

## LINKING LARVAE AND ADULTS OF *APHARYNGOSTRIGEA CORNU*, *HYSTEROMORPHA TRILOBA*, AND *ALARIA MUSTELAE* (DIPLOSTOMOIDEA: DIGENEA) USING MOLECULAR DATA

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**ABSTRACT:** Because the taxonomy of trematodes is based on adults, the larval stages of most digeneans cannot be identified to species based on morphology alone. Molecular data provide a means of linking larval stages to known adults. We obtained sequences from the barcode region of cytochrome oxidase I (COI) from adult and larval parasites of fish, frogs, birds, and mammals across North America. Sequences from adult *Apharyngostriega cornu*, *Hysteromorpha triloba*, and *Alaria mustelae* (Diplostomoidea: Digenea) from definitive hosts matched those of meta- and mesocercariae from fish and frogs. These data provided new information on the distributions of all 3 parasite species. Metacercariae of *A. cornu*, which have not been previously reported in North American hosts, were found in *Notemigonus crysoleucas*, *Pimephales notatus*, and *Catostomus commersonii* in the St. Lawrence River. Metacercariae of *H. triloba* are reported in Canadian waters and in *N. hudsonius* for the first time. *Alaria mustelae* is reported for the first time in frogs from Quebec, Canada, and an additional species of *Alaria* was detected in California. Sequences of internal transcribed spacer rDNA from a subset of specimens support the same species boundaries indicated by COI divergence. There was little divergence in COI sequences from an unidentified diplostomid species sampled at a large spatial scale.

The life cycles of many parasites are poorly known. Among digeneans, for example, it is often difficult to identify the host species used during different phases of a parasite's life cycle. This is because the alpha taxonomy of digeneans is based on the morphology of the adult, and this information generally cannot be linked to larval stages bearing little resemblance to adults.

This problem is acute for metacercariae in the Diplostomoidea, in which even generic level identification is often difficult. For example, species in 6 genera in the Strigeidae (Diplostomoidea: Digenea) are frequently reported in birds (Dubois, 1968; Sulgostowska, 2007), but their metacercariae are often lumped into a single larval genus, *Tetracotyle* (Gibson, 1996; Hoffman, 1999). Niewiadomska (1970) listed morphological characters for distinguishing among genera within *Tetracotyle* metacercariae, but these distinctions are seldom made in practice. The same problem occurs in other diplostomid taxa. Characters exist for distinguishing among genera of metacercariae (Shoop, 1989), yet specimens are identified as larval types such as *Neascus*, *Diplostomulum*, and *Prohemistomulum* (Gibson, 1996; Hoffman, 1999). As a result of these uncertainties, the second intermediate host species used by most diplostomid species remain poorly known.

The classical method of elucidating trematode life cycles is the experimental infection of animals in the laboratory (Bosma, 1934; Ostrowski de Núñez, 1989). In addition to clarifying life cycles, these studies can provide information on important parasite life-history parameters, such as the proportion of infective stages that survive within the host (Voutilainen et al., 2010). However, such research is logistically demanding, and it is usually feasible to study only a single parasite species and a small number of host species. In addition, it is often necessary to use non-natural hosts in experimental studies, which reduces their relevance to parasite–host associations in nature.

Molecular data obtained during parasite surveys provide an alternative means of linking the larval stages of parasites to known adults (Jousson et al., 1998; Overstreet et al., 2002; Jensen and Bullard, 2010; Caffara et al., 2011). Here, we present data from ongoing survey work using molecular data to discriminate and identify species of digeneans that parasitize aquatic wildlife. Our surveying was opportunistic; we sampled fish and frogs that are abundant and easily collected in the St. Lawrence River Valley near Montreal, Canada, as well as host and parasite material contributed by colleagues working in other systems. Thus, our sampling is not designed to examine the life cycle of any particular species and our results probably reflect host–parasite associations that are common in nature. Much of our effort focuses on the diplostomoids because these are common parasites of fishes and amphibians in the St. Lawrence River (Marcogliese et al., 2006; King et al., 2007; Locke, McLaughlin, and Marcogliese, 2010). The results presented here pertain to the life cycles of *Apharyngostriega cornu*, *Hysteromorpha triloba*, and *Alaria mustelae*. In addition, we present data from closely related species to evaluate the capacity of the molecular markers used to resolve species.

### MATERIALS AND METHODS

Host animals (species listed in Results) were collected from Nova Scotia, Quebec, and Ontario, Canada; New Hampshire; and California by us and collaborators provided additional material (Fig. 1). Some animals were necropsied immediately after death, others were killed in the field and then frozen, and some were found dead and then frozen until necropsy. Metacercariae were removed from cysts; fixed in 70, 95, or 100% ethanol, depending on source; and stored at –20 C.

DNA was extracted from individual specimens as described in Moszczyńska et al. (2009). The DNA barcode region of cytochrome oxidase I (COI) and internal transcribed spacer (ITS)1, 5.8S, and ITS2 were amplified and sequenced using the protocols and primers described in Moszczyńska et al. (2009).

Sequence data were subjected to BLAST searches and compared with our own unpublished database, which in all cases produced the closest matches. Interspecific divergence was calculated from net Kimura-2-parameter (K2P) distances by using MEGA 4.0, i.e., mean interspecific minus mean intraspecific K2P (Tamura et al., 2007). Inter- and intraspecific K2P distances were calculated using pairwise deletion.

