.* 2000; Hurst and Jiggins 2005; Benesh *et al.* 2006) and by misleadingly high levels of sequence divergence in geographically isolated populations (Irwin 2002; Kuo and Avise 2005). As a result, it is desirable to obtain sequences from a second marker. In this study, sequences of ITS were obtained from most strigeid species detected with COI data. The merits of ITS and COI sequences for distinguishing strigeid species are discussed in Chapters 1-3.

Molecular discrimination of strigeid species is used to address ecological and evolutionary questions in Chapters 2-4. Because they cannot be identified to the level of species morphologically, strigeid metacercariae are generally treated as a relatively small number of species that occur in a wide assortment of fishes (Margolis and Arthur, 1979; McDonald and Margolis, 1995; Gibson 1996; Hoffman 1999). Little is known of the true diversity and host specificity of metacercariae in strigeids or other groups of trematodes (Chappell 1995; Gibson 1996; Poulin 2007b). There are theoretical reasons for predicting both narrow and broad host specificity in metacercariae (e.g., Dogiel et al. 1966; de Meeûs 2000; Noble *et al.* 1989; Adamson and Caira, 1994). However, without a reliable means of identifying metacercariae to species, evidence related to these predictions cannot be obtained from field data. As a result, evolutionary and ecological mechanisms underpinning specificity in metacercariae can only be tested in laboratory settings, which may not accurately reflect natural processes (Poulin and Keeney 2007) and are logistically limited to smaller numbers of taxa than field studies.

Chapters 2 and 3 differ in taxonomic scope, but both employ molecular identifications to assess the diversity and host specificity of strigeid metacercariae. Chapter 2 is based on molecular data from a large sample of *Diplostomum* metacercariae, which parasitize the eyes of fish. Within the strigeids, *Diplostomum* continues to be a focus of diverse research (e.g., Chappell 1995, Rauch *et al.* 2006), including prior studies of molecular systematics locally and in Europe (Galazzo *et al.* 2002; Niewiadomska and Laskowski 2002). Chapter 3 takes a similar approach but encompasses seven strigeid genera that infect various tissues in the fish host. Both chapters are concerned with both the extent and mechanisms of host specificity in metacercariae. In surveys of parasites, it

is generally impossible to determine whether host specificity arises from physiological incompatibility between certain species of hosts and parasites (i.e., infection cannot occur under any circumstances) or from the ecological availability of certain species of hosts to parasite species (infections can be produced in a laboratory setting, but does not occur in nature because hosts do not encounter parasites) (Combes 2001). Lester and Huizinga (1977) suggested that variation in host specificity in species of *Diplostomum* might arise from differences in immunological activity faced by species inhabiting different tissues of the host. Molecular data obtained in this thesis are used to test this hypothesis in metacercariae of *Diplostomum* (Chapter 2) and other strigeids (Chapter 3). Mechanisms of host specificity (ecological availability versus physiological compatibility) are also evaluated in light of the consistency of parasite distributions across the replicate fish communities sampled in this study (Chapter 3).

Canadian host-parasite records have been used extensively in studies of community and evolutionary ecology of freshwater fish parasites (Poulin 2007b). However, there have been few attempts to duplicate large-scale findings from meta-analyses with original field data. In Chapter 4, the influence of spatial variation on parasite community similarity is compared to that of host phylogeny using both molecular and morphological parasite identifications.

Many studies have examined factors that predict similarity in parasite communities in freshwater fish (Leong and Holmes 1981; Bergeron *et al.* 1997; Fellis and Esch 2005a; Goater *et al.*2005), but few of the factors identified have been shown to have broad applicability. However, one trend that has repeatedly emerged is a tendency for parasite communities that are closer together in space to be more similar than distant

ones (Poulin and Morand 1999; Poulin 2003; Karvonen and Valtonen 2004; Fellis and Esch 2005a, b; Karvonen *et al.* 2005a).

Unlike the well-established influence of geographic distance, little work has been done on how parasite communities vary in more or less distantly related host species. Poulin (2005, 2010) found that the parasite communities of closely related fish species show, at most, a weak tendency to be similar. This is counter-intuitive because most parasites infect a small number of closely related host species (Poulin and Morand 2004; Poulin 2007b). At the community level, this host specificity should translate into closely related host species having similar parasite communities. Our dataset includes spatial replicates of fish species that are closely related but ecologically dissimilar and host species that are ecologically similar but phylogenetically distant, allowing a robust evaluation of the influence these two types of distance at the level of the parasite community.

Chapter 1 Development of primers for the mitochondrial cytochrome c oxidase I gene in digenetic trematodes (Platyhelminthes) illustrates the challenge of barcoding parasitic helminths*

Introduction

The digenetic trematodes (Platyhelminthes) comprise an estimated 24 000 species, many of which have yet to be described (Poulin and Morand 2004). Adults parasitize vertebrates and larval stages typically require a mollusk, usually a snail, as the first intermediate host. Most species also require a second intermediate host, which may be an invertebrate or a vertebrate, depending upon the species (see Figure 3.1). Some digeneans, such as *Schistosoma* spp. and *Clonorchis sinensis*, are important pathogens in humans, while many others have serious impacts on animal husbandry, aquaculture and wildlife management (Roberts and Janovy 2000). Accurate identification of these parasites is important for the diagnosis, treatment and control of pathogenic infections. It is also essential in broader studies relating to digenean diversity, distribution and ecology.

Species-level identification of most digeneans is based exclusively on adult morphology. Difficulties arise because they are small, soft-bodied, have few stable morphological characters and are subject to host-induced phenotypic variation (Blankespoor 1974; Graczyk 1991; Pérez-Ponce de León 1995). These problems are particularly acute for larval stages, which differ morphologically from the adults and, with fewer morphological features than adults, are virtually impossible to identify to

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* Moszczynska A, Locke SA, McLaughlin JD, Marcogliese DJ, Crease TJ. 2009. *Molecular Ecology Resources* 9, 75-82

species. Except for a few well-studied digeneans, larval stages can only be identified to the species level by experimental completion of the life cycle and subsequent study of the adult specimens. This is seldom a practical option and in most cases the identity of digenean larval stages can, at best, only be resolved to the generic level. In this context, molecular markers offer powerful and much-needed tools that have the potential to distinguish between morphologically similar species at any stage in their life cycle.

Many of the difficulties associated with identification of digenean species also apply to other parasitic helminths. Sampling adult helminths usually requires post-mortem examination of the host. However, it is also possible to obtain eggs, larvae, or segments (cestodes) in host excreta and many parasites (digeneans, nematodes) have free-living larval stages that can be collected directly from the environment. In these instances, it can be challenging to determine even higher order classification, particularly for the non-expert. Here again, molecular identification systems hold much promise.

There is ample evidence that sequences from the 5′ end of the cytochrome *c* oxidase I gene (COI), that is, the DNA barcode, can be used to identify species across a broad taxonomic range (Hebert *et al.* 2003a, b, 2004; Smith *et al.*2005, 2007; Ward *et al.* 2005; Cywinska *et al.*2006; Hajibabaei *et al.* 2006; Saunders 2008). To date, most studies employing molecular markers to distinguish digenean species have used the internal transcribed spacer (ITS) regions of ribosomal (r) DNA (Nolan and Cribb 2005; Olson and Tkach 2005). However, the few studies that did use COI sequences have shown that they distinguish congeneric digeneans more clearly than does the ITS region (e.g. Bowles *et al.* 1995; Morgan and Blair 1998; Vilas *et al.* 2005). Most of the digenean COI sequences used to date lie downstream of the barcode region (e.g. Bowles *et al.* 1995; Morgan and

Blair 1998; Morgan *et al.* 2005). However, it seems likely that sequences from the upstream barcode fragment may also provide a useful method for interspecific differentiation in this group.

A principal advantage of the barcoding approach is that use of a standardized marker, a ~600-nt fragment at the 5′ end of COI, ensures that sequence data are comparable across studies. A prerequisite to acquiring these data is the development of primers that amplify this region in the broadest possible range of taxa, thus allowing samples of unknown taxonomic affinity to be identified to species. The most widely applicable primers used in barcoding, those of Folmer *et al.* (1994), are very divergent from many of the published platyhelminth COI sequences (T. Crease, personal communication). Herein, we present the preliminary results of efforts to design primers that will recover barcode sequences in diverse platyhelminths, with particular focus on the Diplostomoidea (Trematoda: Strigeida). In addition, we assess the usefulness of sequences from a small region of the nuclear 18S rRNA gene as a preliminary screening tool in the barcoding of platyhelminth parasites.

Materials and methods

Primer development

Our initial aim was to design primers that would amplify a fragment corresponding to the barcode region at the 5' end of the COI gene in the broadest possible range of platyhelminth taxa. We aligned the barcode region of COI from complete platyhelminth mitochondrial genome sequences available from GenBank in 2006 to identify regions suitable for primer development. Seventeen sequences were aligned from

representatives of two cestode families, three digenean families and one turbellarian family (AF216697, M93388, AF216697, AF540958, AF216698, DQ157223, DQ157222, AF445798, AB107234, AY195858, AB107242, DQ089663, AF216699, AF297617, AF346403, AB049114, AF314223). Based on these sequences, we designed two degenerate primers for the barcode region of COI, MplatCOX1dF and MplatCOX1dR (Table 1.1) and attached 5′ M13 tails so that amplicons could be sequenced with M13 primers. The degenerate forward primer ends 8 nt upstream of the Folmer A primer, and the degenerate reverse primer ends 22 nt downstream of the Folmer B primer, so these primers amplify an additional 30 nucleotides compared to the Folmer primers.

Using sequences obtained with the degenerate primers, we designed a set of primers specific to the family Diplostomatidae, platy-diploCOX1F and platy-diploCOX1R (Table 1.1). The forward primer ends 92 nt downstream of the degenerate forward primer, and the reverse primer ends 66 nt upstream of the degenerate reverse primer, so these primers amplify 158 fewer nucleotides than our degenerate primers. Diplostomid-specific primers were developed for two reasons: first, the degenerate primers did not yield sequences in the majority of these specimens, and second, most specimens examined in this study belong to this family.

Specimen collection, polymerase chain reaction and sequencing

The majority of specimens used in this study came from various avian, amphibian and piscine hosts collected in the Saint Lawrence River basin in Quebec, Canada. Most samples were larval digeneans from fish hosts caught and frozen in 2006. Specimens were identified using morphological characters to the lowest possible taxonomic level, which was generally to genus in the case of larval specimens, and then stored in 95%

ethanol. DNA was extracted using either a glass-fibre extraction protocol (Ivanova *et al.* 2006), a QIAGEN DNeasy Extraction Kit, or a chloroform-isoamyl DNA extraction protocol (modified from Sambrook and Russell 2001).

We attempted to amplify COI from 571 digenean and 20 cestode specimens using the degenerate primers, and from 613 digeneans using the diplostomid-specific primers (Table 1.2). As most of the specimens were small, we suspected low quantities of DNA might affect polymerase chain reaction (PCR) success rates (Ivanova *et al.* 2006). As a positive control in initial samples, a fragment of the nuclear 18S rRNA gene from 90 digeneans and 20 cestodes was amplified and sequenced using both novel and previously published primers (Table 1.1).

For comparative purposes, we also attempted to amplify and sequence the ITS1 + 5.8S + ITS2 region of nuclear rDNA in 102 digenean specimens using previously published primers (Table 1.1).

All PCRs had a total volume of 25 μL and included 1×PCR buffer (20 mm Tris-HCl pH 8.4, 50 mm KCl), 2.5 mm MgCl2, 1.25 pmol of each primer (see Table 1.1), 50 μm of each dNTP, 0.6 U of Platinum *Taq* Polymerase (Invitrogen) and approximately 5 ng (18S PCR) to 50 ng (COI PCR) of DNA template. PCR conditions were 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplicons were visualized on 1% TAE agarose gels stained with ethidium bromide after which 1 μL was sequenced in a 12-μL reaction using 0.5 μL of the BigDye Terminator version 3.1 Cycle Sequencing mix (Applied Biosystems) and 10 pmol of primer. Amplicons generated with the degenerate COI primers were sequenced with the M13(– 21)F and M13(–27)R primers, while those

generated with the diplostomid-specific, 18S and ITS primers were sequenced with the primers used in the PCRs (Table 1.1). The 18S amplicons were only sequenced in one direction, with the 18S9F primer. Sequencing reactions were analysed on an ABI 3730 capillary sequencer (Applied Biosystems) in the Genomics Facility or the Canadian Centre for DNA Barcoding, both at the University of Guelph.

Sequences were assembled in Sequencher version 4.5 (Gene Codes Corporation) and manually edited. The aligned sequences were imported into MEGA 3.1 (Kumar *et al.* 2004) where pairwise sequence divergence estimates were generated using the Kimura 2-parameter model with pairwise deletion. The neighbour-joining algorithm (NJ) was then used to generate a phenogram from the resulting matrix of sequence divergence values. Some specimens analysed in this study were adult trematodes that were identified to species using morphological characters. For example, specimens of *Diplostomum baeri* and *Diplostomum indistinctum* were identified by Galazzo *et al.* (2002). However, most specimens were larval trematodes that could only be identified to genus. In these cases, species were distinguished based on sequence divergence levels and NJ phenograms. Many species distinguished in this way showed narrow host specificity (data not shown), which constitutes independent, additional evidence of their correspondence to species (Roberts and Janovy 2000; Poulin and Morand 2004). The divergence of COI sequences and host specificity of larval trematode species presented here are addressed in more detail in Chapters 2 and 3.

Results

Trematoda

There was no indication that the quantity or quality of DNA in our samples affected the performance of the COI primers in digeneans. Amplicons of the 18S gene were obtained in all 90 trematode samples assayed, including 38 in which COI did not amplify and 58 that failed to yield COI sequences (Table 1.2). In addition, an estimate of DNA quantity (specimen size) showed no relationship with the probability of sequencing success ($R^2 = 0.006$, $P = 0.824$). Only 17 of 102 samples yielded ITS sequences. The degenerate COI primers yielded amplicons of expected length in 314, and sequences in 231 of the 572 digenean samples assayed, including representatives of five genera in the Plagiorchiida, two genera in the Echinostomida, and eight genera in the Strigeida (Table 1.2). The diplostomid-specific COI primers yielded amplicons in eight of nine strigeidid genera tested, and 504 sequences from 610 samples assayed (Table 1.2).

Significantly fewer sequences were obtained with the degenerate COI primers than with the diplostomid-specific primers ($\chi^2 = 289$, d.f. = 1, $P < 0.0005$). In the most intensively sampled taxa, the degenerate COI primers yielded sequences in 27 of 34 (79%) *Diplostomum*, 65 of 128 (51%) *Posthodiplostomum*, 47 of 133 (35%) *Ornithodiplostomum*, 42 of 67 (63%) *Apatemon*, and 9 of 69 (13%) *Ichthyocotylurus* samples. Chromatogram trace signals obtained with the degenerate primers were often unclear for the first 100 to 150 nt at the 5′ end. The diplostomid-specific primers performed best on *Diplostomum* and *Tylodelphys*, in which informative sequences were obtained in 504 of 585 (86%) specimens assayed.

Overall, potentially informative sequences (> 150 nt in length) of the barcode region of COI were obtained in 706 of the 1138 (62%) digenean specimens with one or both sets of novel primers. Here we report sequences only from single representatives of each species detected. Bi-directionally sequenced fragments range from approximately 100 to 650 nt in length; the mean length is 442 nt.

The mean pairwise divergence between congeneric sequences from the barcode region is 19% (3.9–25%, Figure 1.1). Increasing COI sequence length does not affect species-level resolution. The topology of NJ trees based on ~480-nt sequences is identical to those based on fragments that are ~70 nt longer (data not shown). Few closely related specimens yielded both COI and ITS sequences. Barcode-region COI sequences have diverged by 15% and ITS sequences by 3.9% in two species of *Diplostomum* (ITS data from Galazzo *et al.* 2002), while two species of *Ornithodiplostomum* show 6.9% divergence in COI sequences and 4.2% divergence in ITS sequences (Figure 1.2). Short 18S sequences (~380 nt) fail to distinguish two genera (*Posthodiplostomum* and *Ornithodiplostomum*) but resolve higher taxa into distinct clusters (Figure 1.3).

Cestoda

Degenerate COI primers yielded PCR amplicons of expected length in eight of 20 samples comprising representatives of four genera in the Cyclophyllidea. Sequences were obtained from only a single sample (Hymenolepididae: *Cloacotaenia megalops,* Barcode accession FJ477192; data not shown in figures). Amplification of COI was unsuccessful in 11 other cyclophyllidean genera and in two pseudophyllidean genera (Table 1.2). PCR amplicons of the 18S gene were generated in all cestode samples, indicating that the

quality of DNA templates was unlikely to be the cause of unsuccessful COI amplification.

Discussion

It was clear from the initial alignment of flatworm COI sequences that the high level of sequence divergence would make it difficult to design primers that would successfully amplify this gene across the entire phylum. Indeed, the noisiness of the upstream portion of sequences acquired with the degenerate primers suggests that short, non-target amplicons were generated and sequenced along with the primary product. Nonetheless, the taxonomic range of our specimens that did yield amplicons or sequences, despite small sample sizes in many groups (Table 1.2), suggests that the degenerate primers may be useful for barcoding digeneans. In particular, they can be used to generate preliminary data for the design of barcode-region primers specific to lower taxonomic groups.

This approach was fruitful with the Diplostomidae, where amplification and sequencing success was much higher with our family-specific primers. Moreover, as sequences from the barcode region of COI continue to be published for additional platyhelminth taxa (e.g. Park *et al.* 2007), designing primers specific to lower taxa will become easier.

In other studies using molecular markers to distinguish species, primers specific to groups recalcitrant to more generalist primers have also been employed (e.g. Morgan *et al.* 2005; Smith *et al.* 2005, 2007; Ward *et al.* 2005; Hajibabaei *et al.* 2006; Zarowiecki *et al.* 2007; Saunders 2008). This approach requires that samples be partially identified in

order to determine which set of primers to use. If only the higher taxonomy of the sample is known, then a 'cocktail' of primers specific to lower taxonomic groups can be used (Ivanova *et al.* 2007). Alternatively, short (~100-nt) sequences generated with 'mini-barcode' primers can be used to assign samples to family and, in most cases, to species (Meusnier *et al.* 2008). However, it remains to be seen whether mini-barcode primers work with common metazoan parasite taxa such as trematodes and cestodes. With parasite samples, even higher taxonomy can be difficult to determine. In such cases, we suggest that a two-tiered approach to DNA barcoding using short sequences from nuclear rRNA genes could be used to identify a specimen to family. The advantage of these genes is that it is possible to design primers that are more truly 'universal' and that work reliably on DNA samples of low quality or quantity (Frézal and Leblois 2008). The 18S primers used here anneal in highly conserved regions of the gene in flatworms and also work in a broad range of taxa including arthropods (*Daphnia, Drosophila*) as well as vertebrates (lizards, humans) (T. Crease, personal communication). Moreover, longer sequences from rDNA subunits can resolve specimens to even lower taxonomic levels (e.g. Mariaux 1998; Olson *et al.* 2003). We are not advocating the particular 18S gene region or the primers we used for the purpose of preliminary specimen identification. Rather, we suggest that short sequences from nuclear rRNA genes or some other easily recovered target could be amplified with truly universal primers and used to screen samples of completely unknown taxonomic affinity. This would provide enough information to select from multiple primer sets for species-level barcoding using the standard region of the COI gene.

There is an additional matter to consider in designing primers for barcoding parasites. In general, it is desirable that barcoding primers amplify homologous COI sequences in the broadest possible range of taxa, but this may not be so for parasites. Parasites are often too small to subsample and are found within hosts, and it is therefore difficult to obtain uncontaminated tissue. As a result, truly universal primers may amplify DNA from both host and parasite, resulting in lower-quality or unusable sequence data.

Barcode-region COI sequences yielded good species-level resolution in our samples. For example, COI sequences showed better interspecific resolution than ITS sequences, although poor amplification rates with the ITS primers prevented us from comparing the two markers in many sibling taxa (Figure 1.2). There may be more optimal markers than COI for identifying species in *Schistosoma* (Zarowiecki *et al.* 2007) or other flatworms (Littlewood *et al.* 2008), but for barcoding purposes a single marker must be adopted to ensure comparability. Our data suggest that even with imperfectly universal primers, the standard region of COI is a practical target for barcoding digeneans.

From a practical perspective, sequences obtained with these novel primers have enabled us to better understand the diversity in a large sample of larval strigeids from fishes in the Saint Lawrence River. Although the parasites of freshwater fishes in Canada are relatively well studied (e.g. Gibson 1996), little is known of the diversity and biology of some of the most common pathogens, such as *Diplostomum* spp. (Galazzo *et al.* 2002). Cryptic species occur in these taxa and, as discussed in Chapters 2 and 3, a barcoding approach allows us to make considerable advances on questions concerning species diversity, geographical distribution, and host spectra of these problematic pathogens.

Table 1.1 Primers used

PCR primers used to amplify mitochondrial cytochrome *c* oxidase I (COI) and nuclear rRNA gene fragments from samples in the Platyhelminthes. The M13 tails [M13(-21)F and M13(-27)R] at the 5' ends of the degenerate COI primers (MplatCOX1d) are underlined. The Plat-diploCOX1 primers were designed to amplify members of the family Diplostomidae

Primer name	Primer sequence 5'-3'	Approximate product size (nt)	Region amplified
MplatCOX1dF	TGTAAAACGACGGCCAGTTTW CITTRGATCATAAG	650	COI barcode
MplatCOX1dR	CAGGAAACAGCTATGACTGAA AYAAYAIIGGATCICCACC		
Plat-diploCOX1F	CGTTTRAATTATACGGATCC	500	COI barcode
Plat-diploCOX1R	AGCATAGTAATMGCAGCAGC		
18S9F	TGATCCTGCCAGTAGCATATGC TTG[1]		
18S300R	TCAGGCTCCCTCTCCGG[2]	400	18S rDNA
18S637R	TACGCTATTGGAGCTGGAGTTA CCG[3]	600	18S rDNA
BR (F)	GTA GGT GAA CCT GCA GG[4]		
Dig11 (R)	GTG ATA TGC TTA AGT TCA GC[4]	1100	ITS1+5.8S+I TS2 rDNA
D1 (F)	AGG AAT TCC TGG TAA GTG CAA G[5]		
D2 (R)	CGT TAC TGA GGG AAT CCT GG[5]	1100	ITS1+5.8S+I TS2 rDNA

1. Crease (unpublished, based on sequence from *Daphnia pulex*, AF014011)
2. Medlin *et al.* 1988
3. Crease (unpublished, based on sequence from *Daphnia pulex*, AF014011)
4. Tkach *et al.* 2000
5. Hillis and Dixon 1991

Table 1.2 Amplification and sequencing results

Results of PCR and sequencing with primers used to amplify mitochondrial cytochrome *c* oxidase I (COI) and nuclear 18S rRNA gene fragments from samples in the Platyhelminthes

	18S primers		Degenerate COI primers		Diplostomid-specific primers	
Platyhelminthes	N amplified/ N samples	N sequences/ N assayed	N amplified/ N samples	N sequences/ N assayed	N amplified/ N samples	N sequences/ N assayed
Cestoda						
Cyclophyllidea						
Anoplocephalidae						
Anoplocephala perfoliata	1/1[1]	0/1	0/1	-	-	-
Moniezia expansa	1/1[1]	0/1	0/1	-	-	-
Dipylidiidae						
Dipylidium caninum	1/1[1]	1/1[1]	1/1	0/1	-	-
Hymenolepididae						
Cloacotaenia	1/1[1]	1/1[1]	1/1	-	-	-
Diorchis spp.	2/2[1,2]	2/2[1,2]	3/3	1/3	-	-
Diploposthe spp.	2/2[1,2]	2/2[1,2]	0/2	-	-	-
Drepanidotaenia	1/1[1]	1/1[1]	0/1	-	-	-
Fimbriaria	1/1[1]	1/1[1]	1/1	-	-	-
Microsomacanthus spp.	2/2[1,2]	2/2[1,2]	0/2	-	-	-
Retinometra	2/2[1,2]	2/2[1,2]	0/1	-	-	-
Sobolevicanthus	1/1[1]	1/1[1]	1/1	0/1	-	-
Taeniidae						
Taenia spp.	4/4[1]	1/4[1]	0/3	-	-	-
Pseudophyllidea						
Diphyllobothriidae						
Diphyllobothrium latum	1/1[1]	0/1	0/1	-	-	-
Spirometra	1/1[1]	-	0/1	-	-	-

Table 1.2. Continued

	18S primers		Degenerate COI primers		Diplostomid-specific primers	
	N amplified/ N samples	N sequences/ N assayed	N amplified/ N samples	N sequences/ N assayed	N amplified/ N samples	N sequences/ N assayed
Trematoda						
Echinostomida						
Echinostomatidae						
Echinostoma spp.	1/1[1]	1/1[1]	1/5	1/5	-	-
Sphaeridiotrema	1/1[1]	1/1[1]	1/1	0/1	0/1	-
Stephanoprora	1/1[1,2]	1/1[1] 0/1[2]	0/4	-	1/1	-
Paramphistomatidae						
Megalodiscus	-	-	5/11	3/5	-	-
Psilostomidae						
Ribeiroia	-	-	0/3	-	-	-
Plagiorchiida						
Allocreadiidae						
Bunodera sacculata	-	-	0/1	-	-	-
Megalogonia	-	-	2/2	2/2	-	-
Cryptogonimidae						
Centrovarium lobotes	2/2[1]	2/2[1]	1/2	0/1	-	-
Cryptogonimus chili	1/1[1]	1/1[1]	1/5	0/1	-	-
Gorgoderidae						
Gorgoderina	-	-	1/1	1/1	-	-
Phyllodistomum	-	-	0/1	-	-	-
Heterophyidae						
Apophallus	2/2[1]	2/2[1]	1/12	0/1	1/2	0/1
Cryptocotyle	-	-	5/8	5/5	-	-
Macroderoididae						
Alloglossidium gemimum	1/1[1]	1/1[1]	0/1	-	-	-
Choledocystus intermedius	1/1[1,2]	0/1[1,2]	1/1	-	-	-

Table 1.2. Continued

	18S primers		Degenerate COI primers		Diplostomid-specific primers	
	N amplified/ N samples	N sequences/ N assayed	N amplified/ N samples	N sequences/ N assayed	N amplified/ N samples	N sequences/ N assayed
Opecoelidae						
Plagioporus sinitsini	-	-	1/2	1/1	-	-
Plagiorchiidae						
Haematoloechus	-	-	6/10	0/6	-	-
Plagiorchis	-	-	5/5	5/5	-	-
Strigeida						
Azygiidae						
Azygia	-	-	7/15	7/7	-	-
Clinostomidae						
Clinostomum	2/2[1]	2/2[1]	8/9	6/6	2/2	-
Cyathocotylidae						
Cyathocotyle	1/1[1]	1/1[1]	0/1	-	1/1	-
Diplostomidae						
Alaria spp.	7/7[1] 6/6[2]	6/7[1] 1/2[2]	6/6	6/6	2/2	0/2
Fibricola	4/4[1]	4/4[1]	0/4	-	1/4	0/1
Ornithodiplostomum spp.	24/24[1] 15/17[2]	21/24[1] 2/2[2]	68/133	47/68	8/8	0/8
Posthodiplostomum	15/15[1] 13/13[2]	12/15[1] 1/2[2]	83/128	65/83	4/4	0/4
Uvulifer	-	-	7/14	0/7	-	-
Diplostomum	10/10[1] 5/5[2]	9/10[1] 1/1[2]	30/34	27/29	492/548	471/492
Hysteromorpha	-	-	7/9	4/7	-	-
Tylodelphys scheuringi	2/2[1] 1/1[2]	2/2[1] 1/1[2]	0/2	-	34/37	33/34
Strigeidae						
Apatemon (tetracotyle)	9/9[1] 1/1[2]	9/9[1] 1/1[2]	46/67	42/46	-	-
Ichthyocotylurus	2/2[1]	2/2[1]	21/69	9/21	0/2	-
Schistosomatidae	-	-	0/3	-	-	-

[1] 18S9F and 300R
[2] 18S9F and 18S637R
- = not assayed

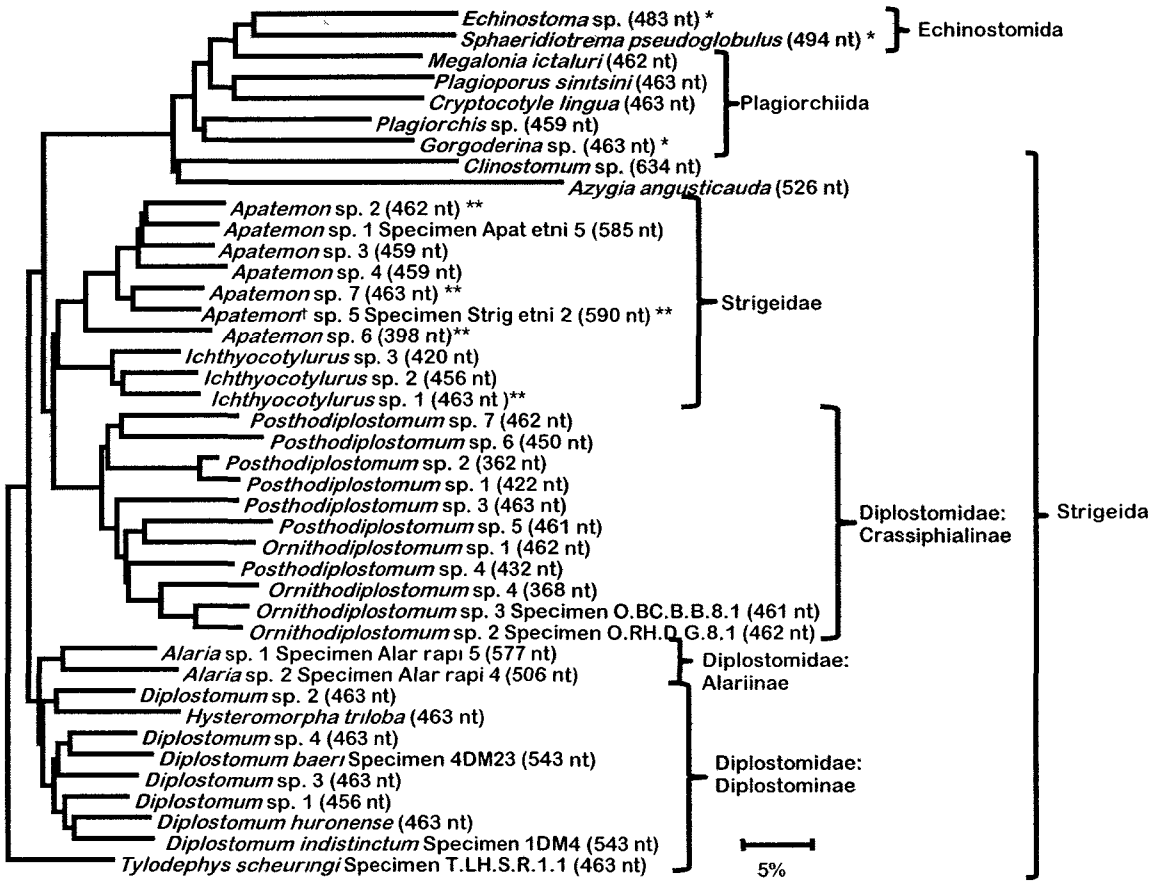

Figure 1.1 Neighbour-joining phenogram of cytochrome c oxidase I sequences

Neighbour-joining tree of barcode-region COI sequences for representatives of trematode taxa obtained in the present study. Sequences are from a single representative among multiple specimens analyzed, with the exception of *Echinostoma* sp., *Plagioporus sinitsini*, *Gorgoderina* sp. and those marked with an asterisk, in which case a COI sequence was obtained from only a single specimen. Sequences have been deposited in GenBank under accession numbers FJ477181-FJ477191 and FJ477193-FJ477223. † Tentative identification. As explained in the Results of Chapter 3, the identifications of species with a double asterisk were revised subsequent to the publication of this chapter.

