

# The Use of Molecular Markers to Distinguish Species of Parasites of Canadian Fishes: Focus on Larval Diplostomatoidea

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## INTRODUCTION

Parasitic helminths (worms) are small, soft-bodied animals with few morphological characters that are stable at the species level, thus species identification represents the major obstacle in evaluating parasite biodiversity. Further, larval stages often bear little resemblance to adults and have even fewer useful species-level characters.

Metacercariae of the Diplostomatoidea are typical in this regard. These highly prevalent larval stages are significant pathogens in fish and, as different species may vary in pathogenicity, species level identification is important.

Metacercariae of *Diplostomum* infect the eyes of virtually all species of freshwater fish (Fig. 1). They cause cataracts, blindness and death, resulting in economic losses to aquaculture and sportfishing interests. Despite their importance, little is known of their diversity, host-specificity, pathology or distribution in Canadian fishes (Fig. 2).

Molecular markers are a powerful tool for connecting developmental stages and assessing diversity in parasites. ITS sequences are known for a number of North American<sup>1</sup> and some European<sup>2</sup> species of *Diplostomum*. However, some species are poorly resolved with ITS. CO1 sequences are more variable than ITS sequences and should provide a better suite of markers for species identification. Once available, these can be used in studies on diversity, distribution and host-parasite relations within this group.

Here we present partial CO1 sequences from previously studied specimens from Quebec<sup>1</sup> and use these as reference sequences to examine the species diversity in a small sample of *Diplostomum* from Manitoba.