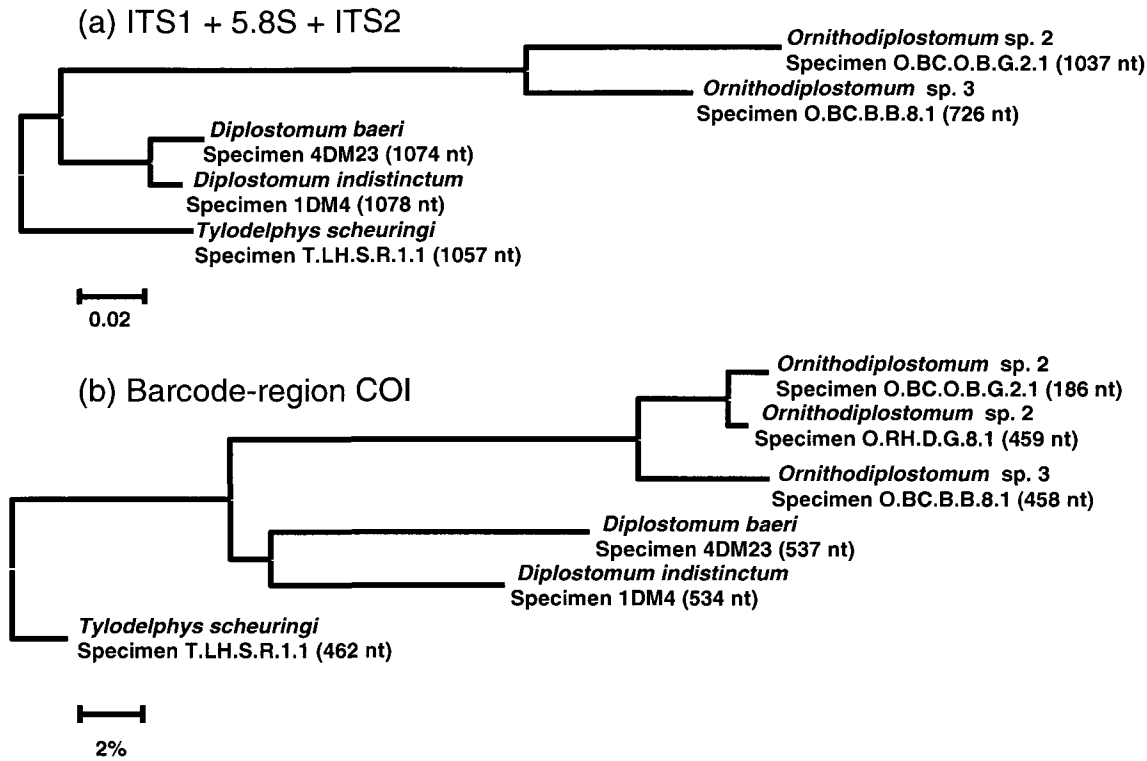

Figure 1.2 Neighbour joining phenograms of cytochrome c oxidase I and internal transcribed spacer sequences

Comparison of taxonomic resolution provided by rDNA and COI sequences from specimens in the trematode family Diplostomidae. (a) Neighbour-joining tree of ITS1+5.8S+ ITS2 rDNA sequences from closely related specimens barcoded in this study. (b) Neighbour-joining tree of barcode-region COI sequences from the same specimens as (a). Sequences of rDNA have been deposited in GenBank under accession numbers FJ469594-FJ469596; rDNA sequences from *D. baeri* (AY123042) and *D. indistinctum* (AY123043) are from Galazzo *et al.* (2002).

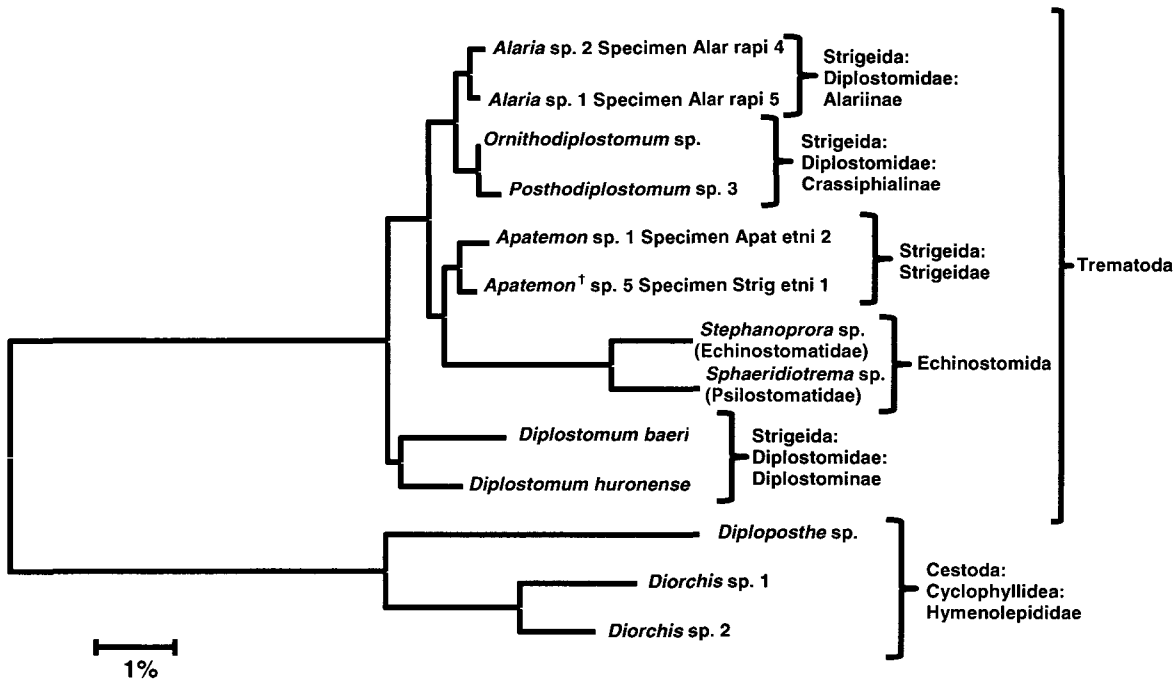

Figure 1.3 Neighbour joining phenogram of 18S rRNA sequences

Neighbor-joining tree generated from partial 18S rRNA gene sequences (380 nt) from representatives of the class Trematoda (subclass Digenea), and the class Cestoda in the phylum Platyhelminthes. Sequences have been deposited in GenBank under accession numbers FJ469581-FJ469593. † Tentative identification, revised to *Conodiplostomum* sp. 5 subsequent to publication of this chapter (see Chapter 3).

Chapter 2 Diversity and specificity in *Diplostomum* spp. metacercariae in freshwater fishes revealed by cytochrome c oxidase I and internal transcribed spacer sequences*

Introduction

Metacercariae of digenetic trematodes belonging to the genus *Diplostomum* parasitize freshwater fishes and (rarely) amphibians throughout the Holarctic. Most *Diplostomum* spp. infect tissues of the eye, with 10 – 100 parasites per eye common in younger fishes while thousands may occur in older hosts (Shostak *et al.* 1987; Marcogliese and Compagna 1999). Metacercariae can impair vision resulting in alteration of feeding and other behaviours and lead to poor growth. Diplostomids have been implicated in losses of wild and farmed fish both by direct mortality and by increasing the susceptibility of infected fish to predation (reviewed by Chappell *et al.* 1994).

Chappell (1995) stressed the need for a reliable means of species-level identification of diplostomid metacercariae because pathology, monitoring and control measures might vary among species. Reliable identification of *Diplostomum* spp. is also important in studies dealing with susceptibility and resistance (Karvonen *et al.* 2004a, b, 2005b), infectivity (Voutilainen and Taskinen 2009), host manipulation (Rintamäki-Kinnunen *et al.* 2004; Seppälä *et al.* 2005a, b, c, 2006), population genetics (Rauch *et al.* 2005) and evolutionary biology (Rauch *et al.* 2006, 2008), especially where multiple species may infect a single site within a host.

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* Locke SA, McLaughlin JD, Dayanandan S, Marcogliese DJ. 2009. *International Journal for Parasitology*, online early, doi: 10.1016/j.ijpara.2009.08.012

Identification of diplostomid species is difficult at all stages of the life cycle but is particularly so for the metacercaria. The simple morphology of these small, soft-bodied larval stages varies with parasite age and host species and is not reliable for discriminating between species (Graczyk 1991; Niewiadomska and Szymański 1991). Consequently, many early records of different *Diplostomum* spp. in fishes from Europe are based on erroneous identifications (Niewiadomska 1996). In Canadian fishes, *Diplostomum spathaceum* has been the most commonly reported species, accounting for 37 of 117 records compiled by Margolis and Arthur (1979) and McDonald and Margolis (1995), but records of this and other species are also questionable (Gibson 1996; Marcogliese *et al.* 2001a, b; Galazzo *et al.* 2002).

Molecular data provide an alternative to morphology for identifying species. In contrast to morphology, DNA sequences can be used to identify species at all developmental stages, a major advantage for studying parasites with complex life cycles (Criscione *et al.* 2005). Molecular studies of *Diplostomum* spp. boundaries have compared sequences of the internal transcribed spacer (ITS) regions of rDNA in species that differ in morphology and life history (Galazzo *et al.* 2002; Niewiadomska and Laskowski 2002). Five European species and three North American species of *Diplostomum* can be distinguished based on divergence in ITS sequences ranging from 1.6% to 4.6% (Galazzo *et al.* 2002; Niewiadomska and Laskowski 2002). However, ITS data are ineffective for distinguishing a pair of *Diplostomum* spp. from Europe (Niewiadomska and Laskowski 2002) and provide ambiguous information for what may be a pair of cryptic species in North America (Galazzo *et al.* 2002). Sequence divergence

can also be used to detect undescribed species, although the practice of delimiting new species based on rDNA sequence divergence alone has been criticized (Kunz 2002).

While most studies using molecular markers to distinguish trematode species have used ITS data, a smaller number of studies have employed sequences of the cytochrome *c* oxidase I (COI) gene (Nolan and Cribb 2005; Olson and Tkach 2005). Platyhelminth COI sequences are more variable than those of the ITS regions and are predicted to provide a greater number of markers useful for species-level identifications (Vilas *et al.* 2005). The first 650 bp of COI have been widely used as a DNA "barcode", an approach that has proven particularly useful for discriminating sibling species in numerous higher taxa (Hebert *et al.* 2003a, b; Frézal and Leblois 2008). Moszczynska *et al.* (2009) developed diplostomid-specific primers flanking the barcode region of COI and presented preliminary data showing that these sequences discriminate between congeneric species in the Diplostomoidea. Here again, however, delimiting species solely on the basis of COI data is controversial (Desalle *et al.* 2005; Hickerson *et al.* 2006; Frézal and Leblois 2008).

A principal goal of this study was to characterize the diversity of *Diplostomum* in fishes of the St. Lawrence River, Canada, using sequences from the barcode region of COI. We employed ITS data to corroborate our COI-based findings, compare the species resolution of the two markers, and link our specimens to *Diplostomum* spp. in our study area that were identified by Galazzo *et al.* (2002) based on adult morphology and ITS data. Galazzo *et al.* (2002) also found that the ITS1 data then available showed that the North American *Diplostomum* fauna is separate from and basal to European species. Herein we re-examine the evolutionary relationship of the European and North American

Diplostomum spp. based on a larger, more dispersed and more diverse sample of sequences.

A further objective was to evaluate hypotheses relating to the specificity of *Diplostomum* metacercariae. A variety of accounts indicate that metacercariae of *Diplostomum* spp. differ in host specificity (Chappell *et al.* 1994; Niewiadomska 1996), but this is impossible to study with conventional approaches in a field setting and taxonomic uncertainty makes it difficult to generalize from the literature. Laboratory studies also show that *Diplostomum* metacercariae are tissue-specific, but beyond a tendency to infect ocular and cerebral tissue, the site-specificity of different species has not been directly compared. In addition, if *Diplostomum* spp. are site-specific, there may be differences in the host-specificity of species inhabiting different tissues. Lester and Huizinga (1977) suggested that species in immunologically privileged sites such as the lens may be able to infect a larger and more diverse spectrum of hosts because of weaker selection for host specificity. Species inhabiting other tissues confront more vigorous immune responses that may vary in different host taxa, and should therefore specialize on a smaller number of more closely related hosts.

Materials and methods

Specimen collection

The majority of *Diplostomum* specimens examined in this study were metacercariae taken from 705 fishes representing six species, namely bluntnose minnow (*Pimephales notatus*), golden shiner (*Notemigonus crysoleucas*), pumpkinseed (*Lepomis gibbosus*), rock bass (*Ambloplites rupestris*), johnny darter (*Etheostoma nigrum*) and

yellow perch (*Perca flavescens*). About 20 fish from each species were caught with a beach seine at each of six sampling localities in the St. Lawrence River near Montreal, Quebec, Canada (Figure 2.1; for sample sizes see Figure 2.2 and also Table 3.2). Fishing took place during a 3-week period in June 2006 and only 1+ year-class, or the smallest sized fish available, were collected. By simultaneously sampling young hosts, we ensured that fish were local to collection localities (not migrants) and had been exposed to the same pool of *Diplostomum* spp. for the same amount of time and during the same period. Metacercariae were also taken from smaller numbers of hosts in 15 other fish species and one amphibian species collected at the same and additional localities in the St. Lawrence River. Fish were killed in an overdose of clove oil/ethanol solution (50 mg/10 ml/L H_2O) and frozen until dissection. Although only *Diplostomum* is discussed herein, each entire fish was examined for parasites using standard techniques (Marcogliese 2002; www.eman-rese.ca/eman/ecotools/protocols/freshwater/parasites). Upon removal from the host, metacercariae were fixed in 95% or absolute ethanol for molecular work. It was not feasible to extract and sequence DNA from all ~5,000 metacercariae encountered and different strategies were adopted to sample metacercariae in different tissues of the various host species. Molecular identification was attempted for all metacercariae in low-abundance niches (the lenses of pumpkinseed, johnny darter, rock bass and yellow perch, and non-lens tissues of bluntnose minnows) and in a sub-sample of parasites in higher-abundance niches (the vitreous humour of yellow perch and lenses of bluntnose minnows and golden shiners).

Adult *Diplostomum* specimens were obtained by feeding fish, or the eyes thereof, to 16 ring-billed gull (*Larus delawarensis*) chicks raised from eggs collected at a colony

near Montreal (see also Galazzo *et al.* 2002). Others were taken from herring gulls (*Larus argentatus*) collected at the Delta Waterfowl Research Station, Delta, Manitoba, Canada and ring-billed gulls culled for pest-control at a landfill north of Montreal. One specimen was obtained from a great black-backed gull (*Larus marinus*) found dead in Kangiqsualujjuaq, Northern Quebec, Canada. Thus, sampling was geographically concentrated in the St. Lawrence River, but specimens from other areas of North America were also collected (Figure 2.1).

Specimen vouchering, DNA extraction and sequencing

DNA was extracted from 497 metacercariae and 135 adult *Diplostomum*. Amplification and sequencing of COI was performed using the diplostomid-specific primers and PCR protocols of Moszczynska *et al.* (2009) at the Canadian Centre for DNA Barcoding (CCDB) in Guelph, Ontario. The ITS region was amplified and sequenced in 21 of these specimens at the CCDB following protocols and using the primers described by Galazzo *et al.* (2002).

For most of the adult *Diplostomum* specimens, only the forebody or a portion thereof was used for DNA extraction (see Galazzo *et al.* 2002). The remainder of the specimen was stained in dilute acetocarmine, cleared in clove oil, and mounted in Canada balsam on a slide to serve as a morphological voucher for sequence data. After further study, these voucher specimens will be deposited at the Canadian Museum of Nature (Ottawa, ON, Canada). In addition, we obtained COI sequences from archived DNA of three vouchered adult specimens each of *Diplostomum indistinctum* and *Diplostomum baeri* studied by Galazzo *et al.* (2002). Some adult specimens were not sub-sampled and were consumed entirely during DNA extraction.

Molecular data analysis

In most cases, forward and reverse trace files provided unambiguous sequences that required only minor post-alignment editing using Sequencher 4.8 (2008, Gene Codes, Ann Arbor MI, USA). In approximately 150 cases, it was necessary to edit trace files before they could be aligned to generate a bi-directional sequence, and in a few (< 30) cases, sequences were obtained from single forward or reverse traces; manual editing of the latter sequences was done in Contig Express (Invitrogen, Carlsbad, CA, USA). Barcode-region COI nucleotide sequences were aligned using ClustalX (Thompson *et al.* 1994). ITS trace files were edited and aligned in Contig Express (Invitrogen, Carlsbad, CA, USA). Distances between sequences of COI and ITS were analyzed using the Kimura 2-parameter model of base substitution (Kimura, 1980) and visualized in Neighbour-Joining (NJ) trees constructed with Mega 4.0 (Tamura *et al.* 2007) with complete deletion of gaps. Maximum parsimony (MP) analysis of ITS sequences obtained in this study and from GenBank was carried out in Mega 4.0 (Tamura *et al.* 2007). Within- and between species divergence in ITS and COI sequences were calculated based on uncorrected p-distances using Mega 4.0 (Tamura *et al.* 2007).

COI sequences were obtained from over 600 individual *Diplostomum* specimens, but herein we present only 28 representative sequences. These include sequences from all species detected and from 21 specimens that yielded both COI and ITS sequences.

Species discrimination and identification

Species were distinguished and, where possible, identified based on at least one of multiple lines of evidence: (i) clustering in NJ trees of sequences of COI and (ii) of rDNA; (iii) patterns of host and (iv) infection-site-specificity observed in species-clades distinguished by sequences; (v) comparison of ITS sequences with those published by Galazzo *et al.* (2002), Niewiadomska and Laskowski (2002) and on GenBank; (vi) comparison with COI sequences obtained from specimens identified by Galazzo *et al.* (2002), and (vii) preliminary examination of adult voucher specimens.

Specificity for host species and tissues

All *Diplostomum* metacercariae encountered in this study occurred in cerebral or ocular tissues and were recorded as being in the lens, vitreous humour or brain. No distinction was made between the sub-retinal space, retina and vitreous humor because metacercariae in these sites often detach in frozen material. The relationship between the infection site of *Diplostomum* spp. and host specificity was examined in two ways. The numbers of specialist and generalist species occurring in a low-immune-activity site (the lens) were compared with the numbers of species in higher-immune-activity sites (non-lens tissues) using a Fisher's exact test. *Diplostomum* spp. were classified as either host specialists or generalists based on the species richness of the host spectrum (i.e., host range) and the mean taxonomic distance between hosts (Poulin and Mouillot 2003). The latter index, S_{TD}, is a measure of the phylogenetic diversity of hosts that varies relatively little with sample size (Poulin and Mouillot 2003). S_{TD} was calculated as the number of steps in a Linnaean hierarchy to a common taxon, based on the classifications of genus, subfamily, family, order, superorder and class in Nelson (2006), such that, for example,

bluntnose minnow (*Pimephales notatus*) was six units from yellow perch and two units from golden shiner (*Notemigonus crysoleucas*) (see inset to Figure 4.5). The effect of lens infection on the host-specificity of metacercariae was also assessed using analysis of covariance (ANCOVA) and multiple regression in order to account for sampling effort. To further ensure that rarity of species did not produce overestimates of host specificity, records of identified metacercariae were randomly reshuffled with host records to calculate the probability of obtaining the observed host specificity by chance using Resampling Stats for Excel (Statistics.com, LLC, Arlington, Virginia, USA). Analyses relating to specificity included only species of *Diplostomum* in which metacercariae were identified from more than one individual host.

Results

Species discrimination and identification

Approximately 5,000 *Diplostomum* spp. metacercariae were found during the course of this study. Among six intensively sampled host species (Figure 2.2), metacercariae were most abundant in the vitreous humour of yellow perch and lenses of bluntnose minnow and golden shiner (high-abundance niches). Metacercariae were much less abundant in the lenses of pumpkinseed, rock bass, johnny darter and yellow perch and in non-lens sites of all hosts except yellow perch (low-abundance niches) (Figure 2.2). Sequences from the barcode region of COI were obtained from 497 of 632 metacercariae in which sequencing was attempted, representing 77% of metacercariae found in low-abundance niches and 5% of those found in high-abundance niches.

Sequences of COI up to 610 bp long were obtained from 632 *Diplostomum* specimens, of which 21 also yielded ITS sequences. Based on sequences and patterns in host- and tissue-specificity, 12 species of *Diplostomum* were detected in the St. Lawrence River (Table 2.1). On average, sequences of both markers varied at least 10 times more between species than within species. The mean interspecific divergence in COI sequences among the 632 specimens and 12 species was 12% (9.9 - 15.1%); mean intraspecific divergence was 0.5% (0.16 - 0.87%) (uncorrected p-distance). Among the 21 specimens and nine species for which ITS data were obtained, and including sequences from an additional 13 specimens reported by Galazzo *et al.* (2002) (total $n = 34$), mean interspecific divergence was 1.97% (0.35 - 3.27%) and mean intraspecific divergence was 0.05% (0 - 0.29%) (uncorrected p-distance, pairwise deletion of gaps).

Intraspecific variation was strongly correlated with the number of specimens sampled for both COI ($r = 0.959$, $P < 0.0005$, $n = 7$) and ITS sequences ($r = 0.925$, $P = 0.025$, $n = 5$) (Figure 2.3). Among 612 COI sequences over 300 bp in length and with less than 1% ambiguous bases, the relationship is best described by a semi-logarithmic curve indicating a threshold of intraspecific variation of less than 1%. Among the 34 ITS sequences obtained in this study and from Galazzo *et al.* (2002), there is no indication that a threshold of intraspecific variation has been reached.

The relationship between intraspecific variation and specimens sampled per species could also be a result of variations in effective population size of different *Diplostomum* spp. To evaluate this, intraspecific divergence in COI was examined in 1,000 rarefactions of samples ($n = 20$) for five species in which more than 20 sequences were obtained. Intraspecific variation in rarefied samples was much the same as when all

samples were included (Figure 2.3). *Diplostomum baeri* appears to be among the most common species in the system (Figure 2.2), and it would therefore be expected have the largest effective population size and show the highest intraspecific variation in COI sequences. Over 60% of the *Diplostomum* metacercariae in all six well sampled host species occurred in vitreous humour of yellow perch, and almost all specimens identified in this site were *D. baeri*. However, the intraspecific divergence of *D. baeri* does not indicate a larger effective population size than other species.

We linked our specimens with the identifications of previous authors through sequence comparison. Nine specimens had rDNA sequences identical or highly similar to consensus sequences of *Diplostomum huronense* or *D. indistinctum* published by Galazzo *et al.* (2002) or to variants of the consensus sequences described by the authors (Tables 2.1 and 2.2). Sequences of the barcode region of COI from archival DNA from specimens of *D. indistinctum* and *D. baeri* studied by Galazzo *et al.* (2002) were identical or highly similar to sequences obtained from specimens collected in the present study (Figure 2.4). One specimen had ITS sequences identical to a variant of *D. huronense* described by Galazzo *et al.* (2002) and several specimens had ITS sequences identical or similar to three specimens that could not be identified by those authors but which resembled *D. indistinctum* (hereafter referred to as *Diplostomum* sp. 1) (Table 2.2, Figure 2.4). The ITS1 sequences of *Diplostomum* sp. 3 differ from those reported for *Diplostomum pseudospathaceum* by Niewiadomska and Laskowski (2002) at two of 573 positions (one deletion and an A-T transversion). No other ITS sequences obtained in the present study matched sequences in GenBank, and there appear to be misidentifications

in that database, particularly among records originating from European material (Figure 2.4).

Diplostomum sp. 1 was clearly distinguishable from *D. indistinctum* based upon NJ analysis of COI (Figure 2.4a) but not of ITS (Figure 2.4b) sequences. One *D. indistinctum* specimen had ITS sequences identical to those in three specimens of *Diplostomum* sp. 1 (see shaded rows in Table 2.2). Including gaps, there were 13 variable sites in ITS sequences between these two species, but no variations were diagnostic. One notable source of variation was a four-base indel (AAAC) in ITS1. In seven of nine specimens examined in the present study, electropherogram traces at these positions had secondary peaks in which deleted or inserted bases could be seen (Figure 2.5). Downstream from these positions, the frame-shifted sequence was visible in secondary peaks. In effect, this indel varied not just between but also within individuals in seven of nine specimens. Two of the nine specimens showed no intra-individual variation. A single *D. indistinctum* specimen showed only the deletion, while a single specimen of *Diplostomum* sp. 1 showed only the insertion. It is possible these represent genotypes native to the two parental species, and the double peaks (or polymorphism) shared by other specimens in both species are a signature of hybridization. Double peaks indicative of intra-individual variation were not observed at other variable positions in ITS traces of the two species, but the overall lack of synapomorphy is also consistent with hybridization between the two species (Table 2.2). Among the seven remaining *Diplostomum* spp. from which ITS data were obtained, there were additional, species-specific variations at the positions of this four-base indel, but only one case of intraspecific variation, and no double peaks in electropherogram traces.

Abundance and specificity of *Diplostomum* spp.

Ten out of the 12 species detected occurred in at least one of the six well-sampled host species (Table 2.1). Most species of *Diplostomum* occurring outside the lenses of hosts were rare, while most species inside the lens were relatively abundant (Figure 2.6, Table 2.1). However, there was no significant relationship between abundance and infection site (two-tailed Fisher's exact test, $P = 0.08$).

The specificity of *Diplostomum* spp. for hosts and tissues was evaluated in seven of the 12 species encountered. In the remaining five (*Diplostomum* spp. 5 - 9), only a single metacercaria was recovered from one individual host (Table 2.1, Figure 2.6), and specificity could not be assessed. These species were therefore excluded from tests of the relationship between host specificity and infection site.

Sequence-based identifications showed seven *Diplostomum* spp. are strictly site specific with respect to the lens (Figure 2.6). Five species were recovered only from the lenses of 258 individual hosts. Two species were found only outside the lens, mainly in the vitreous humour, of 24 individual hosts. Differences in host specificity were obvious in the case of abundant *Diplostomum* spp. and could be inferred for less common species (Table 2.1, Figure 2.6). Four lens-inhabiting species (*D. huronense*, *Diplostomum* spp. 1, 3 and 4) were abundant and occurred in numerous, distantly related host taxa and are clearly host generalists. *Diplostomum baeri* was also common, and a host specialist, representing 63 of 64 metacercariae identified from the vitreous humor of 20 yellow perch. *Diplostomum indistinctum* was recovered from the lenses of only a few host individuals in two species that are distantly related, and it is therefore considered a lens-inhabiting generalist that is rare in our study area. *Diplostomum* sp. 2 is also rare in that

only seven specimens were encountered, all of them outside the lenses of four individual hosts in two species, bluntnose minnow and spottail shiner (*Notropis hudsonius*), which are closely related (Leuciscinae). In a randomization of all records of hosts and identified metacercariae, all seven *Diplostomum* sp. 2 specimens were only assigned to either or both of their actual host species once in 10,000 trials, meaning that the probability of observing this degree of host specificity by chance is exceedingly small. *Diplostomum* sp. 2 is therefore considered a host specialist that is rare in the study area.

The occurrence of five generalist species in the lenses (*D. huronense*, *D. indistinctum*, *Diplostomum* spp. 1, 3 and 4) and two host-specialist species outside this site (*D. baeri* and *Diplostomum* sp. 2) indicates that generalists are significantly more likely to infect the lens (two-tailed Fisher's exact test, $P = 0.048$).

Sampling effort had effects on one of the two measures of host specificity. Based on the seven species of *Diplostomum* in which metacercariae were identified from more than one individual host (i.e., species other than *Diplostomum* spp. 5-9), host range was higher in *Diplostomum* spp. in which more metacercariae were identified ($r = 0.880$, $P = 0.004$, $n = 7$) from a larger number of fish ($r = 0.896$, $P = 0.003$ $n = 7$). In contrast, there was no relationship between either of these measures of sampling effort and S_{TD} (number of identified metacercariae $P = 0.055$; number of hosts in which metacercariae were identified $P = 0.387$). When either measure of sampling effort was taken into account, lens-inhabiting *Diplostomum* spp. did not infect a significantly larger number of species (ANCOVA, $P > 0.10$). In contrast, a significantly elevated S_{TD} indicated the hosts of lens-inhabiting species are more diverse than those of non-lens inhabiting species, taking into account both the number of metacercariae and hosts (ANCOVA, $F_{1,3} = 26.961$, $P =$

0.014). In backward multiple regression, lens infection predicted S_{TD} after the two measures of sampling effort were removed (lens infection coded as 0, non-lens coded as 1; $\beta = -4.44$, $t_5 = -5.989$, $P = 0.002$) and accounted for 88% of the variance in S_{TD} ($F_{1,5} = 35.873$, $P = 0.002$).

Phylogeography of *Diplostomum* spp.

A maximum parsimony (MP) analysis of ITS sequences from European and North American *Diplostomum* spp. did not support an evolutionary history associated with continental separation of the species (Figure 2.7). Two European species (*D. pseudospathaceum* and *Diplostomum paracaudum*) were closely allied with seven North American species, while two North American species fell within a clade composed of six nominal European species.

Discussion

Twelve species of *Diplostomum* are clearly distinguished from each other by barcode-region COI sequences. Notably, COI sequences resolve these sympatric *Diplostomum* spp. more clearly than ITS sequences, which are more frequently used to discriminate congeners in this and other trematode taxa (Nolan and Cribb 2005). The relatively small number of ITS sequences compared (34) most likely explains why no threshold in intraspecific variation is apparent (see Figure 2.3). However, the small number of ITS sequences compared makes the near overlap in mean intraspecific variation (as high as 0.29%) and mean interspecific variation (as low as 0.35%) all the more striking. In contrast, in a sample of over 600 specimens, the maximum mean

intraspecific variation in COI sequences is an order of magnitude smaller than mean interspecific variation, and appears to have reached a threshold (see Figure 2.3).

Rarefactions indicate samples of ~20 individuals are adequate for characterizing mean intraspecific variation in COI sequences in *Diplostomum*. Curiously, intraspecific variation in COI sequences of *D. baeri* is not particularly elevated even though it is by far the most abundant species in our samples, and might therefore be expected to have the largest effective population size. This species is only found in yellow perch, however, and this absolute host specificity may have a homogenizing effect on genetic variation by funneling metacercariae into a smaller number of definitive hosts that specialize on this fish as a prey item. Although lens-inhabiting species appear to be less abundant, they occur in a wide variety of fish taxa, and populations may be fragmented in different definitive hosts foraging differentially on fish intermediate hosts. On the other hand, the low sequence variation of *D. baeri* could also result from a recent increase in abundance, such that variation has not had time to become fixed in the population.

Although one molecular marker outperforms the other, the use of multiple markers and a large sample size provides stronger evidence on which to base taxonomic conclusions. The status of the 12 species is well supported by COI and, in most cases, rDNA sequences and host and/or tissue specificity. We avoided a potential source of error by obtaining sequences of nDNA and mtDNA from the same individual specimens. This lends confidence to the finding of unexpectedly similar or identical ITS sequences in *D. indistinctum* and *Diplostomum* sp. 1, which have distinctive COI haplotypes. Two processes could produce this lack of divergence in rDNA sequences. If the two species recently diverged, mutations unique to each species are expected to become fixed sooner

in maternally inherited mtDNA because it has one quarter of the effective population size of nDNA (Avise 1994). Recently diverged species may also share ancestral polymorphisms, such as the four-base indel in ITS1 observed in these two species. Based on parsimony and similarity-based analyses of both ITS and COI data (data not shown for the MP analysis of COI), and adult morphology (Galazzo *et al.* 2002), *Diplostomum* sp. 1 and *D. indistinctum* do in fact appear to be closely related. On the other hand, introgressive hybridization could also produce these results by mixing the nuclear but not the mitochondrial genomes. The secondary peaks in DNA traces support this scenario (see Sonnenberg *et al.* 2007 for another example of using double peaks in DNA traces to detect hybridization). Intra-individual variation in ITS1 sequences has been recorded in other well-studied trematodes (reviewed by Blair 2006). The presence of alternative, monomorphic character states restricted to either species, as well as specimens in both species displaying a combined character state, suggests hybridization between species (Figure 2.5), but further work is needed to evaluate this possibility.

Identical ITS1 sequences were also reported by Niewiadomska and Laskowski (2002) for *Diplostomum parviventosum* and *D. spathaceum*, which have distinctive morphological and life-history characteristics. Nolan and Cribb (2005) questioned this finding in light of other, distinctive sequences for *D. spathaceum* in GenBank (we presume they were referring to the *D. spathaceum* UK clade in Figure 2.4b). However, it is clear that some European material from which sequences present in GenBank were obtained is misidentified (Figure 2.4b), and the reliability of otherwise unpublished record is difficult to evaluate. By analogy, then, the work of Niewiadomska and Laskowski (2002) and our own provide mutual support for the possibility of separate

Diplostomum spp. having identical ITS sequences, and our data suggest two possible mechanisms, hybridization and recent divergence.

Since congeners can have identical or highly similar ITS sequences, it is difficult to gauge the significance of the near identity of the ITS1 sequences of *Diplostomum* sp. 3 from Quebec with *D. pseudospathaceum* from Poland. There does not appear to be an evolutionary separation of the European and North American fauna (cf. Galazzo *et al.* 2002), and there is no obvious reason why *D. pseudospathaceum* might not be present in Quebec, given the mobility of avian definitive hosts. This question will be further considered in a future communication dealing with the taxonomy of these species based on the morphology of adult voucher specimens. However, even if *Diplostomum* sp. 3 and *D. pseudospathaceum* prove to be morphologically indistinguishable, the situation would not be resolved. Since similar ITS sequences do not necessarily denote conspecificity, this may be another case of cryptic species indistinguishable with rDNA. The most efficient route to answering this question lies in the COI sequences of *D. pseudospathaceum*.

The 12 species of *Diplostomum* we encountered in a single river represent an unprecedented number, but it probably underestimates the true diversity of this genus in our study area. Almost all of 497 metacercariae from which COI sequences were obtained belonged to five common species, and the remaining seven species were represented by seven or fewer individuals. The existence of additional rare species seems likely, and common species in host taxa that were not examined may also exist in the study area. We sampled only six fish species intensively ($81 < n < 137$) and 14 others in small numbers ($2 < n < 20$). This is a small sample of the 56 species and 19 families of

fishes encountered in surveys of the same reach of this river between 1995 and 2005 (Y. Reyjol, Ministère des Ressources Naturelles et de la Faune du Québec, unpublished data), particularly in light of the fact that *Diplostomum* has been reported from over 100 species of fish (Chappell 1995).