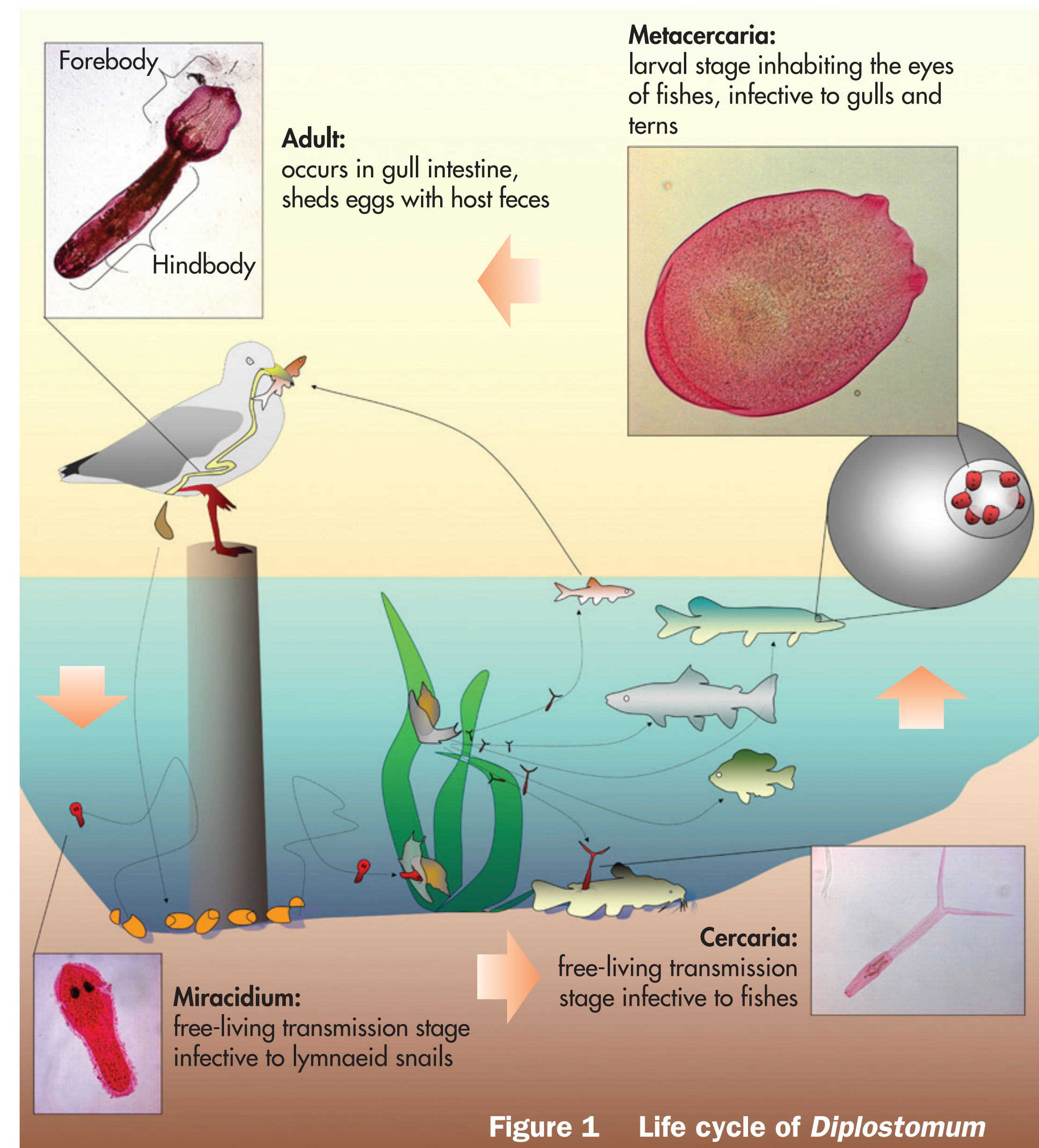
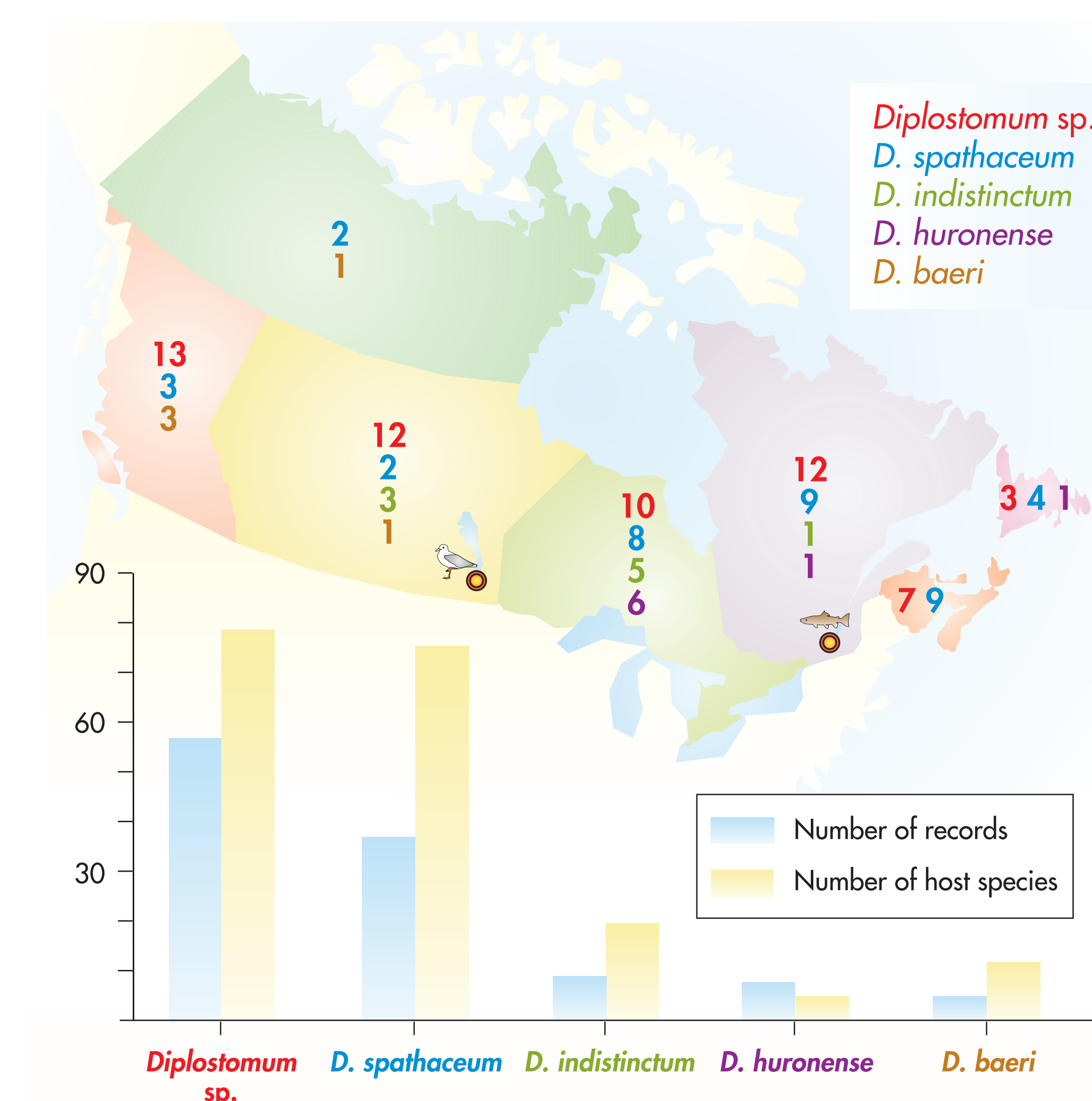