The data allow us to make predictions about *Diplostomum* spp. that we did not encounter in the study area. The marginally significant tendency of lens-inhabiting species to be abundant and of non-lens-inhabiting species to be rare, together with the link between host-specificity and infection site, suggest that undetected species are mostly specialists from non-lens tissues of hosts other than the six that were intensively sampled. This prediction accords with the slower rate of discovery documented for host-specific parasites of Canadian freshwater fishes (Poulin and Mouillot 2005b). The data also suggest that unknown *Diplostomum* metacercariae collected from non-lens tissues of distantly related host taxa are likely to be different species. In consequence, a promising direction for future work in the study area would be to target eight additional fish species in which *Diplostomum* metacercariae have been reported from the vitreous humour (Marcogliese and Compagna 1999; Marcogliese *et al.* 2001a, b).

Although an incomplete census, the 12 *Diplostomum* spp. detected represent surprising diversity for a 150-km reach of a single river. Using traditional approaches, between five and seven species of *Diplostomum* have been recognized in all of Canada's approximately 200 species of fish (Margolis and Arthur, 1979; McDonald and Margolis 1995; Gibson 1996). Furthermore, estimates of global diversity of the genus ranging from 27 (Chappell *et al.* 1994) to 40 species (Niewiadomska 1996) appear low in light of our

data. Ignoring nomenclature, close inspection of Figure 2.4 shows a comparable number of species-clades (~25) in samples from the United Kingdom, Poland and Quebec.

Our results shed light on previous studies in the St. Lawrence River. The *D. indistinctum*-like group encountered by Galazzo *et al.* (2002) has been shown to be a distinct, cryptic species (*Diplostomum* sp. 1). We identified *Diplostomum* spp. in all fish hosts studied by Marcogliese *et al.* (2001a, b) except walleye (*Sander vitreum*). We have made preliminary examinations of adult voucher specimens of the common species in our system, and based on the difficulty of distinguishing adult voucher specimens from morphology alone (data not shown) and the relative abundance of species in our survey (data not shown), it is likely that what was reported by Marcogliese *et al.* (2001a, b) as *D. indistinctum* was actually *Diplostomum* sp. 1, and that *D. huronense* was *Diplostomum* sp. 4.

Overall, we found lower host specificity among *Diplostomum* spp. That, as metacercariae, infect the lens, a non-vascularized compartment in which immune activity is actively limited in vertebrates (Stein-Streilein and Streilein 2002; Sitjà-Bobadilla 2008). When sampling effort was taken into account, lens-inhabiting diplostomids were found to infect an array of host species that is more diverse but not significantly more species rich. However, host range is relatively sensitive to sampling effort (Poulin and Mouillot 2003) and, as illustrated by *Diplostomum* sp. 2 and *D. indistinctum*, it may not be a good indicator of host specificity in rarer species of parasites.

Immunological privilege is thought to have evolved in certain tissues to prevent the severe pathological changes that can be caused by immune reactions such as inflammation in these sites (Stein-Streilein and Streilein 2002). All tissues colonized by

diplostomids are immunologically privileged, which may explain why diplostomid metacercariae do not form protective cysts, unlike most strigeid metacercariae. It is unlikely that parasites in these tissues are "hidden" from the host; rather, the immune response is subject to active, localized restraint (Stein-Streilein and Streilein 2002). However, the lens capsule forms an additional, physical barrier for cell-mediated immune responses (Shariff *et al.* 1980), which are important in defenses against larval helminths (Meeusen and Balic 2000; Kreider *et al.* 2007). Our results indicate that the comparatively robust immune responses outside the lens may exert increased selective pressure resulting in increased host specificity in non-lens *Diplostomum* spp. Among lens-inhabiting species, lower host specificity augments available habitat and the number of routes to definitive hosts. Immune activity in tissues other than the infection site is less relevant here because *Diplostomum* spp. complete migration to eye tissues within 24 h of penetrating a host, before a full, specific immune response is mounted (Chappell 1995; Voutilainen and Taskinen 2009; but see Rauch *et al.* 2006).

There is some support for the effect of the lens on host specificity in the literature. For example, Niewiadomska (1996) lists seven non-lens-inhabiting, host-specialist *Diplostomum* spp., two lens-inhabiting host specialists, and three lens-inhabiting generalists (nine additional species are unclassified). In itself, this points to a significant association (Fisher's exact test, $P = 0.045$) and the validity of the two exceptional lens-inhabiting host-specialist species is questionable (Niewiadomska 1996). However, as Niewiadomska (1996) and others (Chappell *et al.* 1994; Chappell 1995) have cautioned, the confused taxonomy of *Diplostomum* spp. makes it difficult to form an accurate account of host-parasite relationships among metacercariae in this group. We believe this

is the first demonstration of a relationship between host specificity and immunologically privileged sites in *Diplostomum* or, to our knowledge, in other parasites, but the idea is not new. Lester and Huizinga (1977) suggested it to explain experimentally verified specificity of *Diplostomum adamsi* from the retina of yellow perch, in light of the broad host spectra reported in other species from the lens.

The difficulties of studying *Diplostomum* are not unique to this particular parasite. There is growing recognition that the rigorous application of molecular techniques to taxonomy is important for many aspects of parasitology (Criscione *et al.* 2005; Olson and Tkach 2005; see also Bortolus 2008). Overall, our work reinforces other recent studies (e.g., Vilas *et al.* 2005) showing COI sequences are more suitable for trematode species discrimination than ITS markers. These results underscore the utility of using multiple markers for studying *Diplostomum* spp. boundaries, as well as the desirability of maintaining morphological voucher specimens (standard practice in DNA barcoding) and the taxonomic uncertainty that continues to plague this genus.

Table 2.1 *Diplostomum* species in St. Lawrence River fishes

Basis of discrimination and/or identification of *Diplostomum* spp. collected from hosts in the St. Lawrence River, Quebec, Canada, numbers of parasites from which COI sequences were obtained, and numbers and species of intermediate hosts in which metacercariae were identified

Species of *Diplostomum*	Basis of identif-ication or discrim-ination	*n* specimens sequenced (COI)		*n* intermediate host individuals (intermediate host species[d]) infection site
		Adults	Metacercariae	
baeri	1, 3, 5, 6, 7	11[a]	64	20 (1) Vitreous humour
huronense	1, 2, 4, 5	4	19	15 (1-5) Lens
indistinctum	1, 2[b], 3, 4, 5	3[a]	3	3 (5, 6) Lens
sp. 1	1, 2[b], 4, 5	93	173	81 (1-5,7-18) Lens
sp. 2	1, 2, 5, 6	0	7	4 (3, 7) Vitreous humour, brain
sp. 3	1, 2, 5	8	19	15 (1-4, 8, 9, 19[c]) Lens
sp. 4	1, 2, 5	16	209	120 (1-9, 11, 12, 14, 16, 20, 21) Lens
sp. 5	1	0	1	1 (1) Vitreous humour
sp. 6	1	0	1	1 (3) Vitreous humour
sp. 7	1	0	1	1 (3) Vitreous humour
sp. 8	1, 2	0	1	1 (19) Eye[c]
sp. 9	1, 2	0	1	1 (20) Vitreous humour

[a] three specimens from Galazzo *et al.* (2002)
[b] equivocal resolution of internal transcribed spacer (ITS) sequences for *D. indistinctum* and *Diplostomum* sp. 1
[c] specific tissue of eye not determined in *Rana pipiens*
[d] 1=*Perca flavescens*, 2=*Notemigonus crysoleucas*, 3=*Pimephales notatus*, 4=*Ambloplites rupestris*, 5=*Catostomus commersonii* , 6=*Apollonia melanostomus*, 7=*Notropis*

hudsonius, 8=*Lepomis gibbosus*, 9=*Micropterus salmoides*, 10=*Micropterus dolomieui*, 11=*Etheostoma nigrum*, 12=*Notropis spilopterus*, 13=*Notropis atherinoides*, 14=*Moxostoma macrolepidotum*, 15=*Ictalurus nebulosus*, 16=*Labidesthes sicculus*, 17=*Fundulus diaphanus*, 18=*Pomoxis nigromaculatus*, 19=*Rana pipiens*, 20=*Percina caprodes*, 21=*Carpiodes cyprinus*

Key to Basis of discrimination or identification:

1. Distinctive cytochrome c oxidase I (COI) sequence(s), Genbank accession: GQ292475-GQ292502; see also project DIPLO at www.barcodinglife.org
2. Distinctive ITS sequence(s), Genbank accession: GQ292503-GQ292523; see also project DIPLO at www.barcodinglife.org
3. COI sequence obtained from specimens studied by Galazzo *et al.* (2002)
4. ITS sequence match with sequence from Galazzo *et al.* (2002)
5. Site specificity
6. Host specificity
7. Adult morphology

Table 2.2 Alignment of internal transcribed spacer sequences from *Diplostomum* sp. 1 and *D. indistinctum*

Variable sites between *Diplostomum* sp. 1 and *Diplostomum indistinctum* in a 1,079-bp alignment of internal transcribed spacer (ITS) sequences from 15 specimens collected from hosts in the St. Lawrence River, Quebec, Canada. Blank spaces indicate no sequence for that position and dashes indicate indels. Variable sites contain no purely diagnostic characters but some variations are private (sensu Desalle *et al.* 2005) to one species or the other, allowing some specimens to be assigned to one species or the other when combinations of characters are considered. However, specimens 1, 4, 8, G and H (shaded rows) are identical, although the cytochrome c oxidase I (COI) sequences of 1 and 8 clearly differ (see Figure 2.4a). Specimens are numbered as in Figure 2.4: specimens 1-9 were obtained in the present study and A-I are sequences from Galazzo *et al.* (2002). W = A or T, R = A or G

5' position:	9, 10	25	155-158	308	574	599	612	845	Species and source of specimens
Specimen									
1		-	A A A C	T	A	G	A	A	
2		-	- - - -	C	A	G	A	G	
3		-	A A A C	C	W	G	A	A	
4		-	A A A C	T	A	G	A		*Diplostomum* sp. 1, present study
5		-	- - - -	C	A	G	A	R	
6		-	- - - -	C	A	G	A	A	
7	A C	-	A A A C	T	A	G	A	A	
8		-	A A A C	T	A	G	A	A	*Diplostomum indistinctum*, present study and Galazzo *et al.* (2002)
9		-	A A A C	T	C	A	C	A	
A-C	C A	A	A A A C	T	C	A	C	A	
G, H	C A	A	A A A C	T	A	G	A	A	*D. indistinctum*-like specimens of Galazzo *et al.* (2002)
I	C A	A	- - - -	C	A	G	A	G	

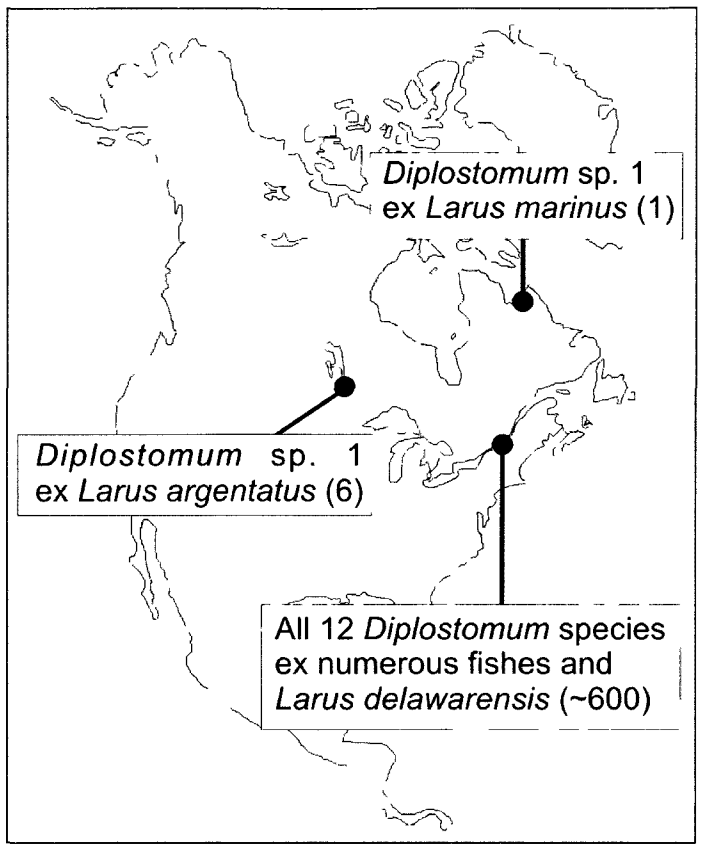


Figure 2.1 Sampling localities

Sampling localities and species of *Diplostomum* detected, with the number of specimens from which cytochrome c oxidase I sequences were obtained in parentheses. Most specimens were metacercariae collected from fish in the St. Lawrence River near Montreal, Quebec, Canada. Thus, the apparent differences in regional diversity are attributable to vastly unequal sampling effort.

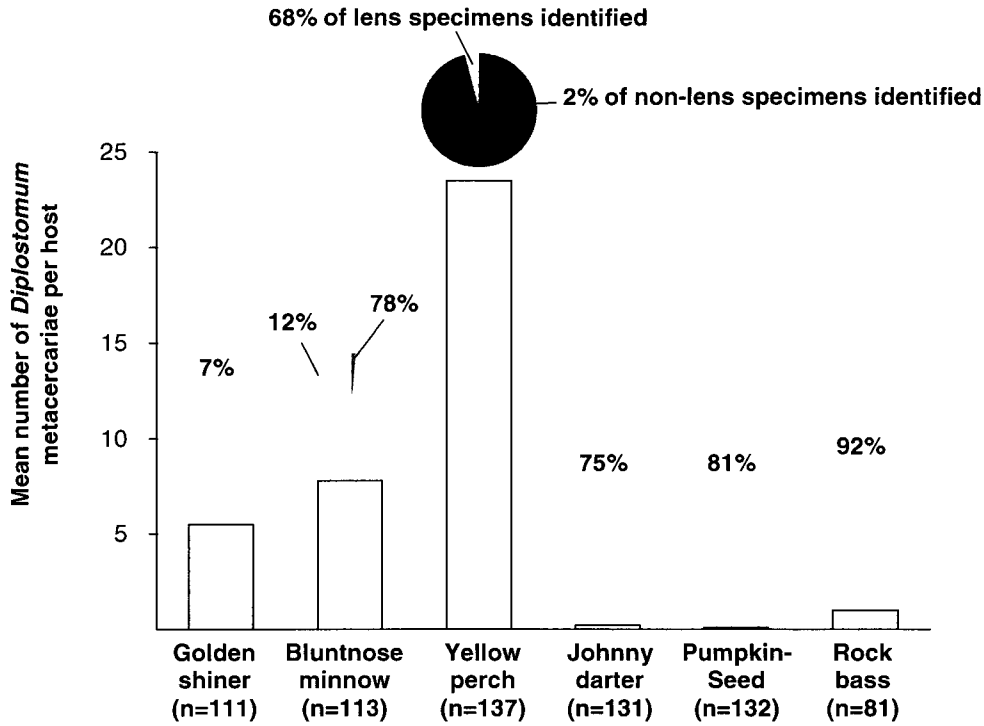


Figure 2.2 Abundance of *Diplostomum* spp. metacercariae in six focal fish hosts

Mean abundance of all *Diplostomum* spp. metacercariae in six fish species from the St. Lawrence River, Canada. The numbers of fish examined are in parentheses. Pie charts show the proportion of all metacercariae found in the lenses (light grey) and other tissues (dark grey) in each host. The percentages of specimens identified with cytochrome c oxidase I (COI) sequences in lens and non-lens tissues are indicated. For example, metacercariae were most abundant in yellow perch, and most of the metacercariae in yellow perch were in the vitreous humour. Only a small percentage of the vitreous-humour metacercariae in yellow perch were identified and almost all were *Diplostomum baeri*. Among the less abundant metacercariae in the lenses of yellow perch, COI sequences were obtained from 95 of 138 (68%) specimens.

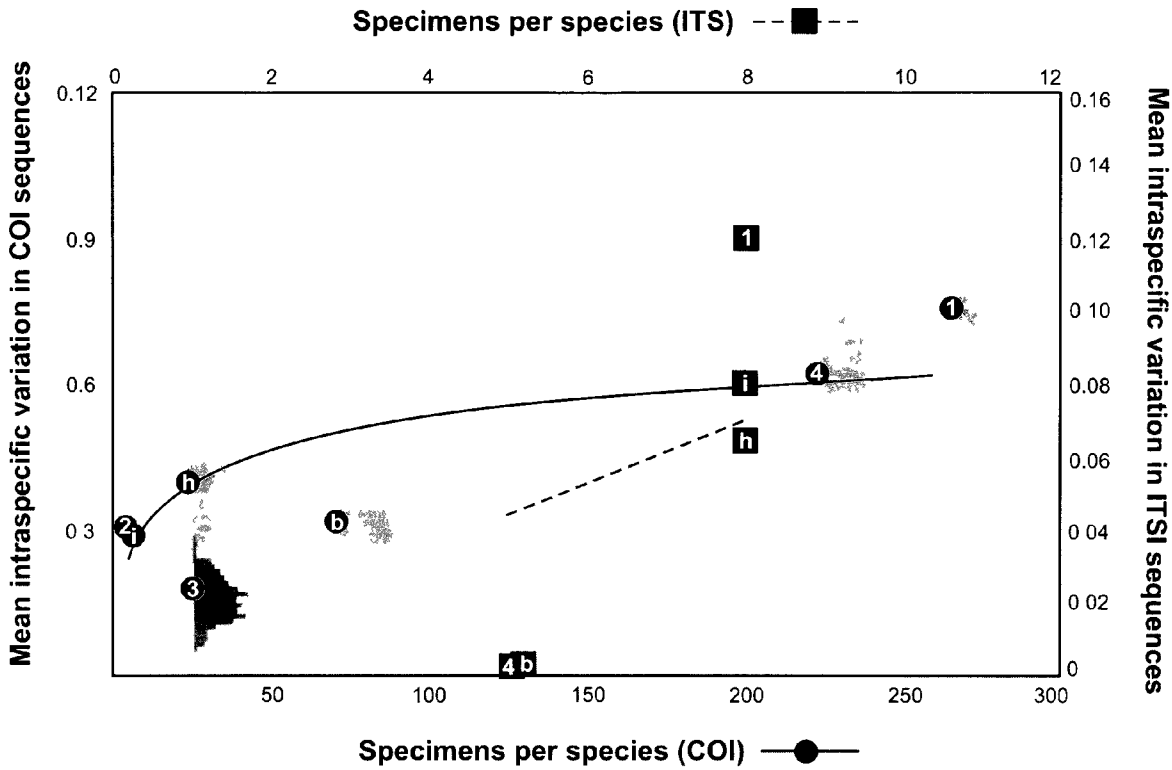


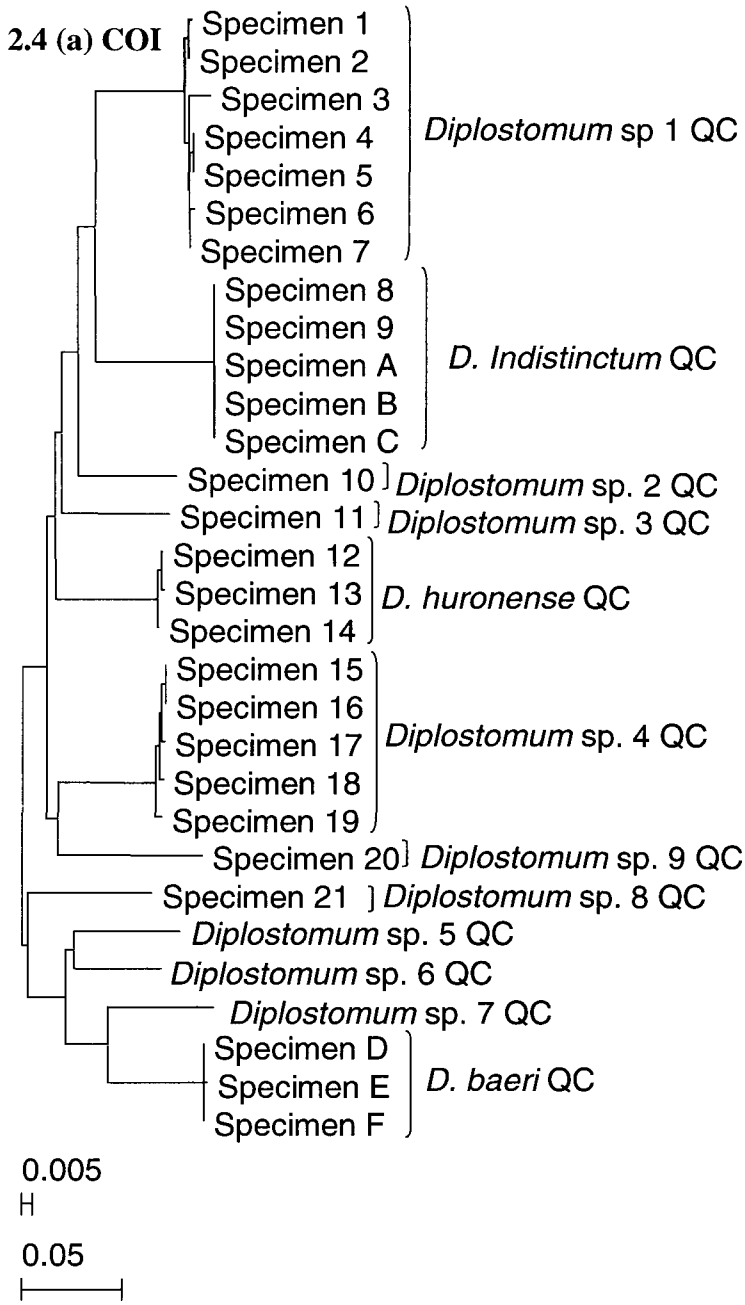
Figure 2.3 Intraspecific sequence variation in mitochondrial and nuclear markers in *Diplostomum* spp.

Mean intraspecific variation in DNA sequences in comparison with number of specimens sampled per species of *Diplostomum* from the St. Lawrence River, Canada. Within-species variation in cytochrome c oxidase I (COI) and internal transcribed spacer (ITS) sequences increases with the number of specimens sequenced. Taking into account that the line must past through the origin, a logarithmic curve indicating a threshold of intraspecific variation provides the best fit to the COI data ($r^2 = 0.69$, $P = 0.020$) and a straight line provides the best fit to the ITS data ($r^2 = 0.75$, $P = 0.040$). ITS data are from 21 specimens obtained in this study and from 13 specimens studied by Galazzo *et al.* (2002). Variations are uncorrected p-distances with pairwise deletion of gaps. The vertical frequency distributions on COI data points represent the mean intraspecific divergence obtained when sequences (including lower-quality sequences) in each species were rarefied to n = 20 for 1,000 iterations. Data points are labelled for each species: b =

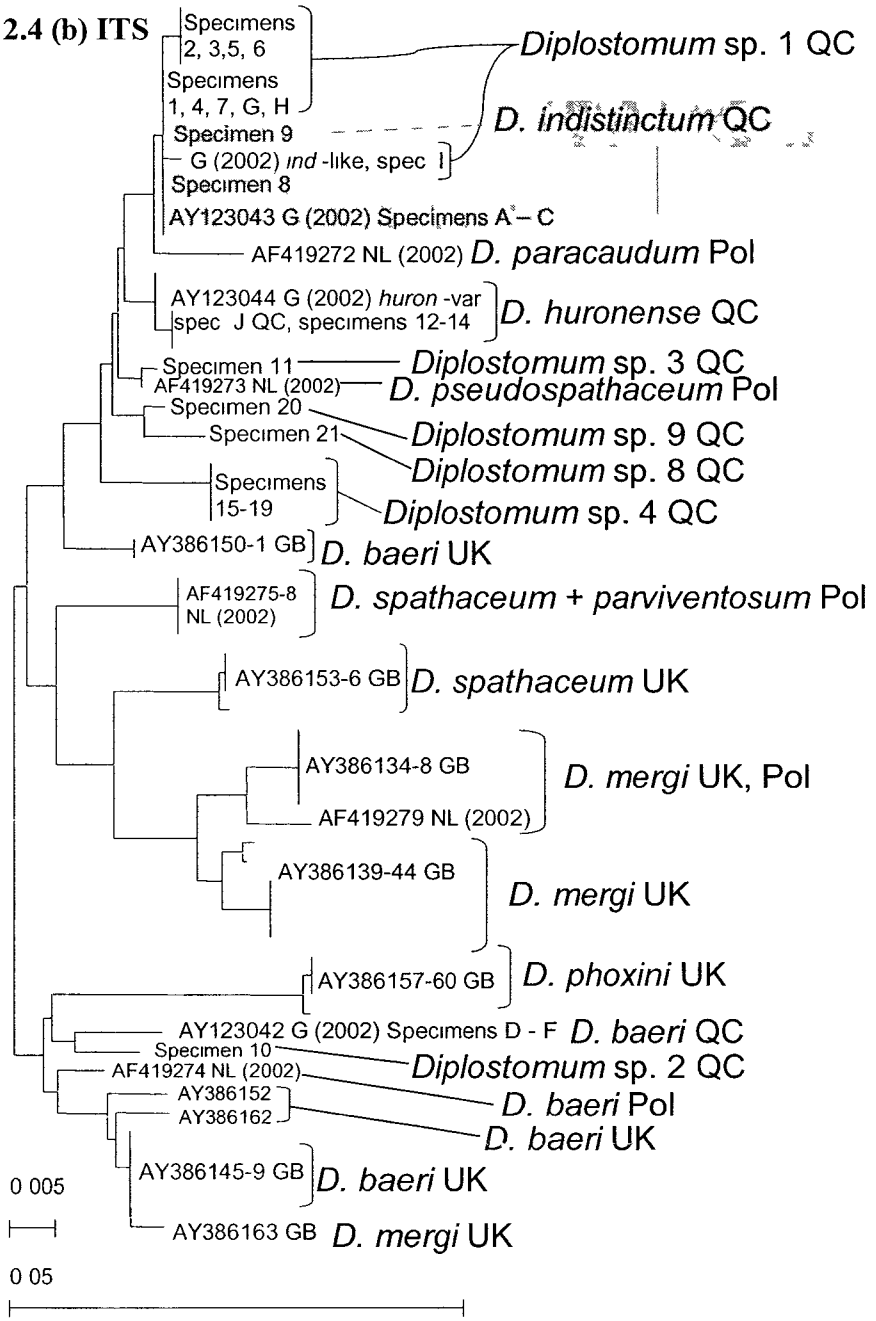
Diplostomum baeri, h = *Diplostomum huronense*, i = *Diplostomum indistinctum*, 1=*Diplostomum* sp. 1, 2 = *Diplostomum* sp. 2, 3 = *Diplostomum* sp. 3, 4 = *Diplostomum* sp. 4

Figure 2.4 Neighbour joining phenograms of cytochrome c oxidase I and internal transcribed spacer sequences from *Diplostomum* spp.

Neighbour-joining analyses of Kimura 2-parameter distances (Kimura, 1980) of (a) cytochrome c oxidase I (COI) sequences and (b) internal transcribed spacer (ITS) sequences from *Diplostomum* specimens (pairwise deletion of gaps) collected from aquatic vertebrates in the St. Lawrence River, Canada, and available in GenBank. Specimens 1–21 were collected during this study, and specimens A–F were collected and identified by Galazzo *et al.* (2002). Both COI and ITS sequences were obtained from specimens 1–21 but only COI sequences were obtained from *Diplostomum* spp. 5, 6 and 7. Geographic origins of specimens are denoted by QC = Quebec, Pol = Poland and UK = United Kingdom. The sources of other ITS sequences are indicated by NL (2002) = Niewiadomska and Laskowski 2002; G (2002) = Galazzo *et al.* (2002); and GB = published only in GenBank. Only consensus sequences were published in GenBank by Galazzo *et al.* (2002), but three variations from the ITS consensus sequences of *Diplostomum indistinctum* (labelled "ind.-like, spec. G–I" above) and one from that of *Diplostomum huronense* ("huron.-var. spec. J") were described in sufficient detail to be reproduced.


2.4 (a) COI
Specimen 1
Specimen 2
Specimen 3
Specimen 4
Specimen 5
Specimen 6
Specimen 7
Diplostomum sp 1 QC
Specimen 8
Specimen 9
Specimen A
Specimen B
Specimen C
D. Indistinctum QC
Specimen 10
Diplostomum sp. 2 QC
Specimen 11
Diplostomum sp. 3 QC
Specimen 12
Specimen 13
Specimen 14
D. huronense QC
Specimen 15
Specimen 16
Specimen 17
Specimen 18
Specimen 19
Diplostomum sp. 4 QC
Specimen 20
Diplostomum sp. 9 QC
Specimen 21
Diplostomum sp. 8 QC
Diplostomum sp. 5 QC
Diplostomum sp. 6 QC
Diplostomum sp. 7 QC
Specimen D
Specimen E
Specimen F
D. baeri QC
0.005
0.05

2.4 (b) ITS


Specimens 2, 3,5, 6
Specimens 1, 4, 7, G, H
Specimen 9
G (2002) ind -like, spec I
Specimen 8
AY123043 G (2002) Specimens A – C
Diplostomum sp. 1 QC
D. indistinctum QC
AF419272 NL (2002) D. paracaudum Pol
AY123044 G (2002) huron -var spec J QC, specimens 12-14
D. huronense QC
Specimen 11
AF419273 NL (2002)
Diplostomum sp. 3 QC
D. pseudospathaceum Pol
Specimen 20
Specimen 21
Diplostomum sp. 9 QC
Diplostomum sp. 8 QC
Specimens 15-19
Diplostomum sp. 4 QC
AY386150-1 GB
D. baeri UK
AF419275-8 NL (2002)
D. spathaceum + parviventosum Pol
AY386153-6 GB
D. spathaceum UK
AY386134-8 GB
AF419279 NL (2002)
D. mergi UK, Pol
AY386139-44 GB
D. mergi UK
AY386157-60 GB
D. phoxini UK
AY123042 G (2002) Specimens D - F
D. baeri QC
Specimen 10
AF419274 NL (2002)
Diplostomum sp. 2 QC
AY386152
AY386162
D. baeri Pol
D. baeri UK
AY386145-9 GB
D. baeri UK
AY386163 GB
D. mergi UK
0 005
0 05

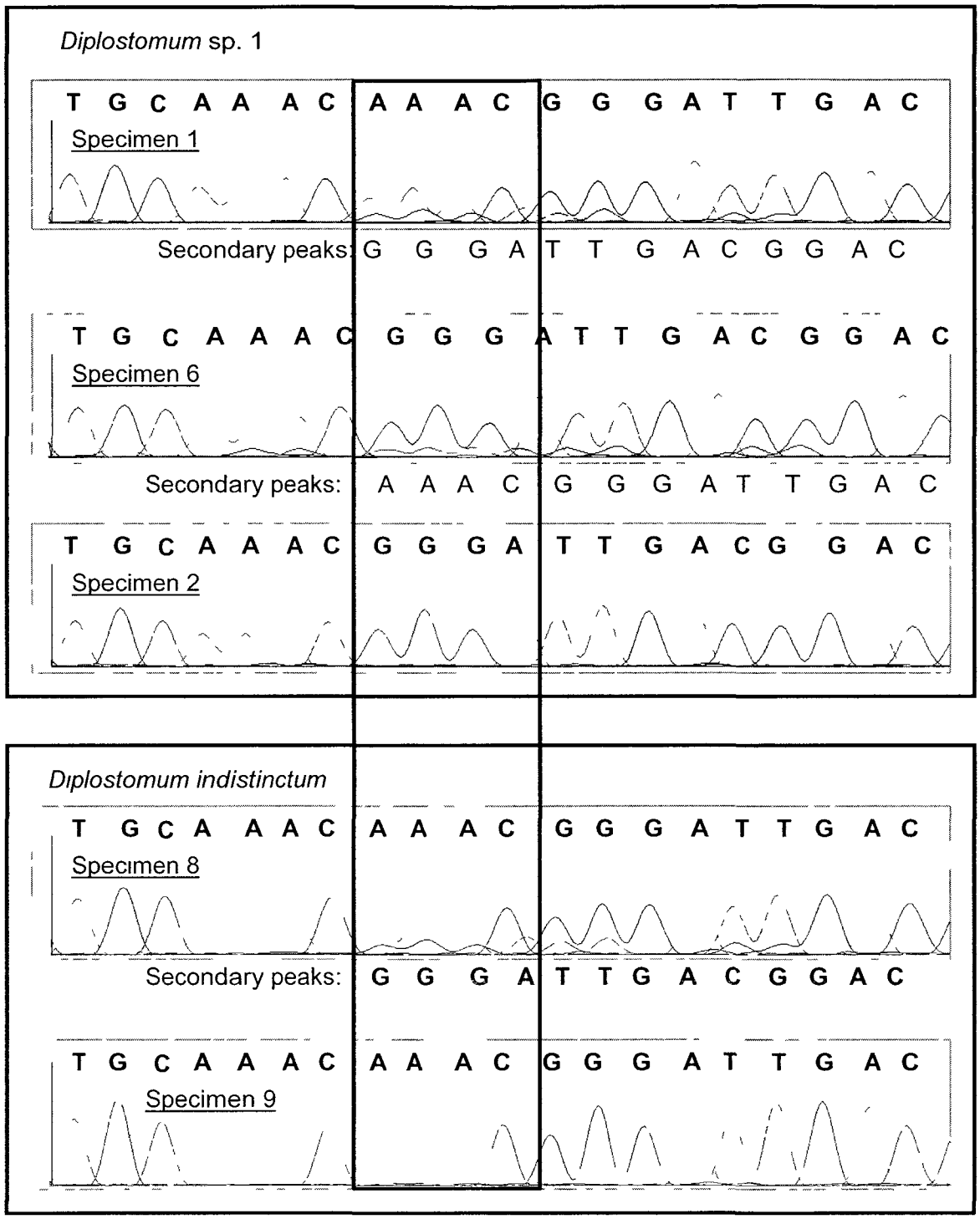


Figure 2.5 Aligned electropherograms of ITS from *Diplostomum* sp. 1 and *Diplostomum indistinctum*

Evidence of hybridization between *Diplostomum* sp. 1 and *Diplostomum indistinctum* in specimens collected from fish and gulls (*Larus delawarensis*) in the St. Lawrence River,

Canada. Forward-read electropherogram traces show a four-base indel in internal transcribed spacer 1 (ITS1) sequences (AAAC/- - - -). Traces from specimen 2 (- - - -) and specimen 9 (AAAC) show different character states without secondary peaks, which may represent the genotype native to each species. In other specimens, both character states are visible (AAAC/- - - -), with the alternative state shown by frame-shifted sequence in secondary peaks in DNA traces. Complete trace files from cytochrome c oxidase I and ITS from additional specimens showing the same patterns are available in project DIPLO at www.barcodinglife.org. Reverse traces are poorly defined in this region but do not contradict the patterns seen here.