Figure 1 Life cycle of *Diplostomum*

## MATERIALS AND METHODS

**Specimens:** Adult *Diplostomum* were obtained from experimental gulls that were fed eyes from fish caught near Montreal (Fig. 2). The ITS1 and ITS2 sequences of these specimens have already been published<sup>1</sup>. Additional adult specimens were obtained from gulls collected at Delta, Manitoba.

**DNA extraction and amplification:** DNA was extracted from the forebodies of adult specimens. Hindbodies were retained for taxonomic study (Fig. 1). Amplification of the CO1 gene was accomplished with previously published primers<sup>11</sup>. When CO1 amplification was unsuccessful, amplification using primers for the 28S region<sup>12</sup> was performed to verify the presence of DNA.

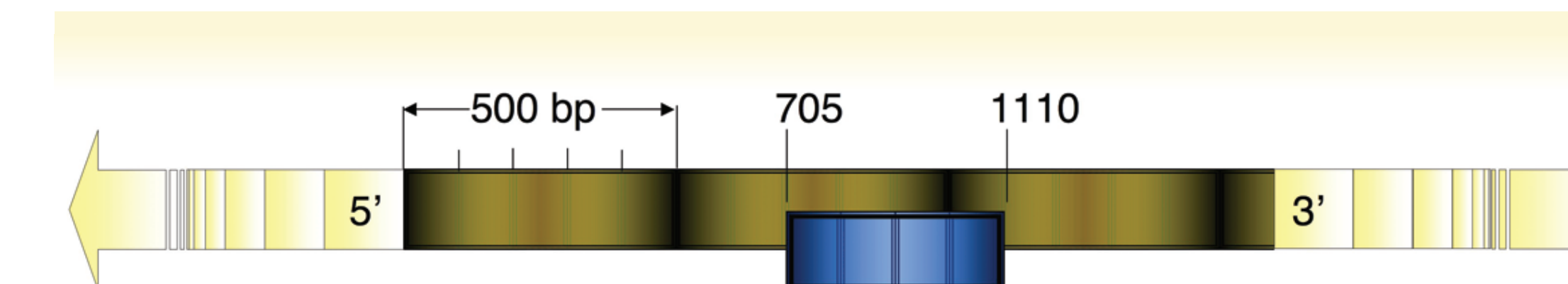


The graph shows the total number of records and total number of host species for each species of *Diplostomum* in Canada<sup>3-10</sup>. The map shows the regional distribution of these records. For example, *Diplostomum* sp. has been reported 13 times and *D. spathaceum* and *D. baeri* have each been reported three times in fishes of BC. Overall, most authors have identified *Diplostomum* only to genus, and records of *D. spathaceum* have been questioned<sup>1</sup>. Sampling localities used in the present study are marked with a ● and hosts collected are illustrated.