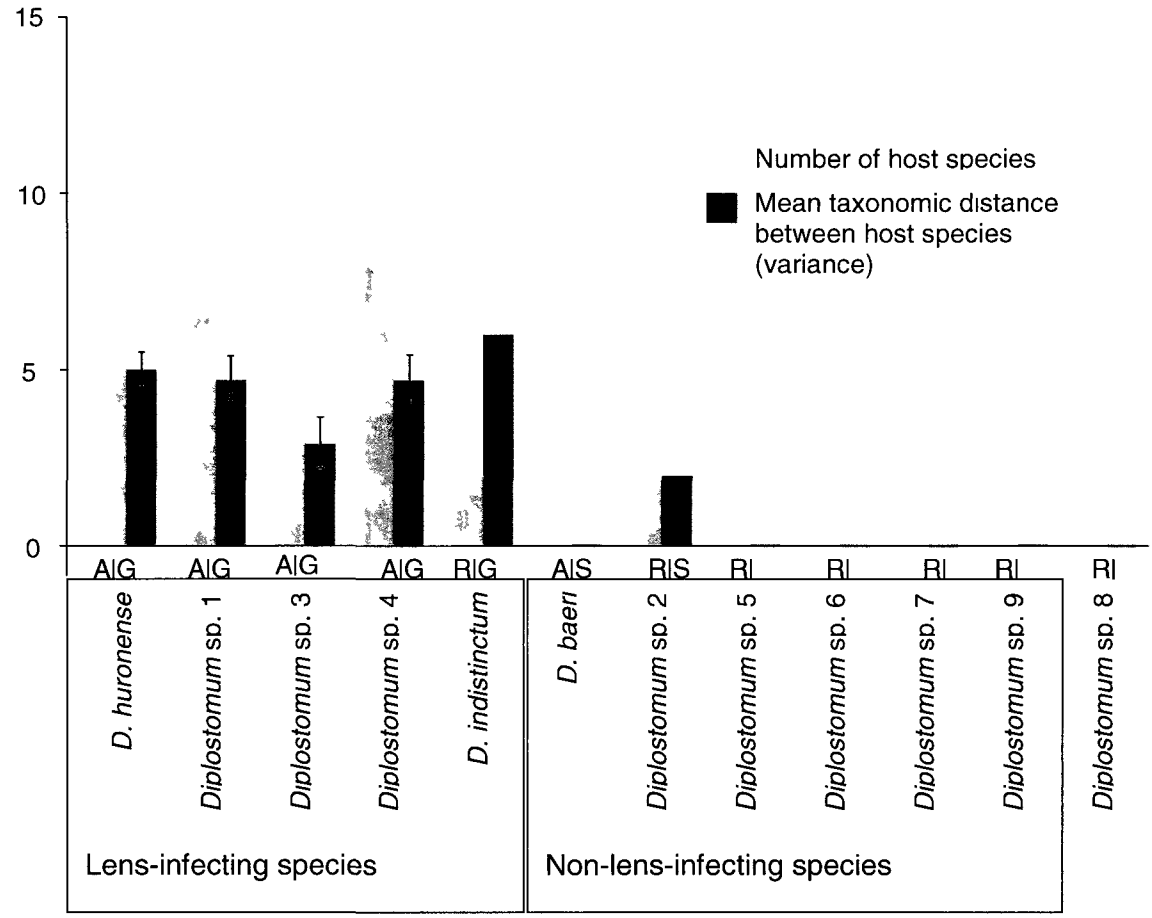


Figure 2.6 Host spectra and infection site in *Diplostomum* spp.

Host specificity and infection site of metacercariae of *Diplostomum* spp. from fishes and one amphibian from the St. Lawrence River, Canada. Lens-inhabiting species have a larger and more diverse range of hosts than species that colonise other tissues. See text and Poulin and Mouillot (2003) for an explanation of taxonomic distance, an estimate of host phylogenetic affinity. The infection site of *Diplostomum* sp. 8 was not determined. A = abundant species; R = rare species; G = species categorised as generalists; S = species categorised as host specialists. *Diplostomum* spp. 5-9 were found only in a single individual host and were not classified as host specialists or host generalists (see also Table 2.1). Inset figure legend indicates parameters measured on the y-axis.

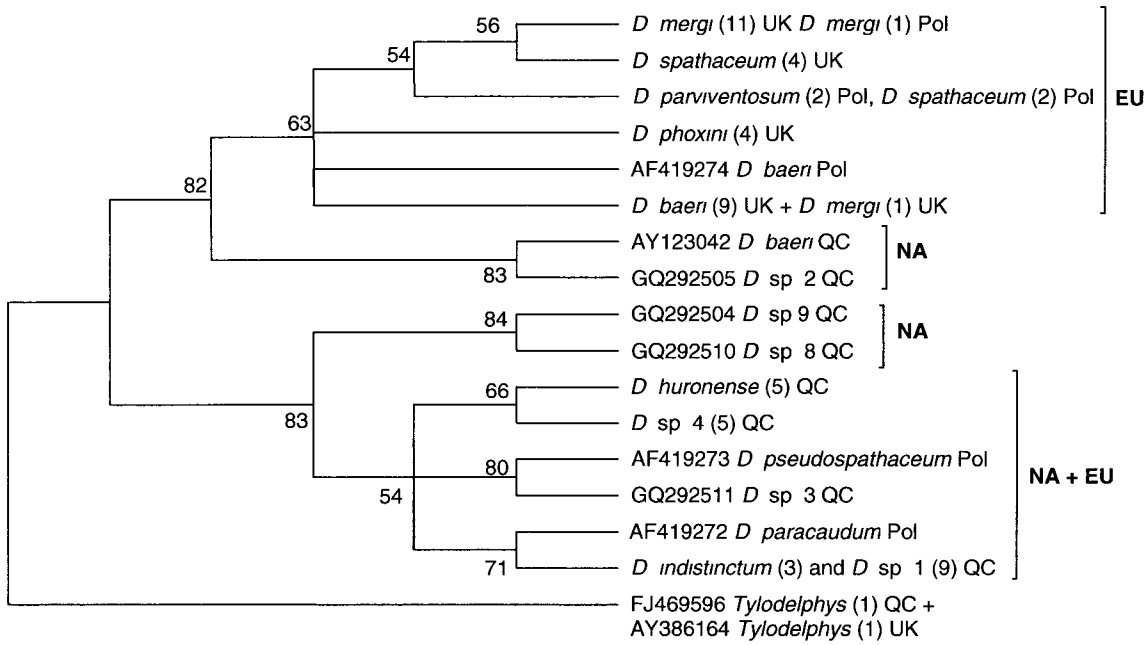


Figure 2.7 Maximum parsimony analysis of internal transcribed spacer 1 sequences from *Diplostomum* spp.

Consensus of the 974 most parsimonious trees of 67 internal transcribed spacer (ITS) sequences from *Diplostomum* and *Tylodelphys* obtained in this study and from GenBank, based on 500 bootstrap replicates, a closest neighbour interchange level of 3, a consensus bootstrap cut-off of 50%, and 163 parsimony-informative sites in a 1,099-nt alignment. Except for two sequences from the outgroup *Tylodelphys*, all sequences used here are the same as Figure 2.4b; one *Tylodelphys* sequence originates from a GenBank record based on UK material and another was obtained from a fish in the St. Lawrence River, Quebec, Canada, in the present study. Accession numbers are given for single sequences and where a clade of multiple sequences has been collapsed to a terminal branch, the numbers of sequences are in parentheses. QC = Quebec, Pol = Poland, UK = United Kingdom, NA = North America and EU = Europe.

Chapter 3 DNA barcodes reveal cryptic diversity and host specificity among strigeid (Platyhelminthes: Trematoda) parasites of freshwater fishes in the St. Lawrence River, Canada

Introduction

The diversity and ecology of many groups of macroparasites is poorly known (Poulin and Morand 2004; Poulin 2007b). Most parasites have been recorded from only a small number of host taxa, but host specificity is often difficult to evaluate (Poulin 2007b). Further complicating matters, many parasites show varying host specificity at different stages of their life cycles.

Strigeid trematodes (Platyhelminthes) are endoparasites with complex life cycles (Figure 3.1). The larval stage that occurs in fish, the metacercaria, causes pathology in populations of natural and farmed fish in freshwater systems around the world (Szidat 1969; Lemly and Esch 1984; Chappell 1995; Sandland and Goater 2001; Overstreet and Curran 2004). Metacercariae cannot be identified to species based on morphology, which has caused them to be treated as a relatively small number of species that occur in a wide assortment of fishes (Gibson 1996; Hoffman 1999). Little is known of the species-level diversity of larval strigeids and the relationships between metacercariae and their hosts are poorly defined (Chappell 1995; Gibson 1996; Poulin 2007b).

Previous surveys indicate that larval strigeids dominate the metazoan parasite fauna of forage fishes in the St. Lawrence River, although only one fish species has been studied in detail (Marcogliese *et al.* 2006). However, such surveys provide no information on the diversity or host specificity of strigeid metacercariae at the species

level. Molecular data offer a solution to these problems and constitute the only practical means of obtaining species-level resolution in a field survey of metacercariae. Results from an earlier study of internal transcribed spacer sequences (ITS) of *Diplostomum* from the St. Lawrence River led us to suspect that additional molecular sampling might uncover a small increase in local strigeid species diversity (Galazzo *et al.* 2002). In comparable studies, sequence data have often revealed an apparently generalist parasite to be a complex of cryptic and, in many cases, host-specific species (e.g., Jousson *et al.* 2000; Miura *et al.* 2005, 2006; Steinauer *et al.* 2007). However, this trend has not emerged in molecular studies of strigeids (Table 3.1).

Most studies using molecular markers to distinguish trematode species have employed sequences of ribosomal genes and of a ~350-bp fragment of cytochrome oxidase 1 (CO1) beginning about 800 bp from the 5' end of that gene (Nolan and Cribb 2005, Olson and Tkach 2005). The first ~650 bp of CO1, the DNA barcode, have been used to distinguish sibling species in numerous other groups (Hebert *et al.* 2003a, b; Frézal and Leblois 2008), including some parasitic helminths (Hansen *et al.* 2007; Elsasser *et al.* 2009; Ferri *et al.* 2009), but very few trematodes (Zarowiecki *et al.* 2007, Locke *et al.* 2009). This study employed DNA barcodes to survey the diversity and host specificity of larval strigeid species in fish in the St. Lawrence River, Canada.

Surveys generally provide no evidence as to whether host specificity arises from physiological incompatibility between parasites and hosts or from parasites being ecologically separated from hosts, what Combes (2001) called the compatibility and encounter filters to infection. In this study, several lines of evidence were used to not only detect host specificity, but to obtain evidence relating to its cause. Firstly, spatial

replicates of multiple host species in separate communities were sampled in ecologically distinctive localities. Fish host species often encounter different parasite species in different habitats, including parasites that circulate through other fish species (Leong and Holmes 1981; Bergeron *et al.* 1997). Consistent patterns of host specificity across the fish communities surveyed in the present study would therefore support compatibility-related mechanisms, while inconsistency would suggest that local ecological availability is more relevant. Secondly, we sampled a large number of host species, including hosts that were ecologically distinctive but closely related, and distantly related but ecologically convergent. If parasite species are distributed in ways that reflect ecological rather than phylogenetic host attributes, this would support the operation of the encounter filter (the availability of hosts to parasites), while the opposite pattern would indicate physiological compatibility to be more important. Thirdly, we looked for evidence suggesting that a physiological mechanism, the host immune response, affects patterns of host specificity in strigeid metacercariae. The immune response varies in different tissues in fishes (Sitjà-Bobadilla 2008). Lester and Huizinga (1977) suggested that in at least one group of strigeid fish parasites, species inhabiting an immunologically privileged site may have access to more host taxa than species inhabiting other tissues. If this pattern is observed among strigeid species in general, this would suggest a physiological basis for host specificity in metacercariae.

Materials and methods

Specimen collection

As stated in the Materials and Methods of Chapter 2, most specimens were metacercariae taken from fishes caught with a beach seine at six ecologically distinctive sampling localities in the St. Lawrence River near Montreal, Canada during a three-week period in June and July of 2006 (Figure 3.2). Six host species were sampled intensively, with a total of 705 fish examined (Table 3.2). These fish species were selected based on their availability throughout the system and represent the species that were most consistently abundant across localities. Only 1+ year-class, or the smallest sized fish available, were collected. By simultaneously collecting only young fish at replicate localities, we ensured that fish were likely to be local to each locality (not migrants) and had been exposed to the same pool of local parasites for the approximately same amount of time and during the same period. Metacercariae were also taken from 14 other species of fish (n = 114) and one amphibian species (n = 11). Most of these hosts were also in the 1+ age-class and were collected at the same time from the aforementioned localities. In addition, metacercariae were collected from older individuals collected on other occasions from these and additional localities in the St. Lawrence River and from elsewhere, including localities > 1000 km from the main study area in the St. Lawrence River. Fish were killed in an overdose of clove oil/ethanol solution (50mg/10ml/L) and frozen until dissection for parasites using standard techniques (Marcogliese 2002; www.eman-rese.ca/eman/ecotools/protocols/freshwater/parasites). Upon removal from the host, metacercariae were fixed in 95% or absolute ethanol for molecular work.

Adult strigeid specimens were also obtained by feeding fish to experimental hosts and from piscivorous birds contributed by colleagues. They will eventually be used for morphological identifications, but that aspect of our research is beyond the scope of this communication. However, nucleotide sequences from adult specimens are included here in calculations of sequence variation.

Molecular data acquisition and analysis

Amplification and sequencing of CO1 were performed using the primers and PCR protocols of Moszczynska *et al.* (2009) at the Canadian Centre for DNA Barcoding (CCDB) in Guelph, Ontario. The ITS region from the same individual specimens was also amplified and sequenced in some specimens at the CCDB following protocols and using the primers described by Galazzo *et al.* (2002) and Moszczynska *et al.* (2009). It is emphasized that sequences of both CO1 and ITS were obtained from the same individual specimens. Except for five samples in which a small number (< 5) of specimens were pooled, all DNA extractions were of single specimens.

Our aim was to obtain CO1 sequences from as many specimens as possible, and as a result every attempt was made to use salvageable regions of even lower quality DNA traces. Base-calling was conducted in isolation, without reference to other sequence data. In most cases, forward and reverse DNA traces were easily aligned and required minimal or no editing. In a small number of cases, substantial base calling was required, and occasionally bases were called before trace files could be aligned to generate bi-directional sequence. In other cases, particularly among the Crassiphialinae, sequences were obtained from single forward or reverse traces, or from traces that aligned on either

side of a problematic poly-T region described in the results. Sequences were manually edited using Contig Express (Invitrogen, Carlsbad, CA, USA), Geneious (Biomatters, Auckland, NZ) and Sequencher 4.8 (2008, Gene Codes, Ann Arbor MI USA). Barcode CO1 nucleotide sequences were aligned using ClustalX (Thompson *et al.* 1994) and subsequent analyses were conducted using Mega 4.0 (Tamura *et al.* 2007). Herein we present only 150 CO1 sequences, including representatives from each of the species detected as well as ITS sequences from the same specimens. The remaining sequences will be published in a future study dealing with the population genetics of the strigeids. Both CO1 and ITS sequences and original DNA trace files are available in projects PRIME, DIPLO and TREMA on www.barcodinglife.org, along with georeference, host and image data for each specimen.

Species delineation and identification

Metacercariae were identified to genus based on morphology, infection site and host species using the keys of Gibson (1996), Hoffman (1999) and Niewiadomska (2002). Potential species were initially distinguished by clustering in neighbour-joining (NJ) analyses of Kimura 2-parameter distances between CO1 sequences. Because barcode sequences are known from only a few strigeid species (*Diplostomum baeri*, *D. huronense* and *D. indistinctum*, see Locke *et al.* 2009), the NJ analysis of barcode sequences amounted to species delineation, rather than species identification. This use of barcode sequences is contentious (Desalle *et al.* 2005; Hickerson *et al.* 2006) and additional evidence of species boundaries was therefore sought. Sequence clusters indicating metacercarial specificity for hosts or tissues were considered strong, independent evidence of species boundaries. CO1 sequences were also analyzed using a

character-based method (maximum parsimony), which has been suggested to be preferable to distance-based NJ analysis for detecting species boundaries (see Desalle *et al.* 2005). In addition, ITS sequences obtained from a subsample of specimens were analyzed in NJ trees to see whether divergence supported CO1-delineated clusters. Representatives of ITS and CO1 sequences from all clusters were submitted to BLAST searches in GenBank, with the aim of matching strigeid species sequenced in other studies. Finally, in a few cases, metacercariae (or adults obtained from experimental infections and/or wild-caught piscivorous birds) showed distinctive morphological features which contributed to species delineation. However, because metacercariae were frozen, detailed morphological examination was not practical. Most metacercariae were too small for subsampling, and no tissue remained for microscopic study after extraction of DNA.

Effect of infection site on host specificity

We compared the host specificity of species of metacercariae inhabiting the lens of the eye with that of species in other tissues, taking steps to remove confounding influences. The lens is one of several immunologically privileged tissues in vertebrates (Stein-Streilein and Streilein 2002; Sitjà-Bobadilla 2008). The membrane encapsulating the lens appears to afford metacercariae a layer of protection from cell-mediated immune responses (Shariff *et al.* 1980) that are particularly important in fighting helminth infection (Kreider *et al.* 2007). A variety of approaches were used to determine whether metacercariae in this site were less host specific, that in turn required host specificity to be quantified.

Several indices of host specificity were used in order to avoid the bias of any single indicator. The simplest and most widely reported measure of host specificity is host range, i.e., the number of species infected by a parasite. Although convenient, host range provides no information on the phylogenetic relationships among host species. For example, *Diplostomum* sp. 2, which occurs in two species in the same subfamily, has a host range equivalent to *Diplostomum indistinctum*, found in members of two superorders (see Results, this chapter). Host phylogeny is relevant because physiological and immunological obstacles for parasites are likely to differ more as the phylogenetic distance between host taxa increases. For example, the histological distribution of eosinophilic immune cells shows distinctive patterns in teleost genera and families (Reite and Evenson 2006).

We therefore also measured the phylogenetic diversity of host spectra using the taxonomic distance indices of Poulin and Mouillot (2003, 2005a). These are based on the number of steps to a common node in a taxonomic tree, using the classifications of genus, subfamily, family, order, and superorder in Nelson (2006) (see inset to Figure 4.5). For example, a bluntnose minnow (*Pimephales notatus*, *Pimephales*, Leuciscinae, Cyprinidae, Cypriniformes, Ostariophysii, Actinopterygii) is six units from a yellow perch (*Perca flavescens*, *Perca*, Percidae, Perciformes, Acanthopterygii, Actinopterygii) and two units from golden shiner (*Notemigonus crysoleucas*, *Notemigonus*, Leuciscinae). Specificity expressed as the mean taxonomic distance between all host species infected by a parasite (S_{TD}) is an index based solely on the phylogenetic diversity of hosts that varies relatively little with sample size (Poulin and Mouillot 2003). The associated variance ($VarS_{TD}$) reflects the heterogeneity of phylogenetic relationships in the host

assemblage. In addition, we calculated S_{TD}*, which weights S_{TD} with differences in prevalence in each host species (Poulin and Mouillot 2005a).

Several analytical techniques were used to test whether lens-inhabiting strigeids differed in host specificity from non-lens species. In each approach, we attempted to control for confounding factors. For example, a rare parasite species will tend to appear host specific because it will occur in fewer individual hosts, which are less likely to represent all host taxa the parasite is capable of colonizing. More abundant parasite species occur in more individual hosts and tend to saturate the range of compatible host taxa (see Poulin and Mouillot 2005b). These expectations raise concerns both from the point of view of species abundance and sampling effort. In addition, since generalist species in the lens were members of a single genus, *Diplostomum*, another set of analyses was conducted to remove phylogenetic influences, i.e., to ensure low host specificity in the lens was not just a *Diplostomum* effect.

Firstly, analyses of covariance (ANCOVAs) were conducted to determine whether lens-infection affected host specificity as measured by the indices described above. Covariates included the number of metacercariae sequenced, and the number of hosts from which sequenced metacercariae were obtained.

Secondly, the effect of the lens on host specificity was tested using multiple regression of phylogenetically independent contrasts calculated with the PDAP module of Mesquite 2.6 (Midford *et al.* 2009; www.mesquiteproject.org). Contrasts were obtained from a phylogeny generated from MP analysis of CO1 DNA sequences rooted with *Clinostomum* sp. Nine equally parsimonious trees all placed species of *Ichthyocotylurus* (Strigeinae) in a clade with diplostominids, rather than with *Tetracotyle* (Strigeinae)

(Figure 3.9). This is unlikely to represent the true phylogeny, according to both taxonomy and cladistic analysis of morphological and life-history characteristics of the adult and metacercarial stages (Shoop 1989; Niewiadomska 2002). This was not surprising, however, because barcode region sequences of CO1 are too short and rapidly evolving for reliable reconstruction of deeper phylogenetic relationships (Hajibabaei *et al.* 2006; Waugh 2007). Consequently, phylogenetically independent contrasts were obtained using both from two phylogenies: the observed CO1 MP phylogeny and one in which *Ichthyocotylurus* species were manually placed at the base of an all-strigeinid clade. The significance and explanatory power obtained using both phylogenies were similar.

Multiple regressions of phylogenetically independent contrasts were conducted to assess how lens-infection affects host-specificity indices, while accounting for sampling effort and species abundance. The independent variable (lens/non-lens) is binary but represents a quantitative difference in immunological activity levels. Lens-infecting metacercariae were coded as 0, denoting residence in a low-immune-activity site, and non-lens metacercariae were coded as 1, representing sites with higher immune activity. Also available for use in the models were the two aforementioned measures sampling effort (N metacercariae sequenced, N hosts from which sequenced metacercariae obtained). However, this resulted in three independent variables to be tested with 36 data points each representing contrasts in host specificity. Because both estimates of sampling effort were strongly correlated ($R = 0.983$, $n = 36$, $P < 0.0005$), and to avoid overfitting the models, multiple regression was limited to lens-infection and a single measure of sampling effort selected based on collinearity tolerance.

Thirdly, the relationship between the low-immune infection site and host specificity was also tested using Pagel's (1994) test of correlated evolution between discrete characters, using Mesquite (Midford *et al.* 2002. PDAP:PDTREE package for Mesquite, version 1.00, www.mesquiteproject.org). Parasite taxa were classified as lens or non-lens inhabitants, and as host generalists or specialists based on a qualitative assessment of host range, S_{TD}, $VarS_{TD}$, and S_{TD}*.

Finally, in strigeids classified as host specific, Fisher's exact tests were conducted to independently assess the probability of the observed host specificity arising by chance.

Results

Delineation of species

Both CO1 and ITS sequences indicated the presence of a large number of strigeid species, many of which were supported by host- and tissue-specific distribution that was consistent across localities. Clustering of CO1 sequences indicated that at least 49 strigeid species occur as metacercariae in fish and amphibians in the St. Lawrence River (Figure 3.3; Table 3.3). Overall, CO1 sequences provided better inter- and intraspecific resolution than ITS sequences.

Sequencing of CO1 was successful in 1088 of 1955 specimens processed (225 of 441 adults and 863 of 1514 metacercariae). Most (735) of the CO1 sequences from metacercariae were from specimens obtained from the six intensively sampled fish species (Table 3.1). The rest were from 16 less intensively sampled fish species and one species of amphibian. Mean sequence length was 449 bp and was as high as 634 bp in some specimens. Usable information could be obtained from sequences as short as 65 bp,

but only 20 sequences shorter than 200 bp were used for identifications (see also Meusnier *et al.* 2008). Nearly all taxa shared a > 14-bp region rich in Ts approximately 180 bp from the 5' end of CO1. In some Crassiphialinae this T-rich span contained no other bases and trace file quality was negatively affected downstream (in forward reads) and upstream (in reverse reads) of this region. In consequence, sequences from some crassiphialinid specimens are effectively based on unidirectional reads of trace files flanking either side of the T-rich span.

The mean and/or maximum intraspecific variation in CO1 was greater than expected in nine species (Table 3.3). In four species (*Ornithodiplostomum* spp. 3 and 4, *Ichthyocotylurus pileatus*, *Posthodiplostomum* sp. 3), DNA traces were of poor quality and/or based on single-direction reads because of the poly-T region, which likely inflated variation within species. In five other species (*Diplostomum* spp. 1, 2 and 4, *Hysteromorpha triloba* and Diplostomoidea fam. gen. sp.), trace file quality was not an issue. CO1 sequence from one specimen of Diplostomoidea fam. gen. sp. differed by 3.5% from the other eight specimens, which differed by < 1.3%. Additional specimens with the same, equivalently divergent haplotypes (≥ 3.5%) would support a second species, but for the moment we are treating this as a single species. In contrast, in *H. triloba* and *Diplostomum* species with high maximal intraspecific variation (Table 3.3), the high values are not the result of outlier haplotypes, and a gradient of divergence up to the maximum values was observed.

No geographic pattern was evident in species that displayed high intraspecific divergence despite good quality trace files. For example, CO1 sequences were identical or not particularly divergent (~1%) in conspecifics of *Diplostomum* spp. 1 and 4 from

avian and fish hosts collected in localities separated by 1700 – 2200 km in Manitoba, Ontario, Quebec, New Brunswick, and Nova Scotia. The highest levels of divergence in CO1 sequences of *Diplostomum* spp. and *H. triloba* were obtained from metacercariae from sympatric fishes in the St. Lawrence River. There was no significant relationship between the number of specimens sequenced per species and mean intraspecific divergence in CO1 sequences ($R = 0.117$, $n = 37$, $P = 0.117$). The relationship between maximum intraspecific divergence in CO1 sequences and specimens sequenced per species was best described by a logarithmic curve indicating a threshold of maximum intraspecific variation at ~3% ($R^2 = 0.836$, $F_{32} = 163.6$, $P < 0.0005$), whether outlier values such as *Ornithodiplostomum* sp. 4 were included or not.

Despite high intraspecific variation in some species, the species boundaries indicated by clusters of CO1 sequence were unambiguous (Figure 3.4). Sequences from even the most divergent specimens were more similar to conspecifics than to members of other species. In addition, a character-based parsimony analysis of CO1 sequences over 400 bp assigned all 955 strigeids to the same clades as the clusters produced in distance-based NJ analysis. Moreover, the species boundaries of 31 clusters were supported by host-specific distribution among multiple individual hosts (Table 3.3). Finally, most species boundaries indicated by CO1 sequences were supported by ITS sequence data, as explained below.

ITS sequences were obtained from 122 specimens, including 105 from which CO1 sequences were also obtained. These represent 32 species indicated by CO1 data (Table 3.3). Topologies produced by NJ analyses of ITS generally supported those derived from CO1 sequences from the same specimens (Figure 3.5), with two exceptions.

Mean divergence in ITS sequences between *Diplostomum* sp. 1 and *Diplostomum indistinctum* was only 0.37% (range 0 – 0.48%), which does not suggest distinct species. CO1 sequences from these species diverged by 11.7% (range 10.2 – 12.6%, see Galazzo *et al.* [2002] and Locke *et al.* [2009], for detailed discussion of these data). The other exception occurred in 35 *Ornithodiplostomum* metacercariae from leuciscinids *P. notatus* and *N. crysoleucas*, in which CO1 sequences formed host-specific clusters with a net mean interspecific divergence of 5.4% (*Ornithodiplostomum* spp. 2 and 3). The ITS sequences from seven of these 35 specimens presented a different picture. Sequences of ITS from three specimens of *Ornithodiplostomum* sp. 3 are essentially identical to those of three specimens of *Ornithodiplostomum* sp. 2, but the ITS sequence from a single specimen of *Ornithodiplostomum* sp. 3 differs from the other six (Figure 3.5). The divergent ITS sequence may represent a "bad" sequence, although ITS trace files were of reasonably good quality.

In summary, despite the minor ambiguities described above, in most cases CO1 sequence quality was high, requiring little editing and forming clear, discrete clusters. Species boundaries were generally clear, and in the cases where there was less clarity, this was often attributable to low trace-file quality. Species boundaries were supported by character-based MP analysis of CO1 sequences, by ITS sequence clustering and by host- and/or tissue-specificity.

Identification of species

Species labelled *Tetracotyle*, a generic name given to certain larval morphotypes, were initially identified as *Apatemon* based on the keys to North American fish parasites by Gibson (1996) and Hoffman (1999) (see also Chapter 1). The placement of these

specimens in *Apatemon* was reconsidered in light of the overall diversity and dissimilarity seen among the clusters of both nuclear and mitochondrial sequences. Although they do not appear in the keys of Gibson (1996) and Hoffman (1999), metacercariae of related taxa such as *Strigea* and *Apharyngostrigea* may also be present and are difficult to distinguish morphologically from those of other genera once frozen. We therefore use the more conservative larval genus name, *Tetracotyle*, with some exceptions. Species labeled *Apatemon* are in a CO1 cluster in which ITS1 and 2 sequences differed by only ~2% from *Apatemon gracilis* (Bell and Sommerville 2002). Identification of *Ichthyocotylurus pileatus* was based on a 99% similarity with an ITS sequence published by Bell *et al.* (2001) and on the key of Gibson (1996).

Specimens of *Diplostomum baeri, D. indistinctum* and *D. huronense* obtained in this study were identified by comparing ITS sequences with those published by Galazzo *et al.*(2002) and using CO1 sequences from archived DNA from the specimens studied by those authors (Locke *et al.* 2009). Sequence from ITS1 in *Diplostomum* sp. 3 differs by only two bases from that of *D. pseudospathaceum* from Poland (Niewiadomska and Laskowski 2002) but more data are needed before the relationship of these North American samples to the European species can be determined (see Locke *et al.* 2009).

The identification of *Conodiplostomum* sp. 5 is tentative for several reasons. No cyst was observed in these metacercariae from the brain of johnny darter. It is possible freezing the hosts destroyed the fragile cyst typical of this genus, but freezing did not have this effect in other species that form the same type of cyst (neascus). This metacercaria lacked pseudosuckers and resembled a neodiplostomulum, which is not encysted, but that larval morphotype is known only from amphibian hosts. The

relationships between *Conodiplostomum* and neodiplostomula *Neodiplostomum* and *Fibricola* and their links to other diplostomoid groups have been repeatedly revised (Shoop 1989; Hong and Shoop 1994; Niewiadomska 2002). However, none of these taxa would be expected to show the high similarity in CO1 sequences with *Tetracotyle* spp. that we observed in *Conodiplostomum* sp. 5 (Figures 3.3, 3.4b).

Strigeid diversity and specificity in the St. Lawrence River

Strigeids dominated parasite communities of fishes in the St. Lawrence River. In the six host species in Table 3.2, individual fish were infected with an average of 35 individual metazoan parasites, over half of which (19) were strigeids. The 47 strigeid species detected in fish (Table 3.3) represent nearly a fourfold increase in diversity known from Canadian fishes (Figure 3.6). Forty-three of these species occurred in one or more of the six well-sampled host species. Species accumulation curves suggest that additional strigeid species remain undetected in at least one of the focal hosts (*P. flavescens*) in the study area (Figure 3.7).

Host specificity was evaluated in the 31 species of metacercariae identified by CO1 and ITS sequences that were found in two or more individual hosts. Exact tests indicated that the distribution of host-specific metacercariae among host species was unlikely to be a sampling artefact. For example, metacercariae identified by sequences as *Posth· liplostomum* sp. 3 were recovered from 19 centrarchid hosts (two *A. rupestris*, 17 *L. gibbosus*). There were 194 hosts in these two host species in which *Posth·diplostomum* sp. 3 was not detected and it was not found in any of the 599 fish in variou other species. This distribution (19/194, 0/599) between these two centrarchid speci and this parasite is unlikely to be random (Table 3.3). Most strigeid species

inhabiting non-lens tissues occurred in a single host species or in a small number of close relatives (Table 3.3). This pattern was consistent across localities. Locality-by-locality infection levels of each species are not presented here, but an example is given in Table 3.4.

Species of metacercariae inhabiting the lenses of fish were significantly less host specific than those in non-lens tissues, controlling for sampling effort and phylogenetic influences (Figure 3.8, Table 3.5). In multiple regressions of contrasts, lens-infecting species were significantly less host specific than non lens species, although it was difficult to separate the effect of lens infection on host range from that of sampling effort.

Although several quantitative descriptors of host specificity were used, they still present an incomplete picture of this attribute. For example, the index designed to capture ecological aspects of host specificity, S_{TD}*, only provides relevant information when there are three or more host species (Poulin and Mouillot 2005a). For example, 22 specimens from 14 johnny darters and one specimen from a golden shiner were identified by CO1 sequencing as *Apatemon* sp. 1. Unidentified metacercariae of the same morphotype (*Tetracotyle*) had a similar distribution, occurring in 55% of johnny darter and 2.5% of golden shiner. The parasite is much more common in one host than in other and, in this sense, it is more host specific than if it achieved equal success in both of these hosts, which are distantly related. However, in this case ($S_{TD} = S_{TD}* = 6$). Nearly a quarter of the strigeid species we detected were found in only two host species (Table 3.3), meaning this aspect of host specificity was not reflected in any of the indices used. Thus, an additional test of the effect of lens infection on host specificity was warranted. In this analysis, species were characterized as specialists or generalists based on a

qualitative assessment of the distributional data. There was a significant, phylogenetically independent correlation between lens-infection and low host specificity, regardless if arguably ambiguous cases such as *Tetracotyle* sp. 1 (see gray boxes in Figure 3.9) were classified as specialists or as generalists (difference between four- and eight-parameter log likelihoods ranged from 4.244 to 4.483, $P \leq 0.04$ after 100 simulations).

Essentially, the spectrum of hosts infected by larval strigeids inhabiting the lens was significantly larger, more phylogenetically diverse, and characterized by more variable diversity than that of species infecting other sites (Table 3.5, Figure 3.8).

Discussion

Strigeids dominated the parasite communities of a diverse sample of fishes from the St. Lawrence River and CO1 sequences revealed an unexpectedly diverse assemblage of metacercarial species, most of which were host specific. Species inhabiting the lens of the eye were less host specific than those in other tissues, and these patterns of host specificity were consistent across localities and among hosts with varying ecological and phylogenetic affinity. Overall, there is evidence that host specificity in strigeid metacercariae is more strongly influenced by whether or not host species are physiologically compatible than whether or not they are ecologically available to these parasites.

Strigeid diversity and specificity

Our findings represent a large increase in the known diversity of these widely occurring fish pathogens. The magnitude of diversity observed was unexpected because the parasites of freshwater fishes in Canada have been well studied using traditional

methods (Margolis and Arthur 1979; McDonald and Margolis 1995; Gibson 1996) and we examined a comparatively small number of fish species and limited geographic area. Previous molecular studies of strigeids have not uncovered similarly increased diversity, although they encompass a similar number of host taxa and much larger geographic scope (Table 3.1). This may be because many specimens were pooled in molecular samples in those studies, which effectively reduces the sample size. In two other studies of larval trematodes in which specimens were not pooled in molecular analyses, Miura *et al.* (2005, 2006) also found a high degree of cryptic diversity in a survey of larval trematodes.

Most larval strigeids in non-lens tissues were highly host-specific. This information can be used in the management of disease in wildlife and aquaculture, in which pathogen identification is a first step to control and treatment. It should also be considered in studies of the community ecology of parasites of freshwater fishes, in which larval parasites are usually ignored or pooled at high taxonomic levels. Specifically, the data suggest two rules-of-thumb for strigeid metacercariae from fish, although molecular verification of our results from other systems is desirable. Firstly, morphologically indistinguishable strigeids occurring outside the lens of distantly related hosts are likely to be different species. In this sense, the strigeids residing outside the lens are no longer cryptic species, because they can be distinguished based on their host. Secondly, the data also show that what appears to be a single, morphologically indistinguishable type of metacercaria specific to single host species, often inhabiting the same tissue, may actually represent more than one species (e.g., *Diplostomum* sp. 5 and *D. baeri* both inhabit the humour of yellow perch; *Ornithodiplostomum* spp. 3, 4 and 8 in

brain of bluntnose minnow; *Posthodiplostomum* spp. 1, 2 and 3 in viscera of pumpkinseed and rock bass).

DNA sequences and species

Sequences of CO1 were effective for uncovering cryptic diversity. One difficulty with the barcoding approach to delimiting species is deciding what level of divergence indicates species boundaries (Desalle *et al.* 2005). There is probably no universally applicable ratio of CO1 divergence within and between species, and additional markers may be useful in groups where this ratio is unknown, or when CO1 sequence quality is poor. In the present case, the species indicated by CO1 sequences were generally supported by ITS sequences. However, the ratio of ITS sequence divergence within and between species was less clear, and in some cases ITS sequences provided no interspecific resolution. For example, the divergence in CO1 sequences and host specificity of *Ornithodiplostomum* spp. 2 and 3 are strong evidence for separate species, yet some specimens in these lineages shared identical ITS sequences. In this and similar cases, the species boundaries that are less clear in our data would be better addressed by additional samples of cleaner CO1 data than by ITS data. This is a technical issue that could probably be addressed with CO1 primers designed specifically for particular subgroups of strigeids (see Moszczynska *et al.* 2009).

In five species, intraspecific divergence in CO1 was higher than expected but not attributable to low-quality traces. However, the most divergent conspecific specimens still clustered unambiguously (Figure 3.4) and species boundaries were supported by character-based analysis and host and tissue specificity. Overall, the use of CO1 data alone appears to be an appropriate and powerful tool for initial assessment of diversity

and host-parasite relationships in this important group of fish pathogens. Ferri *et al.* (2009) reached similar conclusions after comparing the resolution of 12S rDNA and barcode-region CO1 sequences in a diverse sample of parasitic filarid nematodes, despite finding as little as 2% variation in CO1 between species. In their study and in ours, the second ribosomal marker provided corroboration in some cases but was equivocal in others. Delimiting species on the basis of sequence from any single marker is controversial (Kunz 2002; Hickerson *et al.* 2006; Frézal and Leblois 2008; see also Poulin and Keeney 2007). However, in the presence of high divergence in even a single marker, it may be most reasonable to conclude the existence of separate species (Nolan and Cribb 2005), although other sources of variation should also be considered, such as geographic isolation (Irwin 2002; Kuo and Avise 2005). The latter is not an issue in the present case because samples were sympatric in the strictest sense (congeneric strigeid species were often found occupying the same tissue in a single individual fish) and strigeid species are probably widely dispersed by avian definitive hosts.

One of our aims in obtaining rDNA sequences was to determine whether any of the CO1-delineated species we encountered were the same as those studied previously by other authors using ITS, which has been more frequently employed to identify trematode species (Nolan and Cribb 2005; Table 3.1). Only two species encountered had ITS sequences of high similarity to any strigeids on GenBank. *Ichthyocotylurus pileatus* and, less certainly, *Diplostomum* sp. 3 are both probably conspecific with European species, the latter having only two differences in ITS1 with *Diplostomum pseudospathaceum* from Poland (Bell *et al.* 2001; Niewiadomska and Laskowski 2002; Locke *et al.* 2009). If this is true, these are the first strigeids that have been shown by molecular data to reside on

both continents. Our lack of matches with other taxa further underscores the extent of uncharted diversity in this group.

Lens versus non-lens infections

Our data suggest that generalist metacercariae are more likely to inhabit the lens than other tissues, while metacercariae inhabiting non-lens tissues are more host specific. The effect of the lens on all measures of host specificity was large if either phylogeny or sampling effort were taken into account, but reduced in some cases if both confounding factors were removed. However, since the host specificity of metacercarial species was not likely a product of chance, it is arguable that sampling effort should not have been included in multiple regression analyses at all. Omitting sampling effort greatly augments the effect of the lens on host specificity (data not shown).

The most plausible explanation for lower host specificity in metacercariae inhabiting the lenses of fish is the limited immune response in this site. The membrane encapsulating the lens appears to provide a protective barrier from immune cells (Shariff *et al.* 1980; Whyte *et al.* 1990), and cell-mediated immunity is particularly important for fighting helminth infection (Whyte *et al.* 1990; Reite and Evenson 2006). In mammals, the central nervous system as a whole is often considered to be immunologically privileged (Sitjà-Bobadilla 2008). In teleosts, however, the rodlet cells that carry out anti-helminth immune responses also occur in the central nervous system (Reite and Evenson 2006; Dezfuli *et al.* 2007; Sitjà-Bobadilla 2008). This may explain why the strigeids in non-lens tissues of the central nervous system (*Ornithodiplostomum* spp., *D. baeri*, *Diplostomum* sp. 2) did not show lower host-specificity.

Residence in the lens may also benefit parasites in ways unrelated to the host immune response. Szidat (1969) suggested that the lens capsule might protect parasites from gastric juices in the stomach of the definitive host, allowing a higher proportion to survive and reach the intestine, but this idea has not been tested. In addition, high numbers of metacercariae in the lens impair vision and anti-predator behaviour in fish (Seppälä *et al.* 2005a, b, c). However Brassard *et al.* (1982) and Seppälä *et al.* (2006) found no evidence that this leads to increased transmission to appropriate hosts. Thus, there is no evidence that these alternative, potential benefits of lens-infection have real effects on parasite fitness and, most importantly, none of these hypotheses are mutually exclusive. In contrast, the immunological privilege of the lens appears to provide access to a significantly wider range of habitats, which does suggests it may increase parasite fitness. It seems that additional fitness benefits, if any, may have been secondarily derived.

Mechanisms of host specificity in strigeid metacercariae

Three aspects of our study suggest that host specificity in strigeid metacercariae is attributable to physiological rather than ecological factors. Firstly, the link between host specificity and the lens can be plausibly explained by a physiological mechanism (immune evasion), while ecological explanations are not obvious. Secondly, the same patterns of host specificity recurred in separate, distinctive fish communities. In separate habitats, differences in fish community composition and abiotic factors bring hosts into contact with parasites that mainly use other hosts (e.g., Leong and Holmes 1981; Bergeron *et al.* 1997). However, in our study, most parasites outside the lens were limited to the same host species across localities, despite significant differences in fish

community composition in each of the three lakes (data not shown; Y. Reyjol, Ministère des Ressources Naturelles et de la Faune du Québec, personal communication). Thirdly, among non-lens parasites that infect multiple host species, host spectra are generally better explained by the phylogeny of hosts than by ecological convergence. The distribution of *Ornithodiplostomum* sp. 1 provides an example in which host phylogeny bridges an obvious ecological gap. Even though its principal host, johnny darter, is benthic, *Ornithodiplostomum* sp. 1 only occurred in other percids, including yellow perch, which is pelagic. Neither this species nor any *Ornithodiplostomum* were observed in other benthic fishes. Similarly, golden shiner is a midwater feeder while bluntnose minnow consumes organic detritus (Scott and Crossman 1973; data not shown, present study), but *Tetracotyle* sp. 6 was nonetheless found only in these two minnows. Finally, there were few cases of ecologically similar but distantly related hosts sharing parasites, except those inhabiting the lens. For example, although yellow perch shares many ecological attributes with the centrarchids sampled here (Scott and Crossman 1973), no specialist species occurred both in yellow perch and centrarchids. Several relatively abundant, specialist strigeids (*D. baeri*, *Ornithodiplostomum* spp. 2 and 3, *Posthodiplostomum* spp. 3, 4 and 7) were specific to one or two closely related hosts, even in localities where these optimal hosts were less abundant (Table 3.1). If ecological rather than compatibility-related factors governed host specificity in these metacercariae, then these species should have been detected in ecologically similar, but phylogenetically distant hosts.

A small but growing number of studies of host specificity have combined field surveys with DNA-based identifications (e.g., Jousson *et al.* 2000; Steinauer *et al.* 2007;

Poulin and Keeney 2007). To our knowledge, this approach has not been applied previously to trematode metacercariae. Few molecular surveys of any helminth parasites have been conducted on a comparable scale. The breadth and design of the study, along with the actual data, suggest most strigeid metacercariae are host specific and that their distribution among host species has more to do with physiological than ecological factors. In contrast to comparable studies of strigeids, substantial cryptic diversity was uncovered and in at least one host additional species probably remain undetected in our study area. It is clear that much remains to be learned about this group in North America and globally and that DNA barcoding provides a practical means of moving forward.

Table 3.1 Review of molecular systematic studies of strigeids

Summary of molecular studies of strigeids that infect freshwater fish as metacercariae. In some studies, cryptic species have been detected with molecular data, while in others, molecular methods did not distinguish valid species or led to synonymization of invalid species. These studies encompass a vast geographic scope and comparable host diversity to the 22 fish and five avian host species surveyed in the present study. However, in several studies, morphologically similar specimens from the same individual host or host species were assumed to be conspecific and pooled in molecular analyses. This approach reduces the collective sample size from ~1500 to somewhat over 200 specimens, less than 20% of the individual specimens sequenced in the present study. This may explain why there was no net change in diversity detected by traditional methods and that subsequently recognized with molecular data in these studies, while we encountered substantial cryptic diversity in a small geographic area (see Figure 3.6).

Taxon Locality	Species detected		Molecular data	Specimens used in molecular analysis / pooled into *n* samples	*n* host species: mollusc / fish / bird	Source[‡]
	Morphology, life-history	Molecular methods				
Diplostomum			SDS-PAGE			
UK	4	4	electrophoresis	250 / 4	0 / 4 / 0	1
Continental Europe	2	2	RAPD	40 / 40	2 / 2 / 0	2
Continental Europe	6	5	ITS1	15* / 15	3 / 3 / 0	3
Quebec, Canada	3	4	ITS1-2	19 / 19	0 / 3 / 0	4
Ichthyocotylurus						
UK + cont. Europe	4	4	ITS1-2, CO1	48* / 24	0 / 4 / 0	5
Apatemon						
UK	2	1	ITS1-2, CO1	96 / 31	0 / 5 / 0	6
Bolbophorus			ITS1-2, 18S,			
Southern US	1	2	28S, CO1	21 / 21	0 / 1 / 2	7
Southern US	1	2	18S, ITS1-2	1137 / 71	1 / 1 / 2	8
Israel	2	2	ITS1, 2, 18S	?	0 / 0 / 2	9
Total:	21[††]	21[††]		1625 (225)	6 / 14 / 4[††]	

Table 3.1, continued. Notes:

* Estimate.

? = no estimate possible

[††] Totals represent the number of different species across studies, and each species is counted only once. Thus, numbers in columns do not add to up to totals, because the same species appear more than once (e.g., *Diplostomum spathaceum* and *Perca fluviatilis* appear in several studies).

[‡] Source: 1 = Faulkner, 1989; 2 = Laskowski 1996; 3 = Niewiadomska and Laskowski 2002; 4 = Galazzo *et al.* 2002; 5 = Bell *et al.* 2001; 6 = Bell and Sommerville 2002; 7= Overstreet *et al.* 2002; 8 = Levy *et al.* 2002; 9 = Dzikowski *et al.* 2003

Table 3.2 Numbers of fish collected in six focal species sampled at six localities in the St. Lawrence River

Number of fish in six intensively sampled host species examined at six localities in three lakes of the St. Lawrence River during June-July 2006. Localities are numbered as in Figure 3.2. Every attempt was made to collect at least 20 hosts in each species at each locality, and cells < 20 indicate that species were more difficult to collect, thus less abundant locally. In addition to the 705 hosts in this table, 112 fish in 16 additional species were collected from the same or nearby localities, during the same season or in 2007-2008.

			Lake St. Francois		Lake St. Louis		Lake St. Pierre	
			1	2	3	4	5	6
Cypriniformes	Cyprinidae	*Notemigonus crysoleucas*	18	20	14	21	20	20
		Pimephales notatus	4	23	18	26	20	20
Perciformes	Percidae	*Perca flavescens*	20	20	26	24	26	21
		Etheostoma nigrum	9	26	55	41	0	0
	Centrarchidae	*Lepomis gibbosus*	16	21	23	24	22	26
		Ambloplites rupestris	9	10	21	10	12	19

Table 3.3. Striged species detected by DNA barcoding

Molecular and ecological characteristics of species of strigeid metacercariae in vertebrate hosts from the St. Lawrence River, Canada

Larval trematode species	Mean intraspecific divergence (%) in CO1 sequences (SE, range) *n* sequences*	Host(s) † Tissue(s) ††	N metacercariae sequenced	N hosts from which sequenced metacercariae obtained	Probability of observed host specificity arising by chance§
Strigeidae					
Strigeinae					
Apatemon					
sp. 1§§	0.59 (0.18, 0-1.76) 23	**10, 16** V	23	15	**4.7×10^{-9}**
sp. 3	0.49 (0.18, 0-1.33) 10	**3, 5** M V	10	8	**0.00002**
sp. 4	0.15 (0.15, 0-0.22) 10	**3** M V	10	8	**9.4×10^{-9}**
sp. 1x	0.27 (0.19, 0-0.51) 3	**10** V	3	3	**0.00455**
Tetracotyle					
sp. 2	0 (-) 2	12 V	2	1	nc
sp. 6	0.43 (0.23, 0-0.54) 6	**16, 20** V	6	4	**0.00053**
sp. 7	0.24 (-) 2	**20** H	2	2	**0.01684**
sp. 8	0.43 (0.24, 0-0.54) 3	**10** V	3	3	**0.00463**
sp. 9	0.45 (0.29, 0-0.84) 3	**5** V	3	3	**0.00473**
sp. 10	0.69 (0.25, 0-0.84) 5	**10** M	5	4	**0.00076**
sp. 11	1.02 (0.32, 0-1.65) 6	**23** V	6	4	**1.7×10^{-11}**
sp. 16	*n* = 1	10 H	1	1	nc
sp. 17	*n* = 1	12 B	1	1	nc

Table 3.3 continued

Larval trematode species	Mean intraspecific divergence (%) in CO1 sequences (SE, range) *n* sequences*	Host(s) † Tissue(s)††	N metacercariae sequenced	N hosts from which sequenced metacercariae obtained	Probability of observed host specificity arising by chance§
Tetracotyle sp. 18	0 (-) 2	**3** B	2	2	**0.00058**
sp. x	*n* = 1	10 M	1	1	nc
Ichthyocotylurus ***pileatus***	0.79 (0.23, 0.18-2.23) 11	**10, 12** V	11	8	**0.00025**
sp. 2	0.32 (0.31, 0-0.49) 3	12 V	3	1	nc
sp. 3	1.05 (0.39, 0-1.93) 5	3, 12, 18, 20 V	5	4	nc
Diplostomidae Diplostominae *Conodiplostomum* sp. 5	0.33 (0.28, 0-0.55) 3	**10** B	3	3	**0.00002**
Diplostomum **sp. 1**	0.74 (0.24, 0-3.25) 291	1-7, 10, 12-14, 16- 21 L	174	89	nc
sp. 2	0.62 (0.18, 0-2.01) 7	**18, 20** H, B	7	4	**0.00066**
sp. 3	0.14 (0.05, 0-0.55) 27	3, 5, 7, 12, 13, 16, 20, 23 L	21	20	nc
sp. 4	0.58 (0.16, 0-2.79) 242	1, 3, 5, 7, 8, 10-16, 18-20 L	220	131	nc
sp. 5	*n* = 1	12 H	1	1	nc
sp. 6	*n* = 1	20 H	1	1	nc
sp. 7	*n* = 1	20 H	1	1	nc
sp. 8	*n* = 1	23 Eye	1	1	nc
sp. 9	*n* = 1	11 H	1	1	nc
baeri	0.33 (0.10, 0-1.31) 75	**12** H	64 (3107)	20 (113)	**7.7×10^{-16}**

Table 3.3 continued Larval trematode species	Mean intraspecific divergence (%) in CO1 sequences (SE, range) *n* sequences*	Host(s) † Tissue(s)††	N metacercariae sequenced	N hosts from which sequenced metacercariae obtained	Probability of observed host specificity arising by chance§
Diplostomum					
huronense	0.41 (0.14, 0-1.78) 28	3, 12, 13, 16, 20 L	23	14	nc
indistinctum	0.68 (0.26, 0-1.53) 6	8, 13 L	3	3	nc
Diplostomidae gen. sp.	*n* = 1	23 B	1	1	nc
Hysteromorpha					
triloba	0.80 (0.24, 0-3.12) 20	**13, 17** M	13	6	**0.00001**
Tylodelphys					
scheuringi	0.35 (0.11, 0-1.54) 13	3, 12, 22, 23 H	36	13	nc
Crassiphialinae					
Ornithodiplostomum					
sp. 1	0.52 (0.20, 0-1.32) 16	**10-12** B	16	10	**0.00003**
sp. 2	0.75 (0.23, 0-1.79) 21	**16** H V	21	13	**4.8×10^{-12}**
sp. 3	2.26 (0.47, 0.23-4.50) 13	**20** V B H	13	8	**6.6×10^{-8}**
sp. 4	1.71 (0.35, 0-3.10) 2	**20** B	2	2	**0.01684**
sp. 8	3.18 (-) 2	**20** B	2	2	**0.01684**
Posthodiplostomum					
sp. 1	0.66 (0.27, 0-1.2) 6	**3** V	6	5	**7.0×10^{-6}**
sp. 2	0.29 (0.27, 0.34-0.17) 7	**5** V	4	4	**0.00078**
sp. 3	0.51 (0.15, 0-2.26) 46	**3, 5** V	43	19	**4.9×10^{-12}**
sp. 4	0.32 (0.13, 0-1.09) 16	**20** V	13	9	**8.0×10^{-9}**
sp. 5	0.62 (0.30, 0.44-0.95) 3	**5** M	3	2	**0.02835**
sp. 6	0.96 (0.27, 0.25-1.75) 6	**5, 9** M	3	3	**0.0048**
sp. 7	0.85 (0.25, 0-1.76) 8	**12** M	8	5	**0.00023**
sp. 8	0.38 (0.17, 0-0.55) 4	**6, 7** M V	4	2	**0.00010**

Table 3.3 continued

Larval trematode species	Mean intraspecific divergence (%) in CO1 sequences (SE, range) *n* sequences*	Host(s) † Tissue(s)††	N metacercariae sequenced	N hosts from which sequenced metacercariae obtained	Probability of observed host specificity arising by chance§
Diplostomoidea fam. gen. sp.	1.5 (0.34, 0.22-3.5) 9	**4, 5** M	9	3	**0.0051**
Clinostomum sp	0.32 (0.11, 0-1.84) 29	2, 5-7, 10, 12, 16 M	24	13	nc

Table 3.3 notes:

nc = not calculated because parasite was found in only a single individual host or because the host assemblage is diverse and parasite is not considered host specific.

*Based on Kimura 2-parameter distances (Kimura 1980) between sequences obtained from adults and metacercariae, pairwise deletion of gaps, and including sequences from lower quality DNA traces. Standard error calculated from 500 bootstrap replicates (Nei and Kumar 2000).

†Bold indicates the distribution is deemed host-specific, or potentially so. Only intermediate hosts shown. Hosts: 1 = *Labidesthes sicculus*, 2 = *Fundulus diaphanus*, 3 = *Ambloplites rupestris*, 4 = *Pomoxis nigromaculatus*, 5 = *Lepomis gibbosus*, 6 = *Micropterus dolomieu*, 7 = *Micropterus salmoides*, 8 = *Apollonia melanostomus*, 9 = *Morone americana*, 10 = *Etheostoma nigrum*, 11 = *Percina caprodes*, 12 = *Perca flavescens*, 13 = *Catostomus commersonii*, 14 = *Moxostoma macrolepidotum*, 15 = *Carpiodes cyprinus*, 16 = *Notemigonus crysoleucas*, 17 = *Notropis atherinoides*, 18 = *Notropis hudsonius*, 19 = *Notropis spilopterus*, 20 = *Pimephales notatus*, 21 = *Ictalurus nebulosus*, 22 = *Esox lucius*, 23 = *Rana pipiens*

††Tissues: B = brain, M = musculature, V = viscera, H = vitreous humour, L = lens

§ Bold values indicate the probability of the observed host-specific distribution of sequenced metacercariae arising by chance was less than 0.05 in Fisher's exact test.

§§ ITS sequence also obtained for species in bold, see Figure 3.5.

Table 3.4 Abundance of *Ornithodiplostomum* sp. 1 across localities and host species in the St. Lawrence River

Example of the distribution of a strigeid parasite across hosts and localities. CO1 and/or ITS sequences were obtained from specimens of *Ornithodiplostomum* collected from one or both of these two percid hosts at all six localities, and all were shown to be the same species (*Ornithodiplostomum* sp. 1). These identifications were used to estimate the mean abundance of *Ornithodiplostomum* sp. 1 (mean number of worms per host), in each host population, as seen in cells in the table. The qualitative abundance of fish hosts at each locality reflects capture effort: "■" indicates the host was abundant, "□" indicates the host was present but not abundant, and "-" indicates the host was not found in multiple expeditions during a three-week period in June-July 2006. Infection levels in the alternate host (*Perca flavescens*), which was consistently abundant, do not approach those found in the optimal host (*Etheostoma nigrum*) regardless of the abundance of the optimal host. In addition, *Ornithodiplostomum* sp. 1 was never observed in sympatric non-percids although these were also abundant throughout the system (see Table 3.2). Two of three logperch (*Percina caprodes*: Percidae) examined from locality 4 were infected with *Ornithodiplostomum* sp. 1, with a mean abundance of 20.

	Lake St. Francois		Lake St. Louis		Lake St. Pierre	
	1	2	3	4	5	6
Perca flavescens	■ 0	■ 0	■ 0.31	■ 0.33	■ 0.26	■ 1.8
Etheostoma nigrum	□ 6.0	■ 4.31	■ 16.33	■ 4.00	-	-

Table 3.5 Regression of phylogenetically independent contrasts in lens-infection and host specificity

Standard multiple regressions of phylogenetically independent contrasts in lens-infection, sampling effort and indicators of host specificity. The negative slope of lens-infection terms in all models indicates that strigeids inhabiting the lens have a larger and more diverse assemblage of hosts (lens = 0, non-lens = 1), i.e., lower host specificity. (a) Host Range: The number of metacercariae sequenced explains more variance in host range than whether or not strigeid species inhabit the lens, but lens infection also explains a significant and unique portion of variance. b) Taxonomic diversity of hosts (S_{TD}). Sampling effort was not included in the model because neither measure (N metacercariae sequenced, N hosts in which sequenced metacercariae occurred) showed significant correlation with S_{TD} ($P > 0.158$). c) Variance in taxonomic diversity of hosts ($VarS_{TD}$). Both sampling effort and lens infection make significant contributions to the model, but lens-infection explains a larger unique portion of the variance in $VarS_{TD}$. S_{TD}* is not presented because the results are nearly identical to those of S_{TD}.

a) Host Range

Model	Sum of squares	*df*	Mean square	*F*	*P*	Adjusted r^2
Regression	225.760	2	112.880	125.865	<0.0005	0.874
Residual	30.492	34	0.897			
Total	256.253	36				

Coeffic ents

Model		β	*t*	*P*	Zero-order *r*	sr^2
Sampling e	t (N					
metacercar		0.831	12.882	0.002	0.917	0.762
sequenced)						
Lens=0/no	ns=1	-0.217	-3.363	<0.0005	-0.548	-0.199

b) Taxonomic diversity of hosts (S_{TD})

Model	Sum of squares	*df*	Mean square	*F*	*P*	r^2
Regression	10.747	1	10.747	6.335	0.017	0.153
Residual	59.378	35	1.697			
Total	70.125	36				

Coefficients

Model	β	*t*	*P*
Lens=0/non-lens=1	-0.391	-2.517	0.017

c) Variance - taxonomic diversity of hosts ($VarS_{TD}$)

Model	Sum of squares	*df*	Mean square	*F*	*P*	Adjusted r^2
Regression	4.246	2	2.123	10.577	<0.0005	0.347
Residual	6.824	34	0.201			
Total	11.070	36				

Coefficients

Model	β	*t*	*P*	Zero-order *r*	sr^2
Sampling effort (N metacercariae sequenced)	0.831	12.882	0.049	0.474	0.275
Lens=0/non-lens=1	-1.348	-2.964	0.006	-0.555	-0.399

Figure 3.1 The most common life-cycle pattern among strigeid trematodes

Adult parasites in the intestine of definitive hosts (a bird or mammal) reproduce sexually yielding eggs that pass in host feces. Short-lived dispersal stages hatch from eggs and penetrate snails, the first intermediate host. Larval stages in snails reproduce asexually, yielding large numbers of free-living dispersal stages that penetrate fish, the second intermediate host, where they develop into metacercariae. Metacercariae often form cysts in fish hosts and different strigeid taxa occur in somatic tissues such as the eyes, brain, musculature, skin and viscera. Definitive hosts such as piscivorous birds become infected after feeding on infected fish.

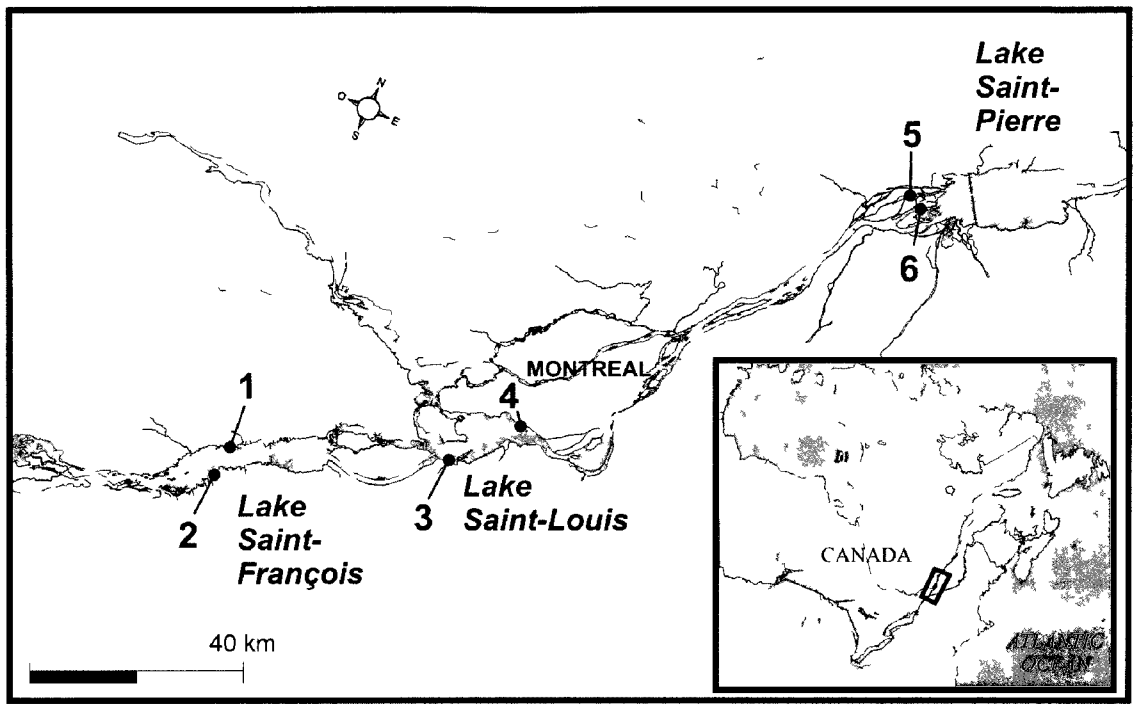


Figure 3.2 Sampling localities in the St. Lawrence River where most fish were collected

Sampling localities where most fish used in this study were collected during a three-week period in June and July 2006. In addition to the fish in Table 3.2, 16 other species of fish from these localities were collected in smaller numbers ($1 < n < 20$). Studies using mark-recapture and population genetics have shown there is little mixing of yellow perch in the St. Lawrence River among all sampling localities except 5 and 6, owing to distance between sites and physical barriers such as regulatory structures and a dredged shipping channel that is impassable to small fish (CSL 1996; Dumont 1996; Leclerc *et al.* 2008). It is likely that populations of the other fish species under study are separated among these localities much like yellow perch, and the fish communities sampled at each locality can therefore be considered independent. Habitat varies at the six localities. The two upstream localities (1, 2) are in a shallow lake where depth is maintained by regulatory

structures, while water levels fluctuate seasonally in the downstream lakes (CSL 1996). Two separate, distinctive water masses meet in Lake St. Louis but do not fully mix until downstream of the sampling area. Locality 3 is in the mouth of a river receiving agricultural and industrial pollutants, 4 is near a major urban centre, while 5 and 6 are in a relatively undisturbed, RAMSAR-protected wetland and UNESCO World Heritage Site, a staging ground for numerous piscivorous birds. Locality place-names and latitude-longitude coordinates are:

1. Creg Quay Marina 45.161, -74.430
2. Pointe Dupuis 45.128, -74.404
3. Beauharnois 45.317, -73.879
4. Dorval Island 45.433, -73.734
5. Ile aux Ours 46.132, -73.051
6. Iles aux Sables 46.115, -73.734

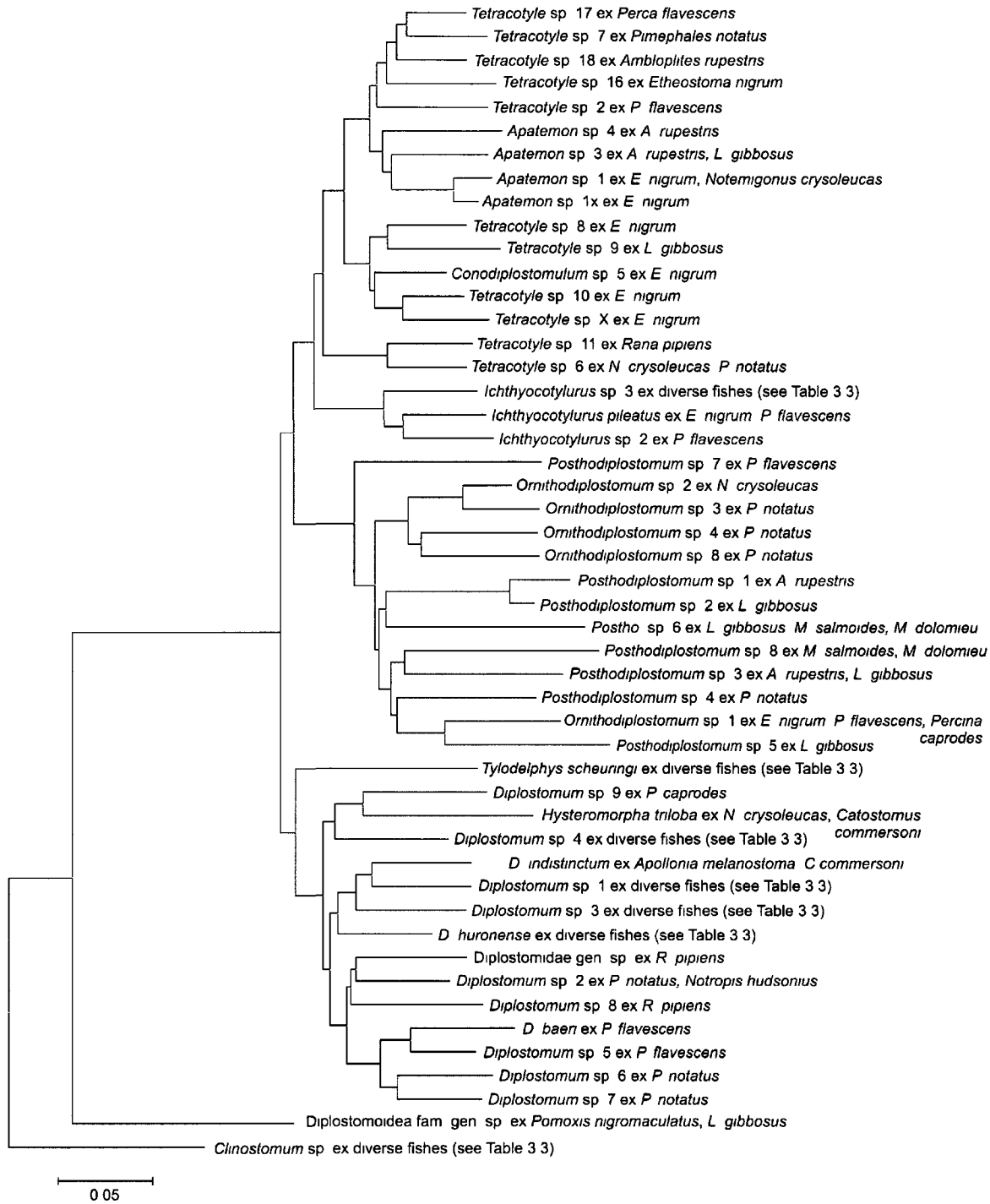


Figure 3.3 Neighbour-joining analysis of cytochrome c oxidase 1 sequences in strigeid species detected in fish and frogs in the St. Lawrence River

Figure 3.3 continued. Neighbour-joining analysis of Kimura-2-parameter (K2P) distances (Kimura 1980) between representative CO1 sequences from strigeids collected from fish and frogs in the St. Lawrence River, Canada. Among the CO1 sequences over 400 bp from 955 strigeid specimens, the mean within-genus interspecific K2P divergence ranged from 3.6 to 23.1% and was on average 13.3%.