Figure 2 Records of species of *Diplostomum* infections in Canadian fishes and sampling localities

## RESULTS

The amplified region corresponds to positions 705–1110 in the CO1 gene of *Fasciola hepatica*<sup>13</sup> (Fig. 3).



Alignment of the 402-bp region amplified in *Diplostomum* species (■) in the present study with the CO1 gene of *Fasciola hepatica*<sup>13</sup> (□), Genbank N° AF216697.

Figure 3 Amplified region of CO1

The four Quebec *Diplostomum* species distinguishable by their ITS sequences<sup>1</sup> can also be distinguished by their CO1 sequences (Table 1, Fig. 4).

Table 1 Comparison of nucleotide (□) and amino acid (■) differences for partial CO1 sequences of four species of *Diplostomum* obtained from fish collected near Montreal

	<i>D. baeri</i>	<i>D. indistinctum</i>	<i>D. sp. QC</i>	<i>D. huronense</i>
<i>D. baeri</i>		9	10	13
<i>D. indistinctum</i>	52–54		1	6
<i>D. sp. QC</i>	46–47	31–33		7
<i>D. huronense</i>	52	35–37	35–36	

CO1 sequences were obtained from 20 of 42 adult specimens of *Diplostomum* from Manitoba. All specimens yielded PCR products for the 28S region. Sequences from Quebec and Manitoba specimens were compared using UPGMA (Fig. 5). Three groups were identified. Most specimens clustered with *Diplostomum* sp. QC; single specimens grouped with *D. baeri* and *D. huronense*.

Thirteen of 14 Manitoba specimens had CO1 sequences identical to those of *Diplostomum* sp. QC; one differed by a single nucleotide. Four other Manitoba specimens yielded shorter sequences also identical to those of *Diplostomum* sp. QC (not shown). The other two Manitoba specimens differed from *D. baeri* and *D. huronense* by 38 and 26 nucleotides and by three and 10 amino acids, respectively.