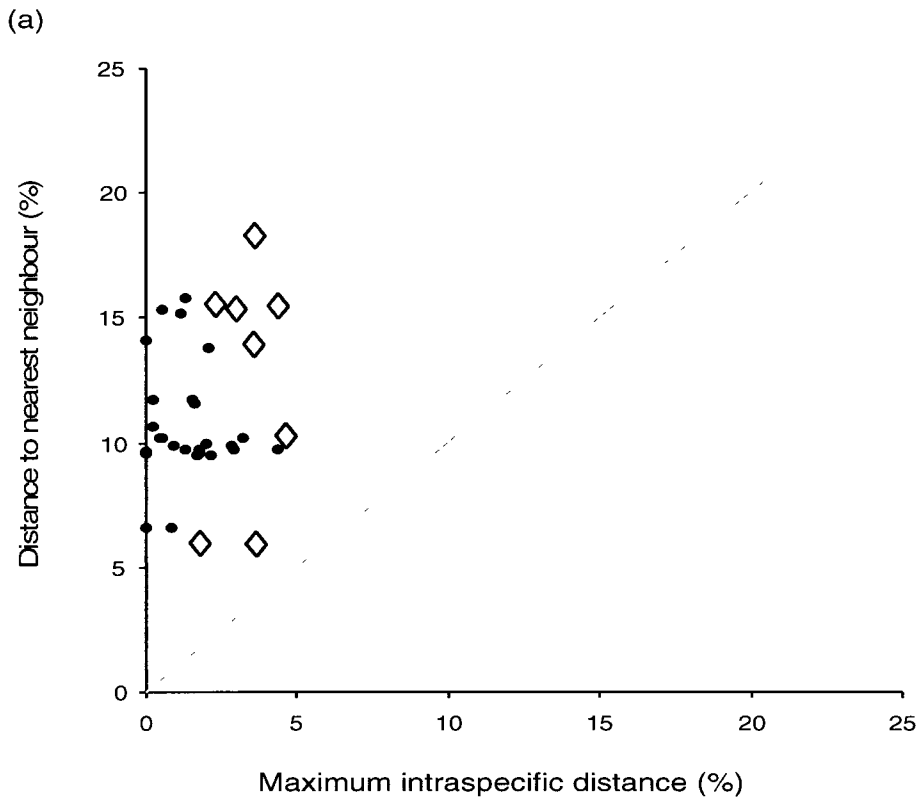
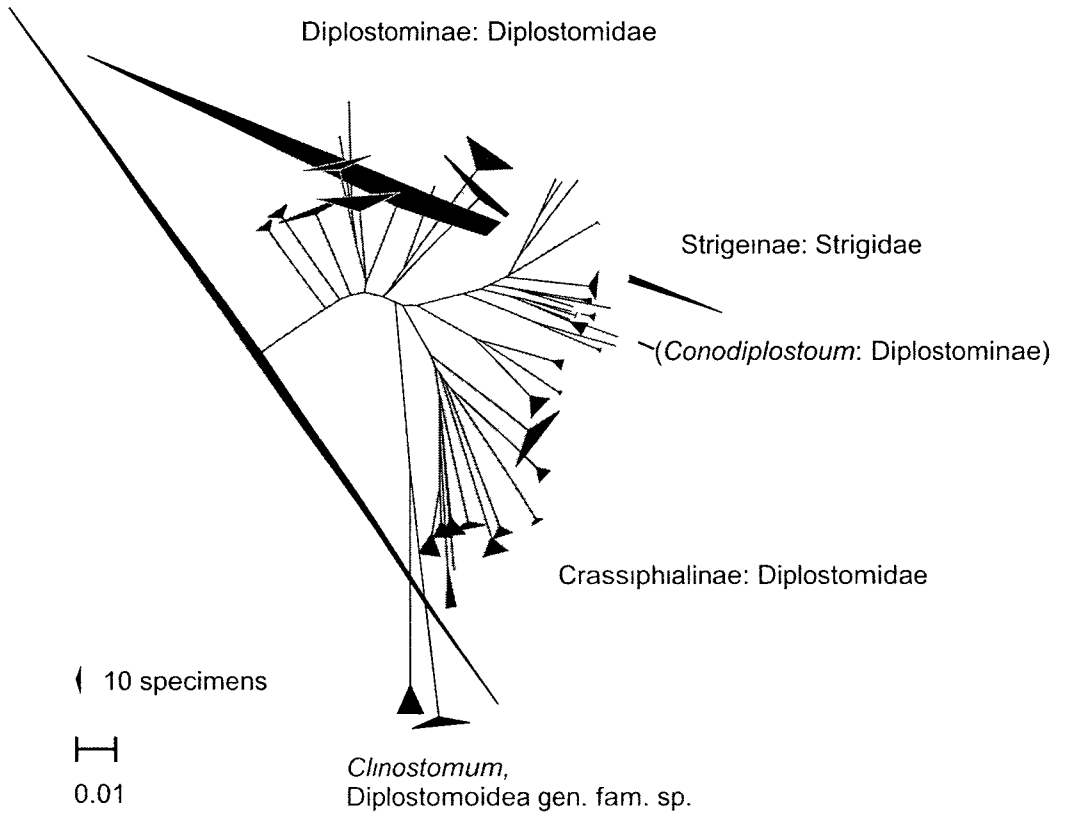


Figure 3.4. Variation of cytochrome c oxidase 1 sequences within and between species in 40 strigeid species detected by DNA barcoding

Two ways of visualizing intraspecific and interspecific divergence in CO1 sequences among 40 strigeid species detected in the present study. In (a) the shortest genetic distance in CO1 sequences between 40 strigeid species is plotted against the maximum distance within species (Kimura 2-parameter distance (%) between 794 specimens with sequences over 420 bp). Diamonds indicate comparisons between species in which poor quality trace files are believed to have inflated intraspecific variation. Although sequences varied more than expected within nine species (Table 3.3, points above > 2% along x-axis), CO1 sequences of the most divergent specimens were never closer to members of another species than to conspecifics. This is indicated by all points occurring

above the y=x line. Nine species that were represented by single specimens are not included here (see Table 3.3). The median number of specimens per species was 6.

(b)



In (b) a neighbour-joining phenogram shows K2P distances between CO1 sequences over 400 bp in length from 955 strigeid specimens. Depth of triangles in (b) indicates intraspecific variation (proportionate to scale bar) and width of triangles is proportionate to the number of sequences (= number of specimens) (see legend). See Table 3.3 for genera included in each subfamilies. Within species, sequences diverged by an average of 0.46% (range 0 – 4.5%). Although sequences varied more than expected within nine species (Table 3.3, see also 3.4a), in most cases this was likely due to poor quality traces and overall clustering was unambiguous.

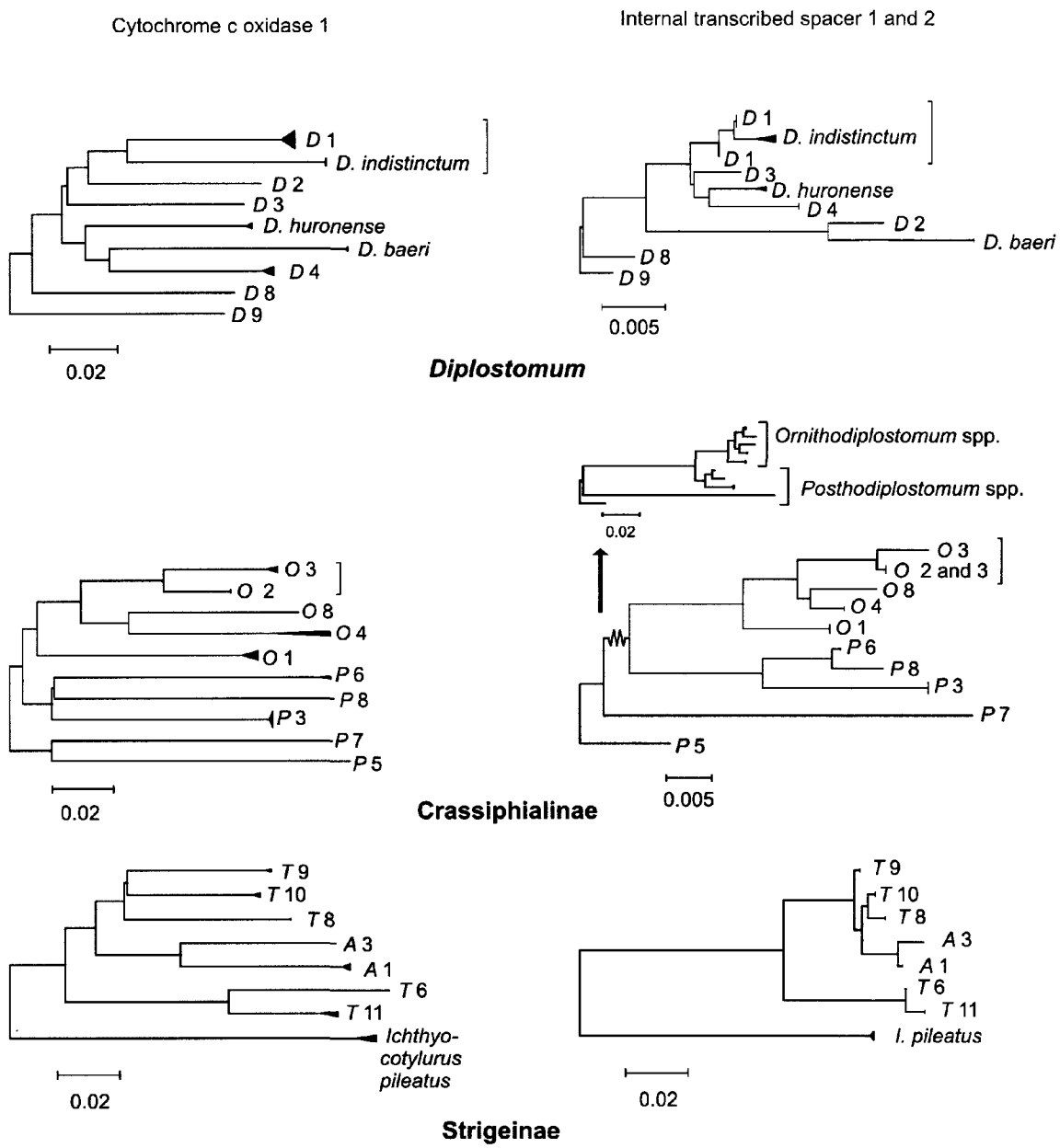


Figure 3.5 Comparison of neighbour-joining analyses of sequences of cytochrome c oxidase 1 and internal transcribed spacer in 27 strigeid species

Neighbour-joining analyses of Kimura-2-parameter distances based on separate alignments of partial cytochrome oxidase 1 (left) and rDNA internal transcribed spacer regions 1 and 2 (right) from 74 strigeid specimens in sibling taxa in three subfamilies collected from fish, amphibians and birds in the St. Lawrence River, Canada. D =

Diplostomum, O = *Ornithodiplostomum*, P = *Posthodiplostomum*, T = *Tetracotyle*, A = *Apatemon*; see Table 3.3 for a guide to subfamilies. Width and depth of triangles on branches represent sample size and intraspecific variation, respectively (see also Figure 3.4b). Topologies are not perfectly congruent, but both markers show the same species boundaries except between *D. indistinctum* and *Diplostomum* sp. 1, and between *Ornithodiplostomum* spp. 2 and 3. Both ITS and CO1 were sequenced in 48 other specimens representing six other species (see Table 3.3) but because no sibling species were encountered for these groups, they are not shown here.

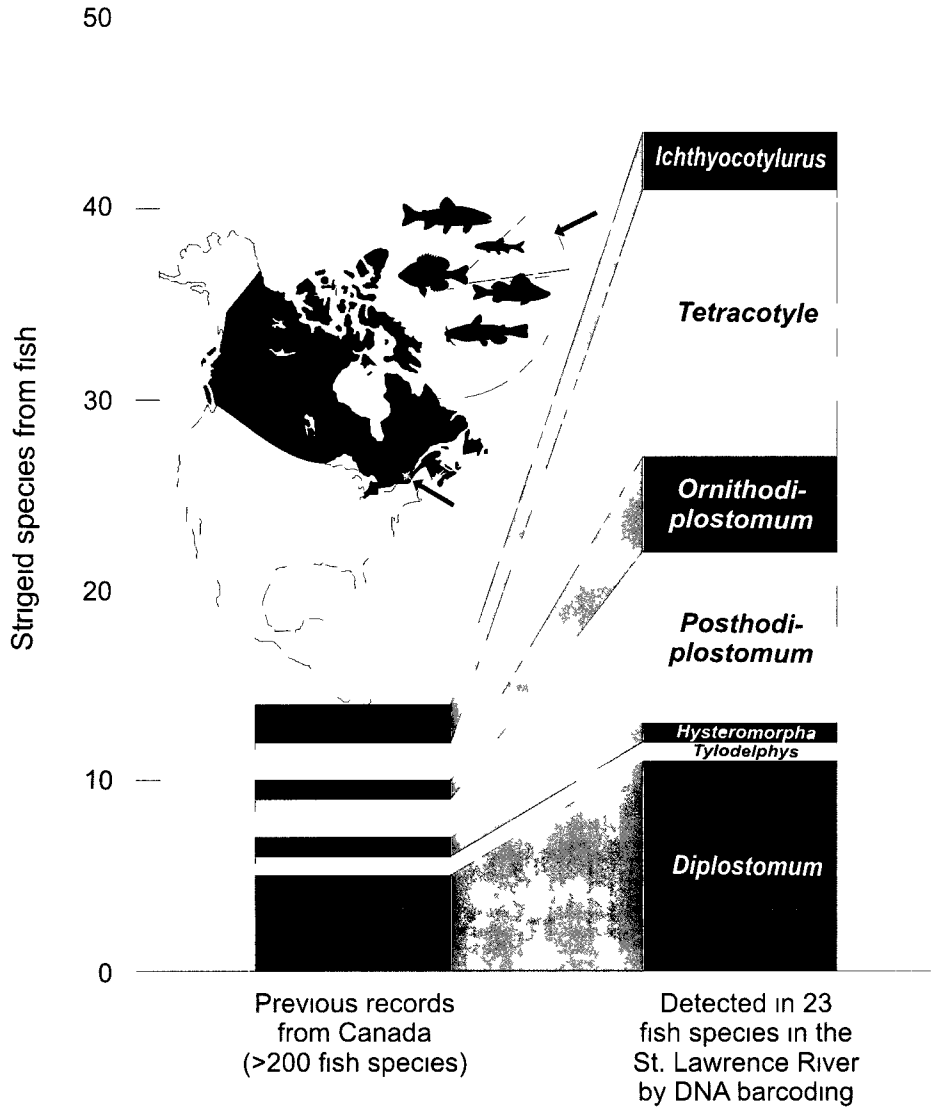


Figure 3.6 Diversity of strigeid metacercariae in fish from the St. Lawrence River detected by DNA barcoding compared to previous Canadian records

The diversity of strigeid metacercariae detected with CO1 sequences in the present study compared to that previously recorded in Canadian fishes (Margolis and Arthur 1979; McDonald and Margolis 1995; Gibson 1996). Molecular data revealed nearly a four-fold increase in strigeid species, even though the survey included less than half the fish species inhabiting a 150-km reach of a single river (Y. Reyjol, Ministère des Ressources

Naturelles et de la Faune du Québec, unpublished data), representing less than a tenth of Canada's fish fauna (pie chart, inset) and a tiny fraction of its surface area (map, inset).

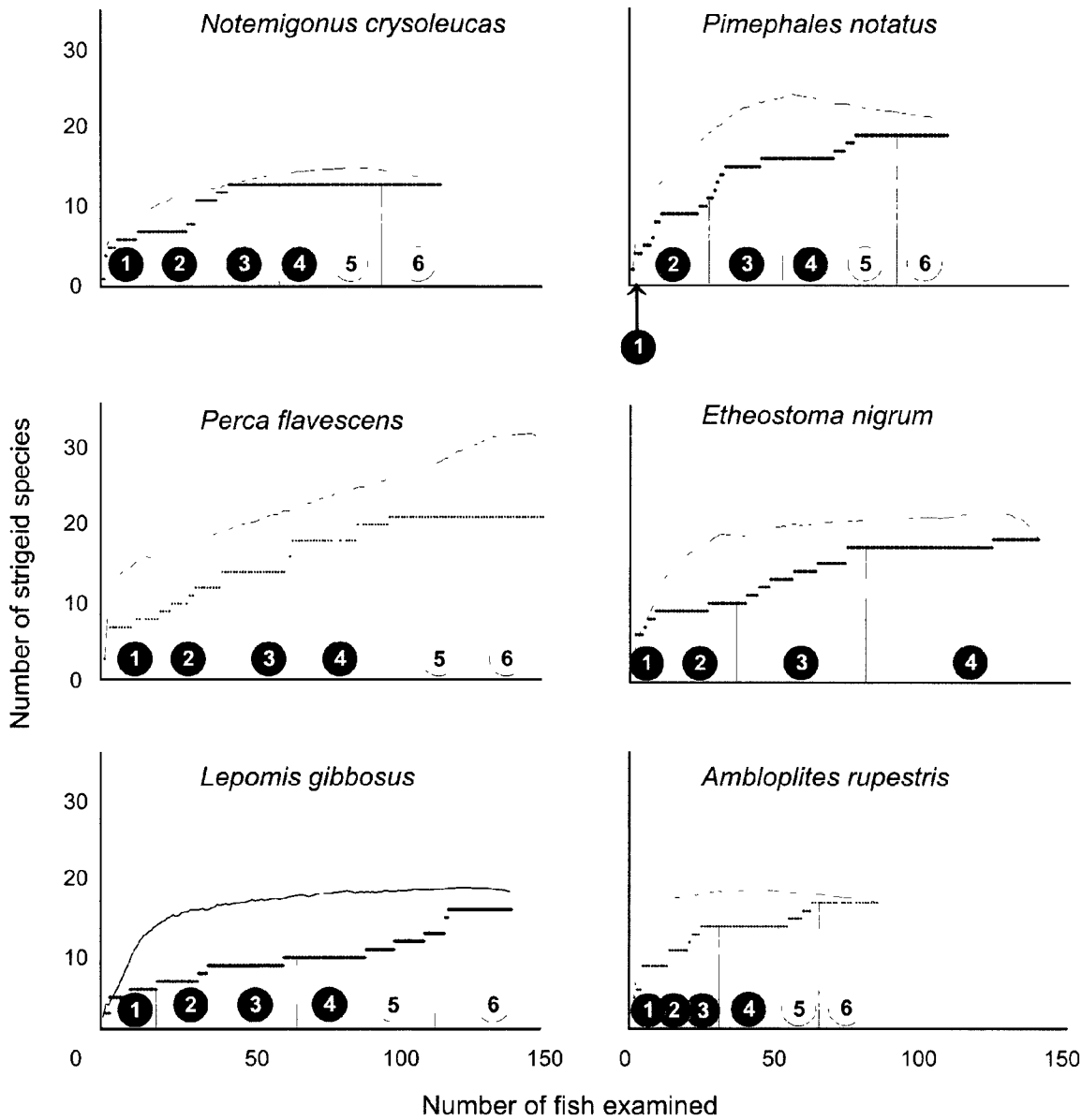


Figure 3.7 Species accumulation curves for strigeids from six focal fish species

Species richness in strigeid metacercariae as a function of number of individual hosts examined in six host species from the St. Lawrence River, Canada. Stepped series of points show the total number of larval strigeid species observed in successively pooled hosts, which are presented in the order in which they were examined in each locality, with localities arrayed in upstream → downstream order (circles are numbered according to localities in Figure 3.2, and upstream lakes are darker than downstream lakes). Lines

are Chao2 estimates of total species richness, which are based on 999 randomizations and the numbers of rare species that occur in only one or two individual hosts (Clarke and Gorely 2006; Dove and Cribb 2006). Chao2 tends to overshoot and then converge on an asymptote of estimated species richness. In *Perca flavescens*, the Chao2 function appears to be beginning to converge on an asymptote, which suggests that < 10 additional strigeid species would be encountered if more individual *P. flavescens* were sampled in the St. Lawrence River. In the other five host species, Chao2 converges on observed species richness, suggesting most strigeid species parasitizing these hosts at these localities in the St. Lawrence River were detected in the present study. Geographically, observed species richness increases with increasing numbers of hosts, but no particular locality or lake is consistently associated with increased diversity.

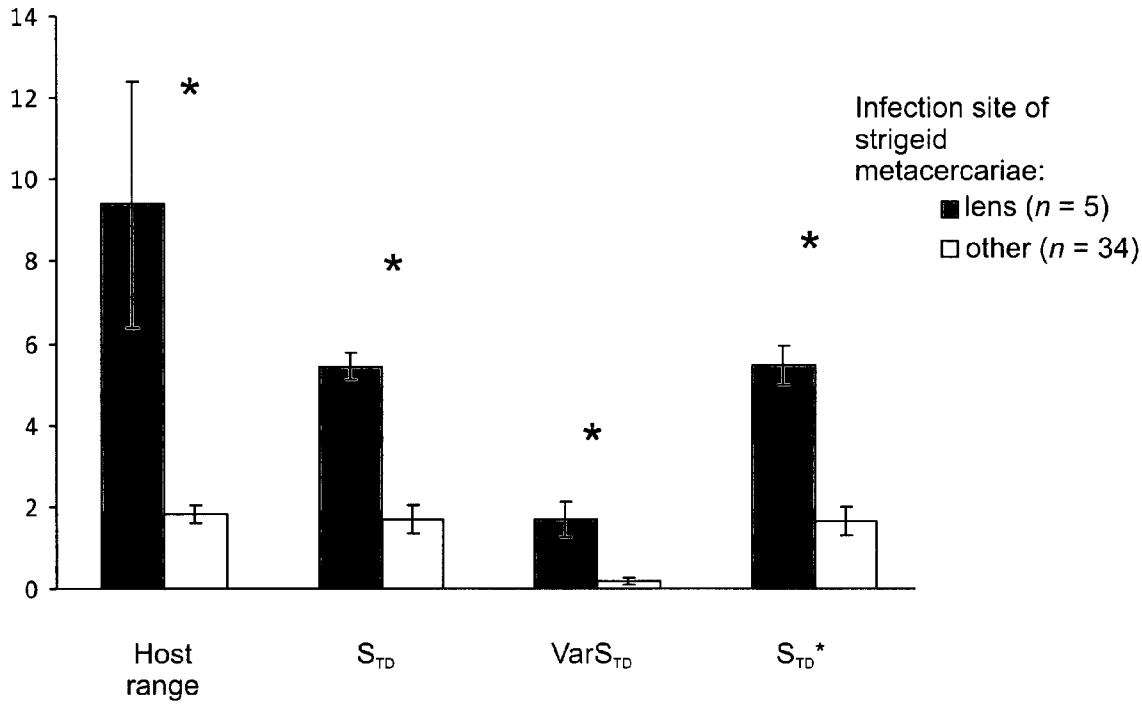


Figure 3.8 Comparison of host specificity in strigeid metacercariae inhabiting the lens and other tissues of fish

Mean values (+/- standard error) of host-specificity indicators in 39 species of metacercariae that inhabit the lens or other tissues of fish hosts. The colors of the bars indicate the infection site of species of larval strigeids (see inset). The index of host specificity measured on the y-axis is indicated under each pair of columns. Lens-infecting species are significantly less host specific than non-lens species. In analyses of covariance (ANCOVA) controlling for the number of sequenced metacercariae and the number of hosts in which they occurred (see Table 3.3), lens-inhabiting species infected an assemblage of host species that was larger (Host range, $F_{1,33} = 11.679$, $P = 0.002$), more diverse (S_{TD}, $F_{1,33} = 9.532$, $P = 0.004$), more taxonomically heterogeneous ($VarS_{TD}$, $F_{1,33} = 9.891$, $P = 0.004$) and in which parasites were more unevenly distributed among different host species (S_{TD}*, $F_{1,33} = 9.806$, $P = 0.004$).

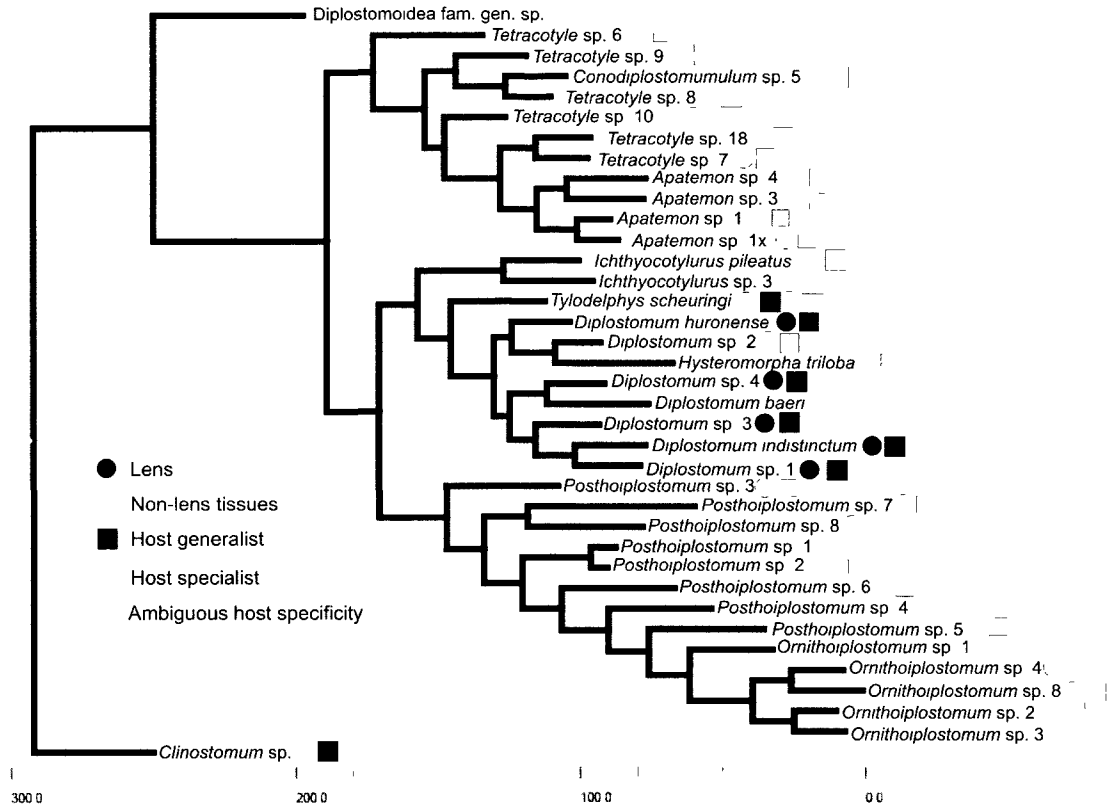


Figure 3.9 Phylogeny used in independent contrast analyses

Phylogeny used for generating independent contrasts between lens infection and host specificity, based on maximum parsimony of partial cytochrome oxidase I sequences from representatives of strigeid species occurring as metacercariae in freshwater fishes from the St. Lawrence River, Canada. Branch lengths are untransformed and based on the best-supported tree in 500 bootstrap replicates of the nine most parsimonious trees with 1018 steps generated from 364 common sites and a closest-neighbour-interchange level of 3. Species with filled circles inhabit the lenses of the eyes of fish hosts and those with empty circles occur in other tissues. Black squares indicate the parasite is a categorized as a generalist in phylogenetically independent correlation of discrete characters; empty squares are host specialists; grey squares have ambiguous host specificity.

Chapter 4 Hosts matter more than habitat for freshwater fish parasite communities

Introduction

Although the structure of parasite communities has been a focus of parasite ecology for decades, few widely applicable generalizations have been discovered (Poulin 2007a), even in well-studied hosts such as freshwater fish (Kennedy 2009). Numerous factors affect parasite communities with variable consistency in these hosts. In this study, we compared the relative importance of host phylogeny and habitat in structuring parasite communities in six species of freshwater fish from the St. Lawrence River, Canada.

A fundamental aspect of a parasite's ecology is its distribution among host taxa, i.e., its host specificity. Although usually studied from the point of view of individual parasite taxa, host specificity can also be considered to be an attribute of parasite communities. Most parasite species are host specific (Poulin and Morand 2004) and specificity should therefore emerge at the community level as well. An absolutely host-specific community of parasites would be limited to a single host species, while the boundaries of less host-specific parasite communities will encompass multiple host species that are more distantly related. In this sense, host specificity is measured with direct reference to the phylogeny of host taxa.

Parasite communities also vary along environmental gradients. In freshwater fish, habitat attributes predictive of parasite community variation are related to water quality, bathymetry, and overall fish community composition (Leong and Holmes, 1981; Bergeron *et al.* 1997; Fellis and Esch 2005a; Goater *et al.* 2005). Much of the similarity of parasite communities in separate host populations can be explained by the geographic

distance between them (Poulin and Morand 1999; Poulin 2003; Karvonen and Valtonen 2004; Fellis and Esch 2005a, b; Karvonen *et al.* 2005a). Essentially, parasite communities that are closer together are more similar than distant ones, and a plausible explanation for this relationship is that proximate habitats tend to be more similar than distant habitats (Soininen *et al.* 2007; Vinarski *et al.* 2007). In the present study, geographic distance is used as surrogate measure of habitat similarity.

Herein we compare the influence of these two fundamental sources of variation in parasite communities, namely the phylogenetic distance between host species and geographic distance between habitats. Both types of distance are tested simultaneously in our analyses, but for rhetorical purposes, we might first consider phylogenetic distance between hosts in isolation. Do closely related host species tend to have similar parasite communities? Poulin (2010) found a weak relationship between the qualitative similarity of parasite communities and phylogenetic distance in a meta-analysis of parasite data from 45 species of freshwater fish. Using a more quantitative approach, Poulin (2005) found no phylogenetic signal in infection levels of individual parasite taxa (rather than communities) among sympatric freshwater fish species. Our dataset includes spatial replicates of host species that are closely related but ecologically dissimilar and host species that are ecologically similar but phylogenetically distant, allowing a robust evaluation of this question at the level of the parasite community.