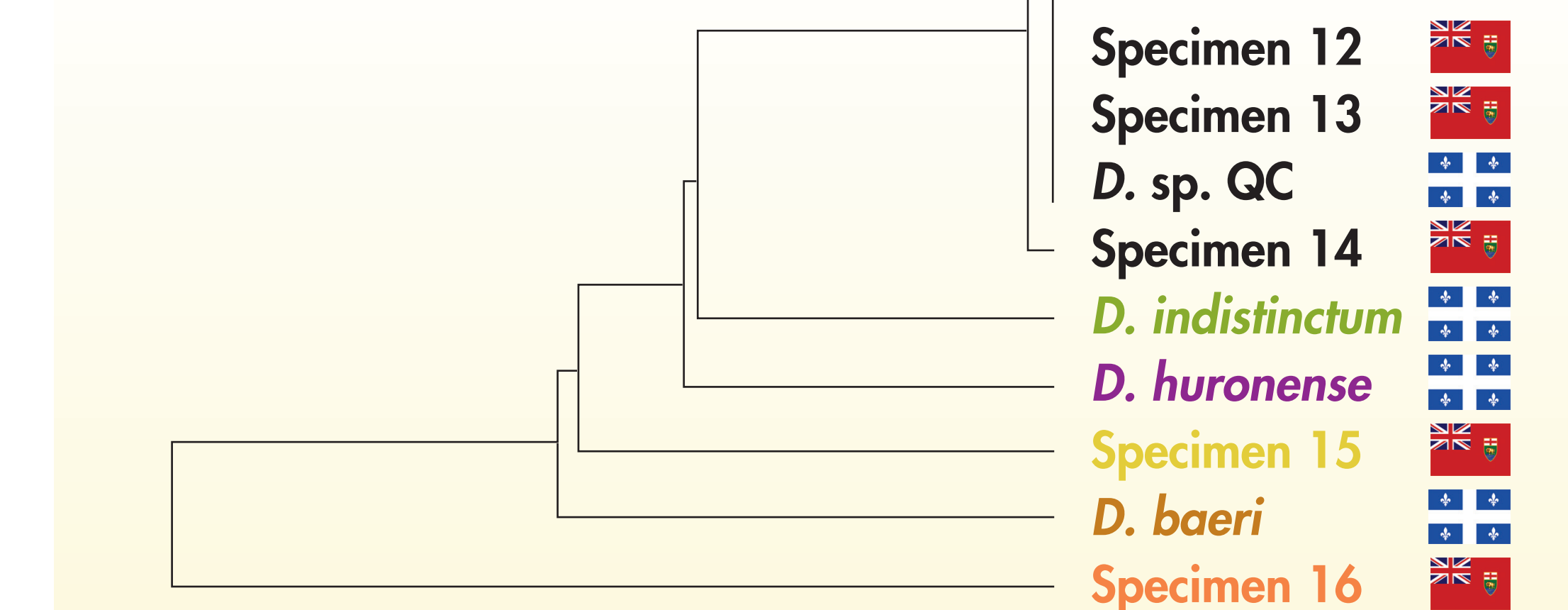


Figure 5 UPGMA grouping of CO1 sequences of *Diplostomum* species in Manitoba (■) and Quebec (□)

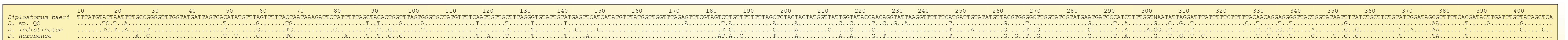


Figure 4 Alignment of partial CO1 sequences of four species of *Diplostomum* obtained from fish collected near Montreal

## DISCUSSION

The CO1 sequences provided more species-specific markers than did ITS regions<sup>1</sup> and confirmed the presence of a cryptic species (*Diplostomum* sp. QC) poorly resolved by ITS sequences.

Of the Quebec taxa, only the cryptic *Diplostomum* sp. QC was found among the Manitoba specimens, and this was the dominant species.

The two unique haplotypes from Manitoba indicate the presence of two other species of *Diplostomum* in this sample.

Amplification of CO1 failed in 22 of 42 Manitoba specimens, while 28S rDNA amplified successfully. Using the same primers, a similar situation has been recorded for another diplostomatoid, *Ichthyocotylurus*, in which CO1 amplicons could not be obtained from one of four species that all yielded ITS products<sup>14</sup>. The failure of the CO1 primers may reflect mutations at annealing sites in individuals of species already characterized or the presence of additional species. Comparison of ITS regions of these specimens with those already known<sup>1,2</sup> may clarify this issue, either by providing the identity of these specimens, or by demonstrating that they are distinct and require further study.

Our results do not support prior identifications of *Diplostomum spathaceum* in Canada, a species that has been widely reported here (Fig. 2) and is common in Europe, but which has been conspicuously absent in recent surveys in Quebec<sup>2,7</sup>.

Although limited in scope, this study demonstrates the value of molecular markers such as barcodes for assessing the diversity and distribution of parasites in general and of *Diplostomum* in particular.

## ACKNOWLEDGEMENTS

This research was supported by funding to the Canadian Barcode of Life Research Network from NSERC, an NSERC grant to J.D. McLaughlin and assistance from the Canadian Wildlife Service.

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