We wanted to test which of these two types of separation, phylogenetic distance between host species, or geographic distance between host populations, is a stronger predictor of their parasite community similarity. No study has examined this particular question, but two related studies found that host phylogeny explains all (Seifertová *et al.*

2008) or much (Vignon and Sasal 2009) of parasite community structure, compared with geographic distance. However, Poulin (2010) suggested that the phylogenetic distance between hosts had a much smaller effect on parasite community similarity than geographic distance.

Our study differs from other surveys of parasite communities in multiple host species in several respects. Firstly, the study is based on original field data that minimizes temporal and seasonal variation, and was designed to isolate spatial and phylogenetic effects on parasite communities with appropriate replication. In addition, the entire metazoan parasite community was inventoried in every fish, rather than only adult helminths, and morphological parasite identifications were done by the same person and thus are consistent within the study. In contrast, most comparable studies have not controlled for one or more of these potentially confounding factors (Poulin and Morand 1999; Poulin 2003, 2005, 2010; Karvonen *et al.* 2005a; Seifertová *et al.* 2008; Pérez-del-Olmo *et al.* 2009; Timi *et al.* 2009). Secondly, molecular methods were used to discriminate species in an important group of larval helminths (Locke *et al.* 2009; Locke *et al.* in preparation - see Chapter 3). Thirdly, we employed non-parametric analyses which have not been widely used in parasitology in order to examine the effects of geographic and phylogenetic factors on parasite communities. These approaches can be used with unbalanced designs and make no assumptions about data distribution or the ratio of cases to observations, which make parasite ecological data problematic for conventional multivariate techniques.

Materials and methods

Sampling

Six species of fish were collected at six localities in three fluvial lakes in the St. Lawrence River near Montreal, Quebec during a three-week period in June-July of 2006 (Table 3.2; Figure 3.2). Seven hundred and five fish were collected using a beach seine and only 1+ year-class, or the smallest sized fish available were retained. Fish were killed in an overdose of clove oil solution and frozen until dissection. This ensured that fish were local and had been exposed to the same pool of parasites for the same amount of time prior to capture. At necropsy fish were thawed and weight and length were recorded. Parasites were identified to the lowest taxonomic level possible using the keys of Beverly-Burton (1984), Kabata (1988), Arai (1989), Gibson (1996) and Hoffman (1999). Strigeid metacercariae were stored in 95% ethanol for DNA analysis as described in Moszczynska *et al.* (2009) (Chapter 1), Locke *et al.* (2009) (Chapter 2) and Locke *et al.* (in preparation - see Chapter 3). DNA was extracted from strigeid specimens and species were distinguished based on sequences from the barcode region of cytochrome oxidase 1 (CO1) and, in some cases, internal transcribed spacer (ITS) regions of rDNA. Larval stages of nematodes, cestodes and acanthocephalans were identified to generic or family level based on morphology and host specificity, using the above-mentioned keys.

Ecological analyses

The effects of host and habitat on parasite community similarity were assessed using a range of non-parametric multivariate techniques with PRIMER v.6 and PERMANOVA+ (PRIMER-E, Plymouth, UK). In many of these techniques the response

variable was the Bray-Curtis similarity (or dissimilarity) between the parasite communities of two individual fish (i.e., infracommunities). Bray-Curtis similarity is both quantitative and qualitative. If calculated based on the untransformed abundance of parasites, this index will reflect the distribution of common parasite species. Transformations can be performed on parasite abundance that place increasing emphasis on rare parasites species. The most drastic transformation (presence/absence) assigns equal weight to abundant and rare species and represents qualitative community similarity. Thus, depending on the transformation of parasite abundance data, analyses based on Bray-Curtis similarity reflect different aspects of parasite community structure, including species richness, composition and abundance.

Similarities between parasite communities were visualized with non-metric multi-dimensional scaling (MDS) prior to subsequent analyses and to aid with their interpretation. Although MDS is not a hypothesis-testing technique, it was clear that parasite communities formed groups reflecting host phylogeny and subsequent analysis sought to compare the influence of host phylogeny with that of habitat.

Two different approaches were used to determine whether attributes related to the host or its habitat were more important for parasite community structure. One set of analyses sought to identify which type of category (host or habitat) best discriminates among parasite communities. The second approach examined which of the two types of distance (host or habitat) were more strongly correlated with parasite community similarity.

Among the categorical analyses, the first test compared whether host species or locality provided better discriminatory power in parasite infracommunities. A model II

(random effects) multivariate permutational analysis (PERMANOVA, Anderson 2001) was used to determine the extent to which host species and sampling locality explained multivariate similarity in parasite communities, while controlling for host length (normalized within species). This technique is analogous to multivariate analysis of co-variance (MANCOVA), but partitions the distances in the multivariate data cloud of dissimilarities that can be attributed to different factors. The stratified sampling design corresponded to a two-way crossed PERMANOVA design (host species × locality) with two empty cells (because johnny darter was caught at four of six localities) and a high number of residual degrees of freedom. PERMANOVA was based on square-root transformed parasite abundance of the entire parasite community, with no dummy variable added (i.e, no uninfected fish, see below). The square-root transformation reduces the influence of abundant species and results in a scale of abundance ranging from 0 to about 6 and therefore it is still a quantitative index of community similarity. By constructing variance terms according to a random effects model, the hypothesis tested regards not only the particular host species and localities surveyed, but any host species from any localities of which these can be considered random representatives (Anderson 2001). In other words, this test could be thought of as an assessment of the effects of these categories in parasite communities in cyprinid, centrarchid and percid hosts in North American waters.

As a further investigation of categorical partitioning in parasite communities, analysis of similarities (ANOSIM) was performed to examine the extent to which parasite communities are structured by different hierarchical levels of categories relating to the host (e.g., host species, family, and order) and its habitat (locality, lake). ANOSIM is

analogous to analysis of variance (ANOVA) and yields a global R statistic that is proportionate to the magnitude of overall difference between factors, much like the *F* statistic of ANOVA (Clarke 1993). Unlike PERMANOVA, which uses actual dissimilarity values, ANOSIM ranks dissimilarities and tests whether ranks are, on average, higher within or between factors.

Canonical analysis of principal coordinates (CAP) was conducted as a complementary method of evaluating parasite community categories (Anderson and Willis 2003). This ordination approach identifies axes through the multivariate cloud of pairwise resemblances in order to maximize discrimination among *a priori* groups. In the present context it was used to determine whether parasite communities are predictive of membership in taxonomic and geographic groups. Because CAP is computationally intensive, the data set was reduced from 705 infracommunities to 34 component communities (all parasites in a host population, see Appendix), based on the average abundance of each parasite species in a given host species at a given locality. CAP was conducted on a Bray-Curtis resemblance matrix (based on square-root transformed abundance) among these 34 component communities, and leave-one-out diagnostics were used to designate the number of axes generated.

The foregoing analyses evaluated which of two types of categories, the host or its habitat, corresponded more strongly to partitions in parasite communities. If host phylogeny has a greater effect on parasite communities, then host-related categories will describe parasite community boundaries. If habitat attributes are more important, then spatial categories will be more important, because parasite communities from a given locality or lake are subject to the same habitat conditions.

The second set of analyses addressed essentially the same host-versus-habitat question, but from the point of view of distances between parasite communities. The geographic distance between parasite communities was used as a surrogate measure of habitat variation (Soininen *et al.* 2007; Vinarski *et al.* 2007). This was compared to the phylogenetic distance between hosts, which was estimated using the classifications of species, genus, subfamily, family, class, order and superorder (classifications followed Nelson 2006). Conspecifics were assigned a taxonomic distance (TD) of zero and TD increased by 1 unit in steps to a taxon common to both hosts. For example, golden shiner (*Notemigonus crysoleucas*) and bluntnose minnow (*Pimephales notatus*) are in the same subfamily (Leuciscinae), which results in all hosts in these species being separated by TD = 2. Both these cyprinid hosts are in the same superorder as the other four hosts and are therefore separated from the latter by TD = 6 (see inset to Figure 4.5).

Nonparametric correlations were used to quantify how strongly phylogenetic and geographic distances between hosts were associated with the similarity of their parasite communities. To avoid a biased view of community similarity, parasite abundance was subject to a series of transformations giving progressively greater emphasis to rare species (no transformation, square-root, fourth root, log (x+1), presence/absence). These transformations were conducted for the entire parasite community as well as for two separate guilds of parasites, namely allogenic parasites (which have at least one terrestrial host) and autogenic parasites (which complete their life cycles in the aquatic environment). Finally, for each of these guilds and transformations, similarities were also calculated with a single individual in a dummy parasite species added to every fish. This was done because Bray-Curtis similarity is undefined between empty communities. By

adding a dummy parasite, two uninfected fish (i.e., having no real parasites) are calculated to be 100% similar. In the present case, including a dummy variable allowed 38 fish with no parasites out of 705 examined to be included in the analysis. This "zero-adjustment" to Bray-Curtis similarity is appropriate when comparing communities that lack species for the same biological reason (Clarke *et al.* 2006), which likely applies to uninfected hosts exposed to the same pool of parasites.

The addition of a dummy parasite, transformations of parasite abundance data and partitioning of the parasite community into guilds resulted in a proliferation of similarity matrices. For each, it was desirable to see which of the two predictor matrices (taxonomic or geographic distance) was the stronger correlate. To visualize the relationships between these matrices, the correlation coefficients between them were themselves treated as a distance measure in 2STAGE, which is essentially an ordination of ordinations (Clarke and Gorley 2006).

Following 2STAGE analysis, three matrices were selected for statistical testing, one of each type of predictor matrix, and one matrix based on community similarity. However, matrix data are not independent because, in the present example, each infracommunity was compared with the 704 remaining infracommunities, and the resulting matrix yielded > 247 000 data points from 705 observations. To control for the non-independence of pairwise comparisons, the relationships between matrices were first evaluated using RELATE, a non-parametric form of the Mantel test (Clarke and Gorley 2006). If the relationship was significant, standard multiple regression was then performed to see which of the two predictor matrices (host phylogeny, geographic distance) explained more variance in the parasite community similarity matrix. Multiple

regression also included Euclidean distances between fish calculated based on another expected correlate of parasite community similarity, fish length (normalized within species) (Poulin, 1995).

Results

In total, 79 species of metazoan parasites were encountered in the six host species (Appendix 4.1). Over half (47) were species of larval strigeids detected by DNA barcoding (Locke *et al.* 2009; Locke *et al.* in preparation - see Chapter 3). On average, fish were infected with 35 individual metazoan parasites and larval strigeids accounted for 19 of these.

Across the six localities and host species, infracommunities and component communities showed a pattern that strongly reflected the phylogeny of their hosts and was less consistent with collection localities (Figures 4.1 and 4.2). A large effect of host phylogeny also emerged in subsequent statistical tests.

Host and habitat as parasite community boundaries

The strongest predictor of parasite community structure in the two-way crossed PERMANOVA was host species, as indicated by the large *pseudo-F* statistic associated with this term (Table 4.1), followed by length and interaction effects. The small *pseudo-F* statistic for locality (1.8617), although significant, shows that this factor describes comparatively little parasite community structure compared with other factors.

There was a significant interaction effect between host species and locality. The nature of the interaction can be visualized in an MDS of the component communities (Figure 4.2b), which shows that the direction of locality effects on parasite communities

differs among host families, but at every locality there are differences between species. To verify this, main effects and pairwise PERMANOVAs (model II) were conducted to detect and assess differences in parasite infracommunities between host species within each locality. These showed that there were significant differences in infracommunities between every host species at every locality, except the two cyprinid hosts at locality 1 (PERMANOVA, *pseudo-t* = 1.4722, $P = 0.093$). However, this marginally significant difference may be related to the small sample size (n = 4) of *P. notatus* at that locality (see Table 3.2).

There was also a significant interaction between host species and fish length, although the low *pseudo-F* value indicates the magnitude of this interaction was comparatively small. Here again, the MDS of the component communities is instructive (Figure 4.2c). Within each species, lengths are distributed heterogeneously among localities. For example, the largest *A. rupestris*, *P. flavescens*, *E. nigrum*, and second largest *P. notatus* were found at locality 1, where *L. gibbosus* and *N. crysoleucas* were small. A series of ANOVAs showed there were significant differences in how lengths within each host species were partitioned geographically at all localities except locality 5 (ANOVA $F_{4,98} = 1.434$, $P = 0.229$), where all fish were uniformly smaller than conspecifics at other localities (normalized lengths ranging from -0.7 to -0.3). However, both main effects and pairwise PERMANOVA tests (see above) indicated significant separation between all host species at this locality as well. In conclusion, the effects of length and locality differ in strength and direction among the six host species, although effects of both are similar within host families. Nonetheless, parasite communities appear to differ among all host species regardless of length and locality.

A series of ANOSIMs indicated that there were differences in infracommunities at the scale of the lake and locality within each host species and, within each locality, among host orders, families and species (Figure 4.3). The pairwise components of these ANOSIMs revealed that all levels of all factors (locality, lake; host species, family and order) differed significantly from one another ($P < 0.0005$). Geographic partitioning was low to strong, with the smallest difference occurring between localities 5 and 6 (pairwise R = 0.075) and lakes 1 and 2 (pairwise R = 0.102) and the greatest between localities 1 and 5 (pairwise R = 0.489) and 2 and 6 (pairwise R = 0.487). Partitioning among host taxonomic categories ranged from moderate to very strong, with R = 0.238 between the two cyprinid hosts and R = 0.915 between *P. flavescens* and *L. gibbosus* and R = 0.909 between *P. flavescens* and *P. notatus*. Overall, ANOSIMs showed that all levels of host taxonomy provided more robust classification of parasite infracommunities than spatial categories. In addition, the global R values showed that parasite communities are separated with greater effectiveness with increasing geographic and host phylogenetic resolution.

In CAP analyses, the 34 component communities were assigned to the correct host species, family and order 100% of the time, and these assignments were significantly non-random according to permutation tests ($0.9508 < Q_{3,9}^{0}\ H\ Q_{3,9}^{0} < 4.52$, $P < 0.0005$). In contrast, only 22 of 34 component communities were assigned to the correct lake ($Q_{6}^{0}\ H\ Q_{6}^{0} = 0.953$, $P = 0.012$), but allocation success of component communities to the correct locality (6 of 34 correctly allocated) did not differ significantly from random allocation success ($Q_{10}^{0}\ H\ Q_{10}^{0} = 1.153$, $P = 0.461$). The number of discriminant axes corresponded

loosely to the number of *a priori* groups, i.e., there were 3 for host order and family, 9 for host species, 10 for locality and 6 for lakes.

Phylogenetic and geographic distance as predictors of parasite community similarity

A pair of 2STAGE analyses showed parasite community similarity was more strongly associated with the phylogenetic distance between hosts than the geographic distance between them (Figure 4.4). This was true regardless of whether or how parasite data were transformed, which guild of parasites was under consideration, and whether or not a dummy parasite was added to each infracommunity. Unlike the PERMANOVA and ANOSIM analyses, in which the parasite community in each individual fish was compared to that in other individual fish in a crossed design that controlled for locality or host species, the correlations in 2STAGE analysis were made across all species and locality boundaries; that is, the parasite community of each fish was compared to that of every other fish. The results show that regardless of the geographic distance separating two fish, the taxonomic distance between them correlated with parasite community similarity with moderate or greater strength (Figure 4.4). In contrast, geographic distance is at most weakly associated with parasite community similarity. The correlation coefficients between host taxonomic distance with the various parasite community similarity matrices are on average $\rho = 0.42$ ($0.17 < \rho < 0.57$) (Figure 4.4). Taxonomic distance was more strongly associated with the allogenic ($0.35 < \rho < 0.57$) than the autogenic ($0.17 < \rho < 0.27$) parasite community. The extremes of these correlations were tested separately in RELATE, and all were significant at $P < 0.0005$. In contrast, the geographic distance separating two hosts was correlated with parasite community

similarity much less strongly (mean $\rho = 0.07$, $0.001 < \rho < 0.10$). Geographic distance was slightly more strongly related to the autogenic parasite community ($0.07 < \rho < 0.10$) than the allogenic parasite community ($0.01 < \rho < 0.06$).

Non-parametric Mantel tests, (the RELATE routine, Clarke and Gorley 2006) revealed parasite community similarity (untransformed abundance, entire parasite community, no dummy) is significantly associated with both phylogenetic ($\rho = 0.558$; $P < 0.0005$) and geographic distance ($\rho = 0.087$; $P < 0.0005$) between hosts. In subsequent multiple regression, phylogenetic distance, geographic distance and length all explain a significant portion of the variance in parasite community similarity (Table 4.2). However, taxonomic distance accounts for nearly all the explanatory power of the model and 31% of the variance in parasite community similarity (Table, 4.2; Figure 4.5).

Because other studies of the relationship of geographic distance with parasite community similarity have been limited to a single host species, for comparative purposes correlations within each host species were also performed (Figure 4.6). In correlations limited to conspecific hosts, geographic distance correlated with parasite community similarity most strongly in yellow perch ($\rho = 0.499$) and least strongly in pumpkinseed ($\rho = 0.167$) (Figure 4.6). Geographic distance was not more strongly associated with community similarity of autogenic parasites in all six host species, as predicted by the dispersal limitation hypothesis (see Discussion). In cyprinid hosts, geographic distance was most strongly associated with allogenic parasites, while in centrarchids the relationship was strongest in autogenic parasites, and in percids the association was inconsistent among guilds.

Discussion

This study assessed parasite community variation arising from habitat and host phylogeny in replicate fish communities within a major waterway. Overall, several types of analyses of a large set of field and molecular data showed that the effect of host phylogeny on parasite communities was much larger than that of habitat. PERMANOVA and ANOSIM showed the most meaningful boundaries in parasite communities reflect the phylogeny of their hosts, not their habitat. CAP analysis demonstrated that parasite communities from multiple localities correctly predict membership in host species, families and orders, but are poor predictors of geographic origin when multiple host species are compared. RELATE routines and regression showed the phylogenetic distance between two individual hosts predicts a substantial amount of variance in the similarity of their parasites, even when comparing fish from different habitats. In contrast, the geographic distance between hosts is only informative of parasite community similarity if comparisons are made within a single host species.

Numerous studies have attempted to identify attributes of host species that are associated with parasite community structure and composition (reviewed in Combes 2005). In our study, both qualitative and quantitative parasite community similarity showed that host specificity is not just an attribute of individual parasite species, it can also be viewed as an emergent property of parasite communities. The infection levels of multiple parasite species were tested simultaneously, and the strong effect of host phylogeny contrasted markedly with results from studies by Poulin (2005, 2010), which examined a much larger number of host species than the present work. These studies found that for a given parasite species, host species with similar infection levels do not

tend to be closely related, and there is only a weak tendency for closely related hosts to have similar parasite communities (Poulin 2005, 2010). However, there are important differences between these studies and the present work. Poulin (2005) included only autogenic parasites, while we inventoried the entire infracommunity. In addition, Poulin (2005) included only parasite taxa present in four or more sympatric host species, which is likely to have skewed his results in favour of generalists and arbitrarily reduced the role of host phylogeny in infection levels. Poulin (2010) limited his analysis to qualitative similarity among communities of parasite genera rather than species. In contrast, the present study is based on original field data and attempts to control for confounding influences that cannot be dealt with in meta-analyses. For example, data compiled by Poulin (2005, 2010) was derived from studies of fish of unknown age classes, collected in different seasons and years (e.g., Leong and Holmes, 1981).

Nonetheless, the relationship we observed between taxonomic distance and autogenic parasites was comparatively weak, and in that sense our results are consistent with Poulin (2005), who found no relationship (and did not consider allogenic parasites). Together these findings suggest that allogenic parasites are more host specific than autogenic parasites. This emerged clearly in 2STAGE analysis, and can be attributed to two basic attributes of the parasite communities surveyed here: first, most allogenic parasites were larval strigeids (in terms of both abundance and diversity) and, second, most strigeids were highly host specific (Locke *et al.* in preparation - see Chapter 3).

Although we examined parasite community similarities in only six host species, it is worth emphasizing that two of them differ markedly in their ecology, but are closely related. Johnny darter is benthic and smaller bodied than the pelagic yellow perch, yet the

overall communities of these two percids were still more similar to each other than to any other hosts. The difference between the two fish was mostly attributable to high levels of *Diplostomum baeri*, found only in yellow perch (data from SIMPER analysis, not shown). If the ecological convergence of hosts was more important than phylogenetic distance, we would expect the parasite communities of yellow perch to bear more resemblance to those of the ecologically similar centrarchids (Scott and Crossman 1973). Instead, the parasites of centrarchids are more similar to those of johnny darter, largely because both johnny darter and pumpkinseed were infected by larval *Dichylene* in the liver and shared low levels of *Diplostomum* spp. in the lens (SIMPER analysis, not shown).

We used geographic distance principally as a surrogate for habitat variation between individual fish, in order to compare its effects on parasite communities with the effects of phylogenetic distance, similar to Seifertová *et al.* (2008). Those authors found that phylogenetic distance was a stronger predictor of parasite community similarity than geographic distance among European populations of *Leuciscus cephalus* (Cyprinidae). In fact, the apparent effect of geographic distance was completely attributable to the genetic distance in sequences of cytochrome *b* between host populations (Seifertová *et al.* 2008). This study examined the same question on a much smaller geographic scale, but among host species, rather than among populations of a single species. Like Seifertová *et al.* (2008), we also found that phylogenetic distance was much more predictive of quantitative and qualitative parasite community similarity.

In this respect, our study resembles that of Vignon and Sasal (2009), who also examined the effects of spatial and host factors on parasite communities in eight tropical

fish species from multiple localities. Those authors found that the amount of variation in parasite communities that could be attributed to geographic and host effects depended on scale. In fish communities on islands 1500 km apart, geography and host species were equivalent in predicting infection levels, while at smaller scales, host species was the most important predictor in most cases. However, at the 1500-km scale, Vignon and Sasal (2009) compared only a single pair of congeneric fish species. To reconcile the implications of their results and ours, it seems that if host populations are separated by geographic distances that are very large and phylogenetic distances that are sufficiently small, the effect of habitat variation may approach that of host phylogeny. On the whole, even though there are no units in which these distances can be compared, it seems reasonable to maintain that host phylogenetic distance has a greater effect on parasite communities than geographic distance (see Seifertová *et al.* 2008).

Two mechanisms have been advanced to explain the tendency of spatially proximate communities to be similar, namely dispersal limitation and environmental gradients (e.g., Soininen *et al.* 2007). Distant communities may tend to be dissimilar because organisms are less likely to disperse across greater distances. If the dispersal mechanism is accurate, the greater mobility of the terrestrial hosts of allogenic parasites should reduce the effect of geographic distance on community similarity in this guild. In our results, the relative strength of the distance-similarity relationship was not consistently associated with any parasite guild in the six host species. This is in keeping with studies to date. In some studies (Poulin 2003; Karvonen and Valtonen 2004; Karvonen *et al.* 2005a), higher-dispersing allogenic parasites showed the expected weaker relationships (or none) with geographic distance than lower-dispersing autogenic

parasite, while in others, the opposite trend was observed (Fellis and Esch, 2005a, b). Taken together, this suggests that something else may explain this widely observed trend in parasite communities (see also Vinarski *et al.* 2007), and our use of geographic distance as a surrogate measure for habitat variation seems well justified in consequence.

Our study did not focus on the nature of the relationship between community similarity and geographic distance in the same manner as recent works (Poulin 2003; Fellis and Esch 2005b). For example, only two recent studies have examined this relationship at the infracommunity level (Pérez-del-Olmo *et al.* 2009; Timi *et al.* 2009) and none have done so in freshwater fish hosts. Nor have any other studies looked the decay of similarity with geographic distance in a replicated manner among multiple fish communities. We suspect the lack of a similarity-distance relationship in some previous studies of allogenic parasite communities may be due to depauperate communities (e.g., a single allogenic parasite species in Karvonen and Valtonen (2004) and Karvonen *et al.* (2005a), the taxonomic imprecision inherent in meta-analyses (Poulin 2003), morphological identifications of metacercariae (Karvonen and Valtonen 2004; Karvonen *et al.* 2005a), as well as pooling of samples collected in different years, seasons, or culled from different studies (Karvonen and Valtonen 2004; Karvonen *et al.* 2005a; Poulin 2003). In contrast, our allogenic data set was rich in species, supported by molecular identifications (Locke *et al.* in preparation - see Chapter 3), morphological identifications were done by a single person, and temporal variation in parasite communities was minimized by simultaneously sampling sympatric of hosts of approximately the same age.

Indeed, the importance to the present work of species-level identification of metacercariae cannot be overstated. The molecular techniques and data which underpin community analyses presented here are discussed elsewhere (Moszczynska *et al.* 2009, Locke *et al.* 2009, Locke *et al.* in preparation - see Chapter 3). Without these data, our results would likely have been strikingly different, as strigeid metacercariae would have been treated as few dominant generalist genera. Molecular data were essential to uncovering the diversity and specificity of this dominant group of parasites. A surprisingly small proportion of field studies employing molecular data do so to study purely ecological processes (Johnson *et al.* 2009). The present study provides an additional example of how molecular information can lead to novel conclusions with far-reaching implications in the community ecology of parasites.

Table 4.1 Multivariate permutational analysis (PERMANOVA) of host and locality as descriptors of parasite communities in freshwater fish

Results of model II (random effects) PERMANOVA based on Bray-Curtis similarities of square-root transformed abundance of parasite infracommunities, with no dummy variable. Parasite communities in three outlier fish excluded. The sums of squares are sequential (Type I) and 999 permutations of residuals were performed under a reduced model.

Factors:	Name	Type	Levels
	Length (normalized within species)	covariate (random)	continuous
	Locality	random	6
	Species	random	6

Source	Sum of squares	*df*	Mean square	*Pseudo-F*	Unique permut-ations	*P*
Length	**30 309**	**1**	**30 309.0**	**4.0679**	**998**	**0.001**
Locality	**188 690**	**5**	**37 738.0**	**1.8617**	**996**	**0.003**
Species	**1 124 500**	**5**	**224 900.0**	**17.5350**	**999**	**0.001**
Length × locality	17 768	5	3 553.6	1.2627	998	0.076
Length × species	**63 510**	**5**	**12 702.0**	**3.1144**	**997**	**0.001**
Locality × species *	**235 300**	**23**	**10 231.0**	**5.8510**	**998**	**0.001**
Length × locality × species	0	0		No test		
Residuals	1 152 600	660	1 746.4			
Total	2 812 700	704				

*Two empty cells

Significant effects in bold.

Table 4.2 Predictive power of geographic and taxonomic distance for the similarity of parasite communities in six freshwater fish species from the St. Lawrence River

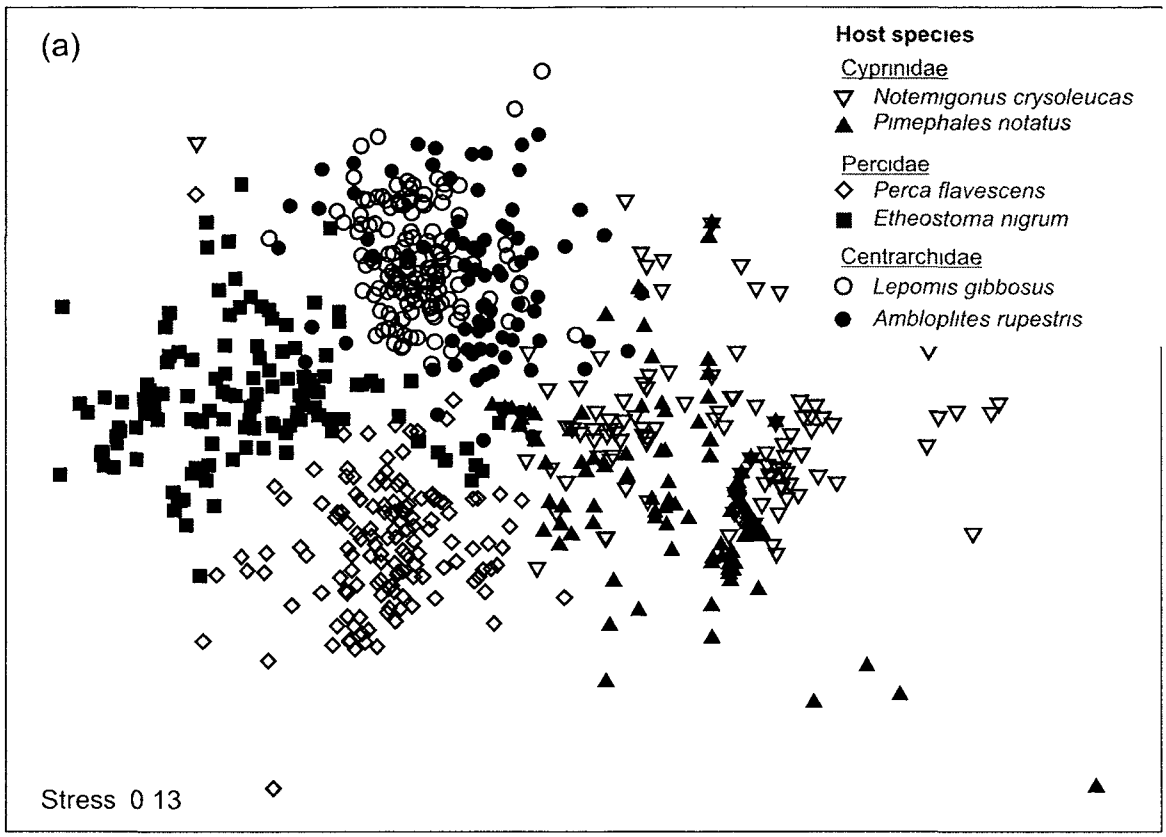
Results of backward regressions with three factors used to predict parasite community similarity (Bray-Curtis similarity based on untransformed abundance, no dummy variable). A model with three predictors (taxonomic and geographic (km) distance between hosts and Euclidian distance between fish hosts calculated from length, normalized within species) explained significantly more variance in similarity than one based on taxonomic distance alone (indicated by the significant changes in R^2). However, the magnitude of change in R^2 was small; the taxonomic distance between hosts accounts for over 96% of the explanatory power of the model. Thus, in comparisons of multiple species of fish from multiple localities, the phylogenetic distance between individual fish predicts the similarity of their parasite communities much more strongly than the geographic distance between them does.

Predictors in model		Sum of squares	*df*	Mean square	*F*	R^2	*P**
Taxonomic distance	Regression	19427797.537	3	6475932.512	39085.698	0.322	<0.0005
Length	Residual	40999202.691	247452	165.685			
Km	Total	60427000.227	247455				
Taxonomic distance	Regression	18853543.844	2	9426771.922	56109.912	0.312	<0.0005
Length	Residual	41573456.383	247453	168.005			
	Total	60427000.227	247455				
Taxonomic distance	Regression	18790053.199	1	18790053.199	111671.824	0.311	<0.0005
	Residual	41636947.029	247454	168.261			
	Total	60427000.227	247455				

* *P*-values indicate the significance of both the model and the change in R^2. *F* and other statistics refer to the model, not the change in R^2.

Figure 4.1 Non-metric multi-dimensional scaling (MDS) of parasite infracommunities in 705 fish from the St. Lawrence River

MDS of Bray-Curtis similarity among infracommunities (untransformed abundance, no dummy parasite). Both plots are identical, but points are coded according to host species in (a) and locality in (b). Points that are close together represent parasite communities that are similar. Stress indicates the degree to which inter-point distances correlate with similarity rankings. Values less than 0.1 leave little room for misinterpretation; values less than 0.2 are useful visualizations of relationships, while stress > 0.3 may show misleading relationships (Clarke 1993). Infracommunities clearly separate into clusters consistent with host phylogeny, forming groups that correspond to both host species and family, while geographic clustering is more difficult to discern.


(a)
Host species
Cyprinidae
Notemigonus crysoleucas
Pimephales notatus
Percidae
Perca flavescens
Etheostoma nigrum
Centrarchidae
Lepomis gibbosus
Ambloplites rupestris
Stress 0 13

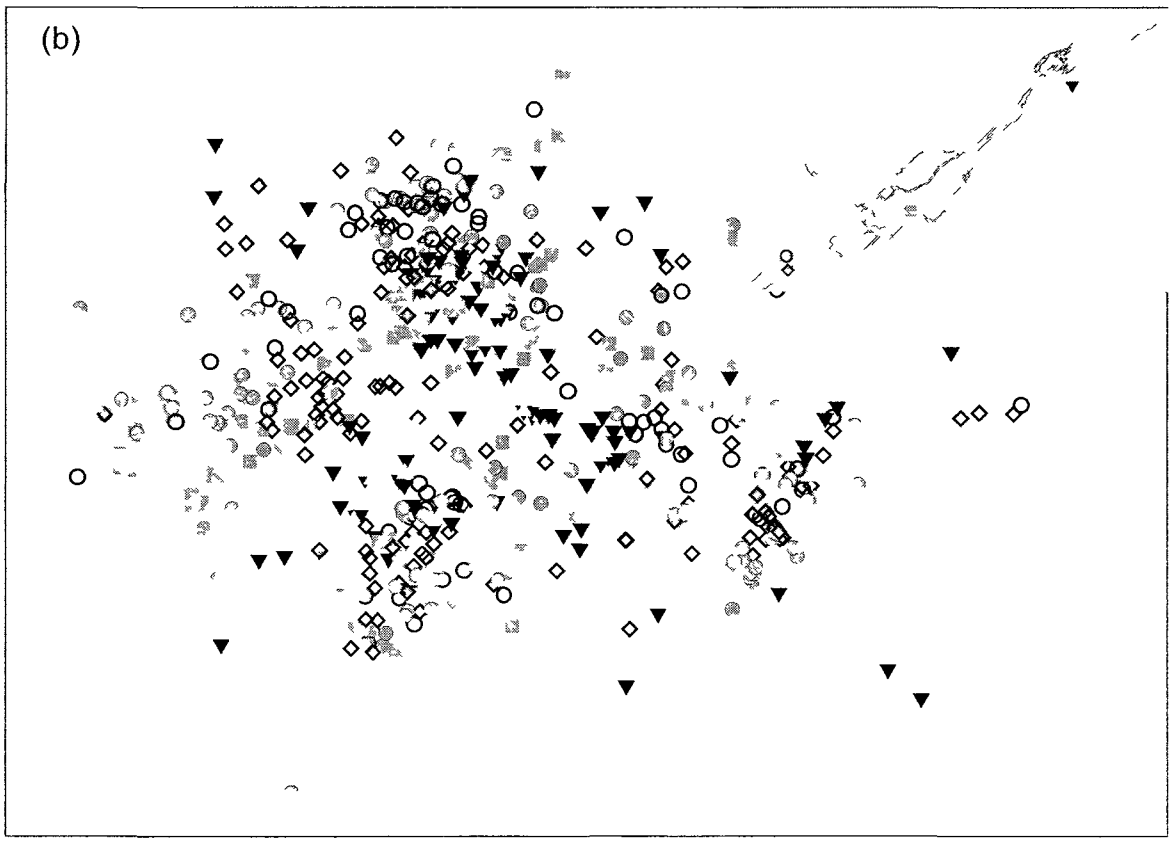
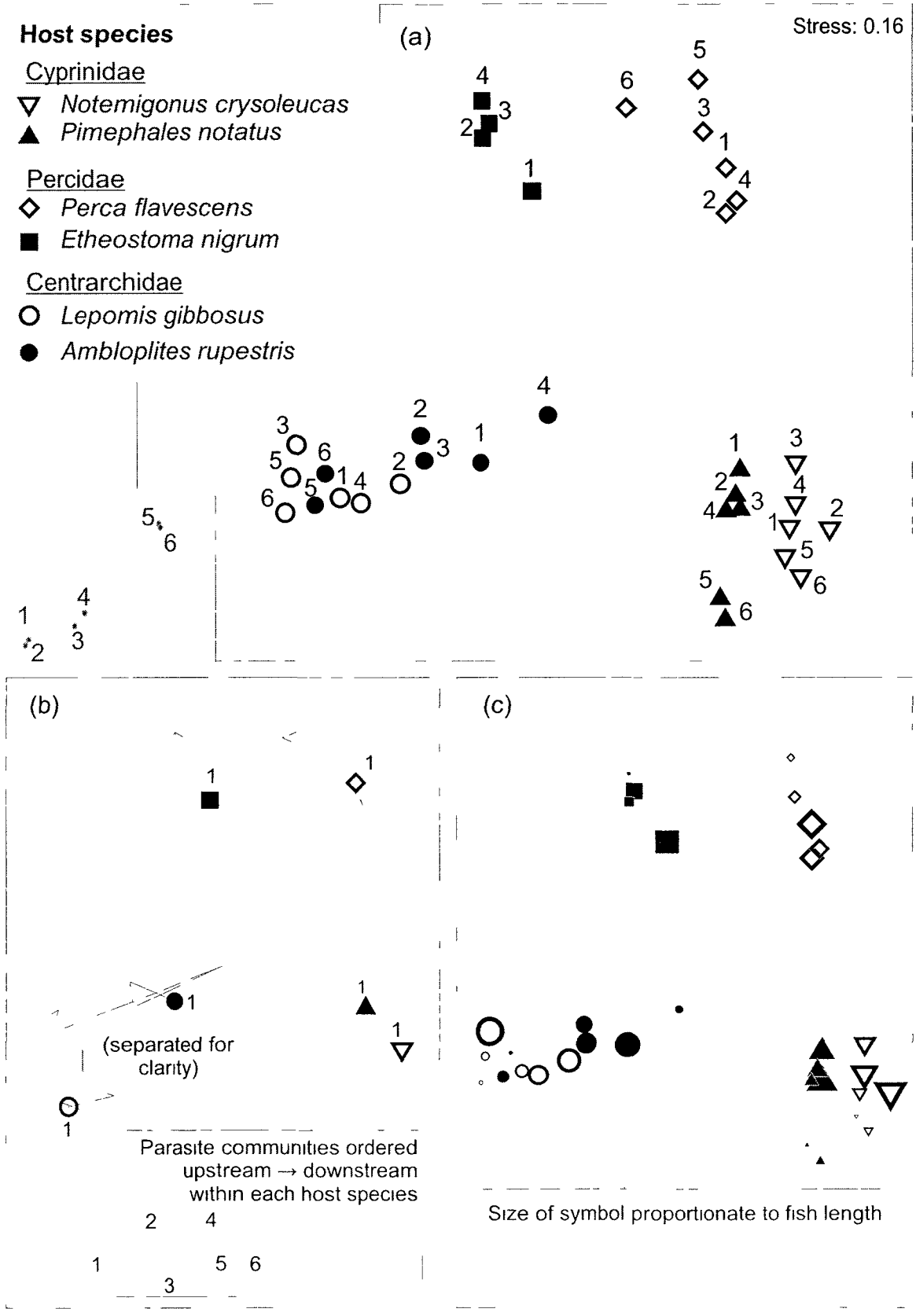

(b)

Figure 4.2 Non-metric multi-dimensional scaling (MDS) of parasite component communities in 34 populations of six fish species from the St. Lawrence River

MDS of Bray-Curtis similarity among 34 component communities (square-root transformed abundance, no dummy parasite). Points that are close together represent parasite communities that are similar. Ordinations in a, b and c are identical, but points are coded or arrayed in lines to emphasize different aspects of how parasites are distributed among hosts and in space. Like the MDS of 705 infracommunities (Figure 4.2), the 34 component communities clearly separate according to host species and family in (a). The same information is shown in (b), but the points are joined in upstream to downstream order within each host species. This shows that locality effects are roughly the same within host families, but the directionality of effects differs among host families. For example, in both cyprinid hosts upstream and downstream communities separate along the vertical axis, with downstream communities mostly found at the bottom of ordination. In percids, localities also separate along the vertical axis, but downstream communities are found at the top. In centrarchids, in which points in (b) have been repositioned slightly, geographic separation is along the horizontal axis. In (c), parasite communities are coded according to host length relative to conspecifics. Within each host family, parasite communities cluster in a similar manner across the range of host sizes, but the direction of this effect differs among families. For example, parasite communities in the largest hosts of both cyprinids are in the upper right, while ordination of those in smaller cyprinids places them in lower left. The direction of large → small sized hosts is different, but generally internally consistent, in the other two families. Overall, these plots shed light on the significant interaction effects detected in PERMANOVA (Table 4.2). Taken together, the analyse show that the manner in which parasite communities differ between host species is affected by host size and locality, but there are differences between host species at every level of host size and locality.


Host species
Cyprinidae
Notemigonus crysoleucas
Pimephales notatus
Percidae
Perca flavescens
Etheostoma nigrum
Centrarchidae
Lepomis gibbosus
Ambloplites rupestris
(a)
Stress: 0.16
(b)
(c)
(separated for clarity)
Parasite communities ordered upstream → downstream within each host species
Size of symbol proportionate to fish length

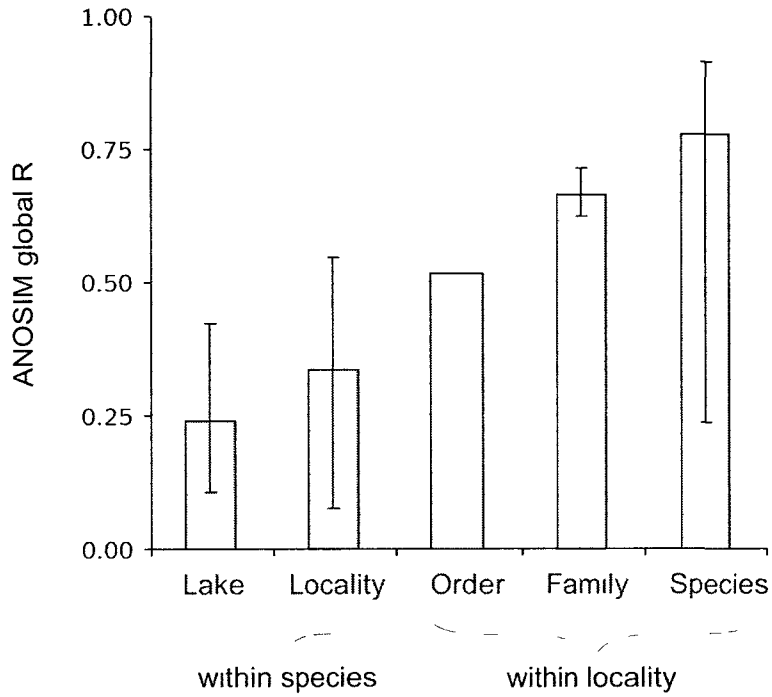
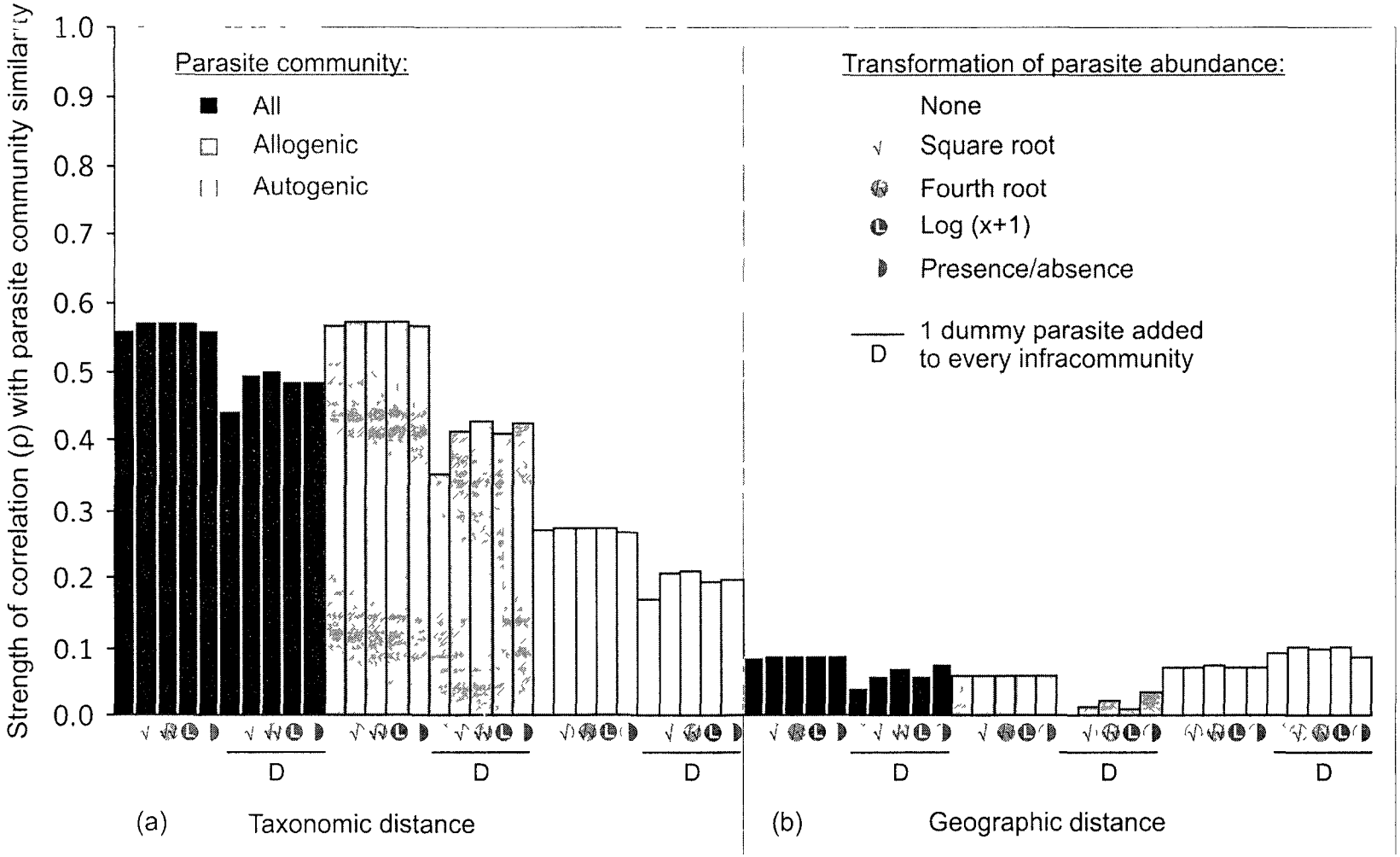


Figure 4.3 Effects of host phylogenetic and spatial categories in analyses of similarity (ANOSIM) in parasite infracommunities

Global R values from two-way crossed ANOSIMs based on untransformed Bray-Curtis distances between parasite communities in 705 fish in the Saint Lawrence River, Canada. The global R values are all significant at $P < 0.0005$. The magnitude of the R values, which vary from -1 to 1, corresponds to the degree of partitioning in the multidimensional cloud of Bray-Curtis resemblances that is attributable to each factor. A global R value = 1 indicates that every community is, on average, more similar to other communities in the same grouping factor than it is to members of any other factor. "Error" bars show the range of pairwise R-values associated with different levels of each factor. For example, differences among lakes within species range from R = 0.107 (Lake St. Francois compared to Lake St. Louis) to R = 0.424 (Lake St. Francois compared to Lake St. Pierre).

Figure 4.4 Second-stage ordination (2STAGE) of correlations between parasite infracommunity similarity and taxonomic and geographic distance between individual hosts

Correlations between parasite infracommunity similarity and (a) taxonomic and (b) geographic distance. The height of each column shows the correlation (ρ) of these factors with the similarity of parasite communities separated into allogenic/autogenic guilds (indicated by colors in legend) (2STAGE, Clarke and Gorley 2006). Five transformations to parasite abundance data recur in the same order (see legend) in a progression from quantitative to qualitative similarity, and a dummy parasite was added to each infracommunity in columns with a line and "D" underneath, which are subject to the same series of five transformations. The taxonomic distance between individual hosts correlates more strongly with parasite community similarity than geographic proximity, regardless of how parasite data are transformed, which guild is under consideration, or whether a dummy parasite is added to infracommunities.


Strength of correlation (ρ) with parasite community similarity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Parasite community:
All
Allogenic
Autogenic
Transformation of parasite abundance:
None
Square root
Fourth root
Log (x+1)
Presence/absence
1 dummy parasite added to every infracommunity
D
D
D
D
D
D
(a) Taxonomic distance
(b) Geographic distance

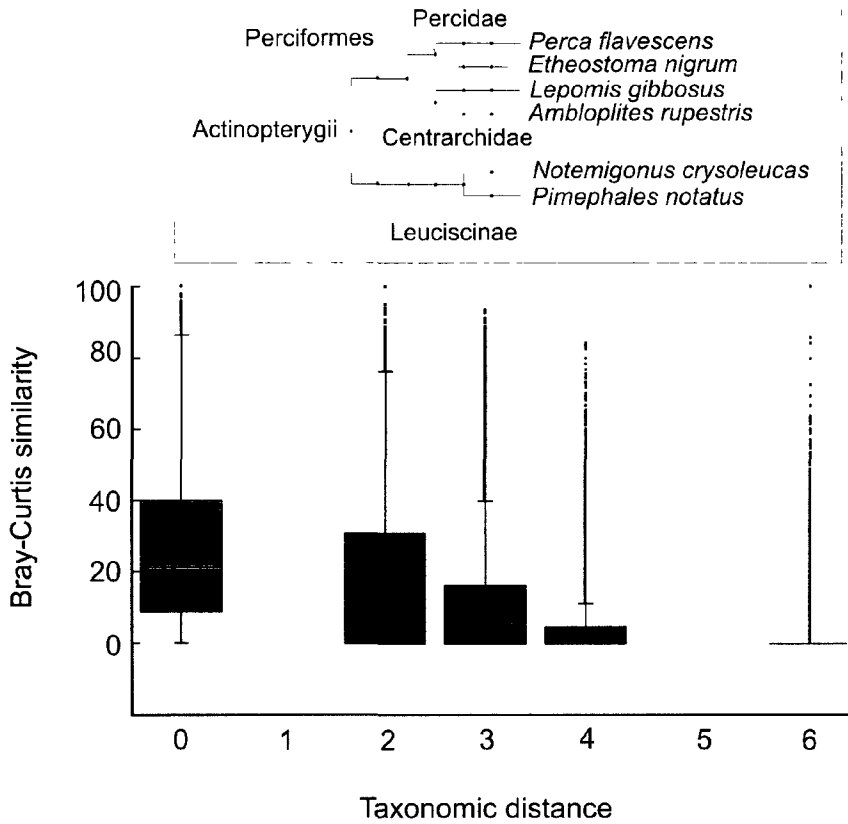


Figure 4.5 Effect of taxonomic distance between hosts on parasite infracommunity similarity in 705 fish from six different species and six different localities in the St. Lawrence River

Boxplot of 247 456 Bray-Curtis similarities between parasite communities in 705 fish in six species from the St. Lawrence River, Canada (based on untransformed abundance of all parasites, no dummy variable). Lines within boxes represent median values in similarity, boxes contain the upper and lower quartiles of the distribution, and whiskers encompass 99% of the distribution. Conspecific fish (taxonomic distance = 0) have the most similar parasite communities, while more distantly related fish have less similar parasites. Inset, the tree used to calculate taxonomic distances between all 705 fish. Taxonomic distance between fish accounts for 31% of the variance in parasite community similarity (see Table 4.2).

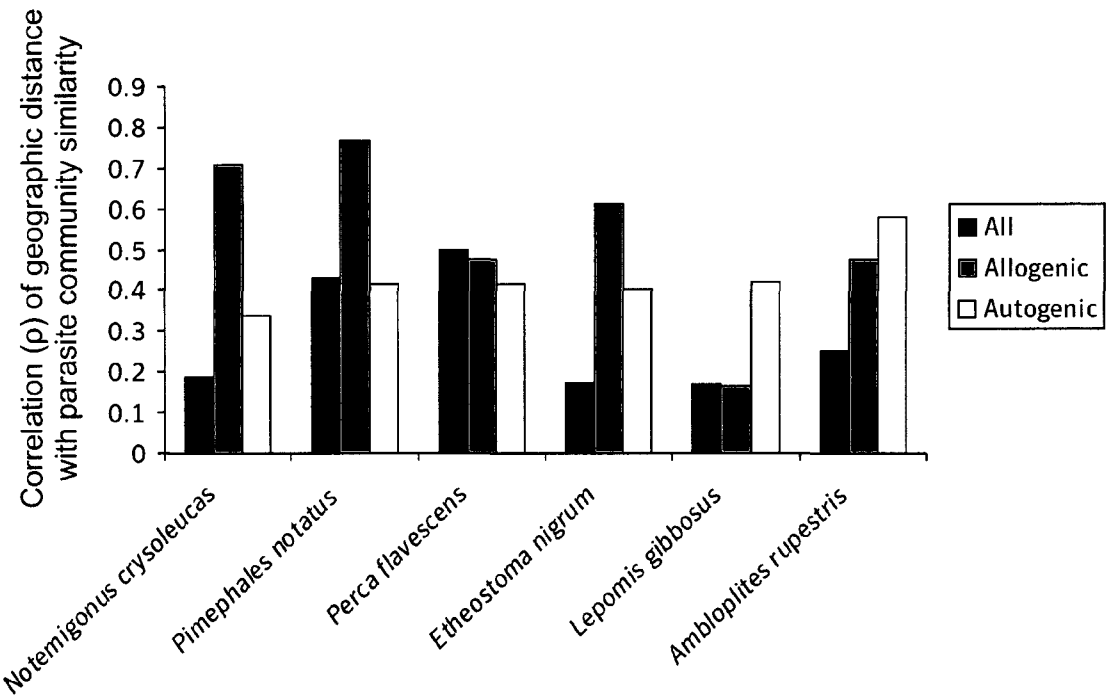


Figure 4.6 Correlation between geographic distance and parasite infracommunity similarity broken down by host species and parasite guild

Strength of correlation between parasite community similarity (untransformed abundance, no dummy variable) and geographic distance (km) in 705 individual fish hosts, partitioned by parasite guild. Absolute values of correlation coefficients are used; parasite communities are increasingly dissimilar with increasing distance. In all cases $P < 0.0005$ (RELATE test, Clarke and Gorley 2006). The effect of distance between localities on community similarity in different guilds varies in the six hosts species, with no clear pattern emerging across hosts.

Appendix 4.1

Mean abundance of metazoan parasites collected from six species of fish at six localities in the St. Lawrence River, QC.

Perca flavescens (Percidae) (n = 137)

Ectoparasites

Monogenea		Copepoda		Hirudinidae
Unidentified	*Urocleidus adspectus*	*Argulus* sp.	*Ergasilus* sp.	Unidentified
0.2928	0.02857	0.01429	0.007	0.0786

Endoparasites

Acanthocephala	Nematoda				Cestoda	
Unidentified cystacanth	Unidentified larval stage	*Dichelyne* sp.	*Raphidascarus acus*	*Philometra* sp.	Unidentified metacestode	*Bothriocephalus cuspidatus*
0.1071	0.5643	0.01429	0.1786	0.036	0.064286	0.01

Digenea								
Apatemon sp. 2	*Apophallus brevis*	*Bunodera sacculata*	*Azygia angusticauda*	*Clinostomum* sp.	*Cryptogonimus chili*	*Crepidostomum farionis*	*Diplostomum* spp.	*Diplostomum* sp. 1
0.0214	10.8571	0.036	0.0714	0.1	0.1000	0.1000	0.3499	0.08571

D. baeri	*D. huronense*	*Diplostomum* sp. 3	*Diplostomum* sp. 4	*Diplostomum* sp. 5	*Ichthyocotylurus* spp.	*Ichthyocotylurus* sp. 3	*Neochasmus* sp.
22.04	0.0071	0.0143	0.557	0.0071	2.3	0.0071	2.028

Ornithodiplostomum sp. 1	*Posthodiplostomum* sp. 7	*Phyllodistomum superbum*	*Proterometra macrostoma*	*Rhipidocotyle* sp.	*Tylodelphys* sp.
0.4286	0.9286	0.1000	0.3499	0.0071	0.5857

Etheostoma nigrum (Percidae) (n = 131)

Ectoparasites

Monogenea	Copepoda		Hirudinidae
Unidentified	*Copepoda* sp.	*Lernea* sp.	Unidentified
0.3817	0.007634	0.03053	0.0840

Endoparasites

Acanthocephala	Nematoda				Cestoda		
Unidentified cystacanth	Unidentified larval stage	*Camall-anus* sp.	*Dichyl-ene* sp.	*Raphidasc-aris acus*	*Bothriocephalus cuspidatus*	*Proteocephalus* sp.	*Triaenophorus* sp.
0.2901	0.0992	0.0153	2.1832	0.6641	0.2519	0.0076	0.0076

Digenea					
Apatemon sp.	*Apatemon* sp. 1x	*Apophallus brevis*	*Clinostomum* sp.	*Conodiplostomum* sp. 5	*Diplostomum* spp.
0.1576	0.4733	0.4733	0.4504	0.0611	0.0611

Diplostomum sp. 1	*Diplostomum* sp. 4	*Ichthyocotylurus* spp.	*I. pileatus*	*Ornithodiplostomum* sp.	*Ornithodiplostomum* sp. 1	*Tetracotyle* spp.	*Tetracotyle* sp. 8
0.0534	0.1298	0.0306	0.0688	8.8626	0.4885	2.5267	0.0153

Tetracotyle sp. 10	*Tetracotyle* sp. x	*Tylodelphys* sp.	Crassiphialinae gen. sp.	*Azygia angusticauda*	*Crepidostomum farionis.*	*Neochasmus* sp.
0.0153	0.4733	0.0076	0.0153	0.7404	0.1527	0.4885

Lepomis gibbosus (Centrarchidae) (n = 132)

Ectoparasites

Monogenea		Copepoda		Hirudinidae
Unidentified	*Urocleidus* sp.	*Actheres pimelodi*	*Ergasilus* sp.	Unidentified
15.6593	0.0149	0.0519	0.0074	0.0148

Endoparasites

Acanthocephala	Nematoda		
Unidentified cystacanth	Unidentified larval stage	*Dichelyne* sp.	*Raphidascaris acus*
2.2815	0.4370	3.0444	0.1037

Cestoda				
Haplobothrium sp.	Proteocephalidae gen. sp. metacestode	*Bothriocephalus cuspidatus*	*Proteocephalus* sp.	*Triaenophorus* sp.
0.0741	0.0444	0.0444	0.0076	0.0076

Digenea						
Apophallus venustus	*Azygia longa*	*Clinostomum* sp.	Crassiphialinae gen. sp.	*Crepidostomum* sp.	*Diplostomum* spp.	*Diplostomum* sp. 1
0.1241	0.2000	0.1000	0.6370	0.01482	0.0370	0.0741

Diplostomum sp. 3	*Diplostomum* sp. 4	*Neochasmus* sp.	*Posthodiplostomum* spp.	*Posthodiplostomum* sp. 2	*Posthodiplostomum* sp. 3
0.0074	0.0741	8.8591	25.5852	0.0074	0.3111

Posthodiplostomum sp. 5	*Posthodiplostomum* sp. 6	*Phyllodistomum superbum*	*Tetracotyle* spp.	*Tetracotyle* sp. 3	*Tetracotyle* sp. 9	*Uvulifer* sp.
0.0222	0.0074	0.0074	0.1630	0.0222	0.0222	0.6370

Ambloplites rupestris (Centrarchidae) (n = 81)

Ectoparasites

Monogenea	Copepoda		Hirudinidae
Unidentified	*Actheres pimelodi*	*Ergasilus* sp.	Unidentified
7.0981	0.0741	0.2346	0.0741

Endoparasites

Acanthocephala		Nematoda			Cestoda	
Unidentified cystacanth	*Neoechinorhynchus* sp.	Unidentified larval stage	*Dichelyne* sp.	*Raphidascaris acus*	Unidentified metacestode	*Proteocephalus* sp.
0.0988	0.0123	0.4370	0.6790	0.1037	0.1728	0.0494

Digenea								
Apophallus venustus	*Apatemon* sp. 3	*Apatemon* sp. 4	*Azygia longa*	*Clinostomum* sp.	*Crepidostomum cooperi*	*Cryptogonimus chili*	*Diplost-omum* spp.	*D. huro-nense*
0.0123	0.0988	0.0494	0.1358	1.1852	0.0988	0.9877	0.0864	0.0247

Diplostomum sp. 1	*Diplostomum* sp. 3	*Diplostomum* sp. 4	*Ichthyocotyl-urus* spp.	*Neochasmus* sp.	*Posthodiplo-stomum* spp.	*Posthodiplo-stomum* sp. 1	*Posthodiplostomum* sp. 3
0.4321	0.0494	0.4321	0.4815	1.6049	7.7037	0.0864	0.0247

Phyllodistomum superbum	*Proterometra macrostoma*	*Rhipidocotyle* sp.	*Tetracotyle* sp.	*Tylodelphys* sp.	*Uvulifer* sp.
0.0123	0.1728	0.0123	0.3210	0.3457	1.0123

Notemigonus crysoleucas (Cyprinidae) (n = 113)

Ectoparasites

Monogenea			Copepoda		Hirudinidae
Unidentified	*Gyrodactylus* sp.	*Urocleidus* sp.	*Actheres pimelodi*	*Ergasilus* sp.	Unidentified
0.0442	0.0177	0.0442	0.0741	0.0177	0.0089

Endoparasites

Acanthocephala		Nematoda		Cestoda
Unidentified cystacanth	*Neoechinorhynchus rutili*	Unidentified larval stage	*Raphidascaris acus*	*Pliovitellaria* sp.
0.0089	0.0123	0.0619	0.0177	0.0354

Digenea						
Apatemon sp. 1	*Apophallus brevis*	*Azygia angusticauda*	*Centrovarium lobotes*	*Clinostomum* sp.	Crassiphialinae gen. sp.	*Diplostomum* spp.
0.0089	0.1150	0.0177	0.0442	0.0089	0.0177	5.1681

D. huronense	*Diplostomum* sp. 1	*Diplostomum* sp. 3	*Diplostomum* sp. 4	*Hysteromorpha triloba*	*Neochasmus* sp.
0.1150	0.2124	0.0530	0.0177	0.7612	0.7611

Neochasmus sp.	*Ornithodiplostomum* spp.	*Plagioporus* sp.	*Posthodiplostomum* sp.	*Tetracotyle* sp. 6
0.7611	3.3800	0.5664	0.0177	0.0354

Pimephales notatus (Cyprinidae) (n = 111)

Ectoparasites

Monogenea	
Unidentified	*Gyrodactylus* sp.
0.0278	0.0370

Endoparasites

Nematoda	
Unidentified larval stage	*Dichelyne* sp.
0.0185	0.0093

Digenea					
Centrovarium lobotes	*Clinostomum* sp.	*Cryptogonimus* sp.	*Diplostomum* spp.	*D. huronense*	*Diplostomum* sp. 1
0.4167	0.0089	0.2778	6.9910	0.0923	0.5463

Diplostomum sp. 2	*Diplostomum* sp. 3	*Diplostomum* sp. 4	*Diplostomum* sp. 6	*Diplostomum* sp. 7	*Ichthyocotylurus* sp.	*Ichthyocotylurus* sp. 3	*Neochasmus* sp.
0.0648	0.0278	0.3611	0.0093	0.0093	0.0278	0.0093	0.8519

Ornithodiplostomum spp.	*Ornithodiplostomum* sp. 3	*Ornithodiplostomum* sp. 4	*Ornithodiplostomum* sp. 4	*Ornithodiplostomum* sp. 4	*Plagioporus cooperi*
0.9722	0.1204	0.0185	0.0185	0.0185	0.4074

Posthodiplostomum sp.	*Posthodiplostomum* sp. 4	*Rhipidocotyle* sp.	*Tetracotyle* spp.	*Tetracotyle* sp. 6	*Tetracotyle* sp. 7	*Uvulifer* sp.
0.8333	0.2593	1.7410	0.0370	0.01816	0.01816	0.0093

General conclusions

Parasites are difficult to identify to the species level using morphology, and this poses a problem in studies of parasite community and evolutionary ecology. Larval stages are particularly problematic in this regard. For example, it is virtually impossible to discriminate among congeneric species of strigeid metacercariae, which are common pathogens in fish from the St. Lawrence River and around the world (Szidat 1969; Lemly and Esch 1984; Chappell 1995; Gibson 1996; Sandland and Goater 2001; Overstreet and Curran 2004; Marcogliese *et al.* 2006).

In Chapter 1 of this thesis, two sets of novel primers were presented that can be used to sequence the barcode region of the mitochondrial cytochrome c oxidase I (COI) gene (Moszczynska *et al.* 2009). COI sequences were used to discriminate species in a large sample of strigeid metacercariae from fish in the St. Lawrence River (Locke *et al.* 2009; Chapter 3). Chapters 2 and 3 presented data showing that these mitochondrial markers resolve strigeid species more clearly than more commonly used internal transcribed spacer (ITS) regions between nuclear genes encoding ribosomal subunits, even though the amount of COI variation was higher than expected within a small number of species. In most cases, species indicated by molecular data were specific to certain tissues of a small number of closely related fish species, which constituted a separate line of evidence that these species boundaries were accurate. Both markers revealed a nearly fourfold increase in the number strigeid species known from Canadian fishes. This is remarkable because only a small number of fish were surveyed in part of a single river, and the parasites of Canada's freshwater fishes are among the most well

studied in the world (Margolis and Arthur 1979; Gibson 1996; McDonald and Margolis 1995; Poulin 2010).

Sequence data presented in Chapters 2 and 3 showed that most strigeid metacercariae are highly host specific, with the exception of species inhabiting the lens of the eye. The diversity and host specificity of strigeid metacercariae described here contrast markedly with the previous conception that strigeid metacercariae consist of a few species capable of infecting diverse fish taxa (Gibson 1996; Hoffman 1999). The limited immune response in the lens is a likely explanation for significantly lower host specificity in strigeid species inhabiting this site. The strigeid species with metacercariae that inhabit other tissues showed consistently higher host specificity across separate and ecologically distinctive fish communities. The distribution of some strigeid species was constrained by the phylogenetic affinity rather than the ecological convergence of their fish host species. Overall, these findings suggest that physiologic interactions between host and parasite are more important than ecological factors in determining host specificity in strigeid metacercariae.

The host specificity of larval strigeids had important effects on parasite communities as a whole. Closely related host species had significantly more similar parasite communities. This effect was much stronger than the tendency of parasite communities in spatially proximate fish to be similar, which is emerging as one of the few truly general features of parasite communities (Poulin and Morand 1999; Poulin 2003; Karvonen and Valtonen 2004; Fellis and Esch 2005a, b; Karvonen *et al.* 2005a; Vinarski *et al.* 2007; Seifertová *et al.* 2008: Pérez-del-Olmo *et al.* 2009; Timi *et al.* 2009). The effect of spatial proximity on parasite community similarity was pronounced only if

comparisons were limited to fish of the same species. Categories relating to host phylogeny were much more predictive of parasite community attributes than spatial categories. Since spatial categories are a reasonable surrogate for habitat variation, this suggests that, in comparison with host phylogeny, habitat has a minor role in determining parasite community composition and structure.

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