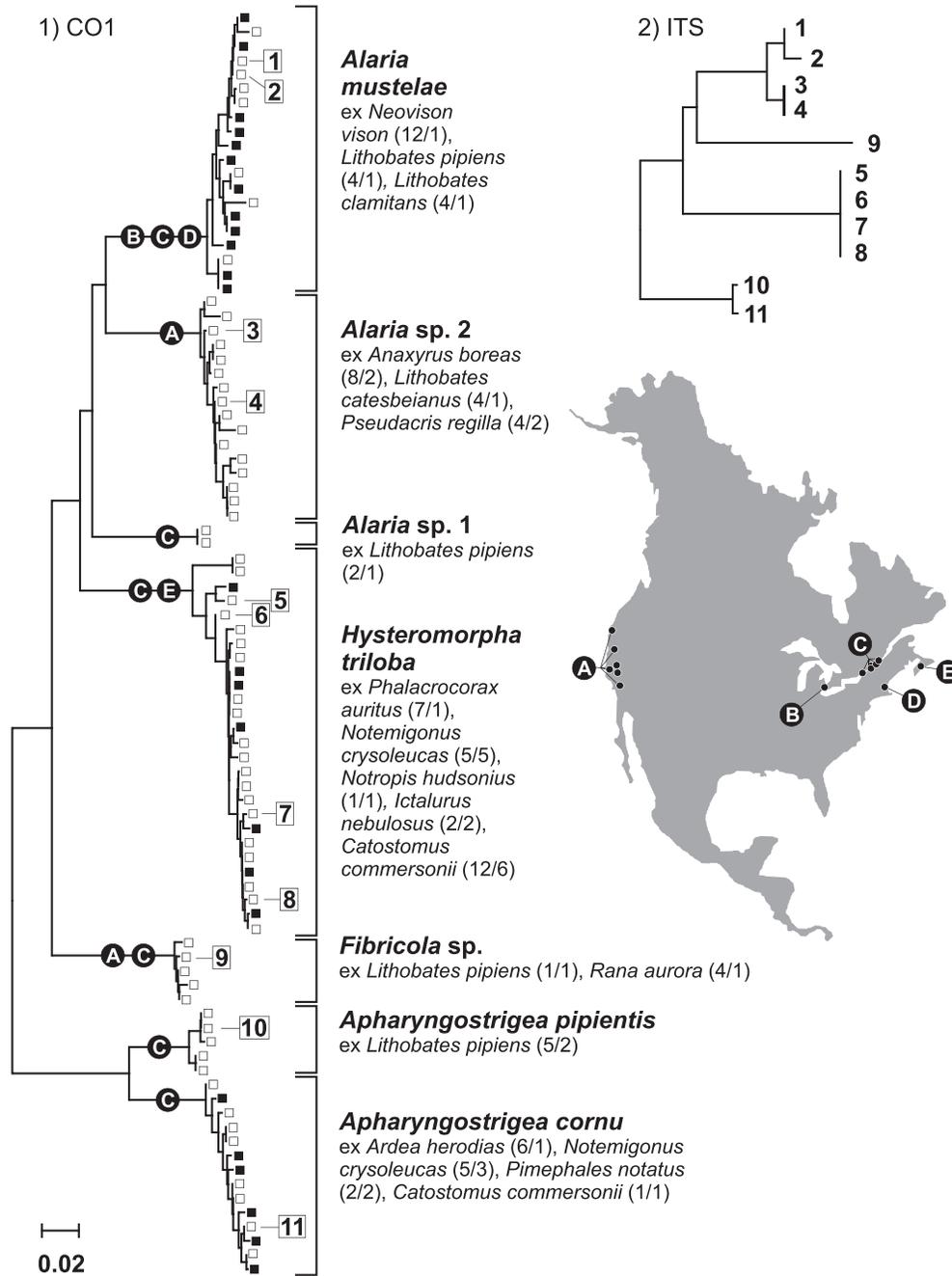
Two types of morphological vouchers for sequence data have been deposited in the U.S. National Parasite Collection (USNPC, Beltsville,

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FIGURES 1–2. Neighbor-joining analysis of K2P distances between partial CO1 (1) and partial ITS1-5.8S-ITS2 (2) sequences from diplostomoids from fish, amphibian, bird, and mammal hosts collected across North America. Empty boxes indicate sequence from meta- or mesocercariae, and solid boxes indicate sequence from adult parasites. Sequences of both CO1 and ITS were obtained from specimens indicated by boxes 1–11, i.e., sequence 1 in Figures 1 and 2 originate from the same individual *A. mustelae*. Identification and hosts are listed beside each cluster in Figure 1. In parentheses next to each host species is the number of specimens sequenced and the number of hosts from which sequenced specimens were obtained, e.g., sequences from 8 mesocercariae collected from 2 different *A. boreas* are included in the *Alaria* sp. 2 cluster of sequences. Encircled letters on branches in Figure 1 correspond to collection localities of all specimens in the corresponding cluster; localities are shown on the map. Both phenograms are on the same scale and based on pairwise comparison of sites common to 80% of all sequences (416 sites, 89 sequences of CO1; 875 sites, 11 sequences of ITS). Six short, non-overlapping ITS sequences (2 from *A. pipientis* and 4 from *Alaria* sp. 2, identical to conspecifics in Fig. 2) are not shown because they cannot be aligned.

Maryland; accessions listed in Results). True morphological voucher specimens were obtained in cases where DNA was extracted from part of a specimen, and the remainder were stained and mounted on a slide. Generally, however, this was not possible because of the small size of these organisms. We therefore also collected “bulk lot vouchers,” i.e., a specimen destined for DNA extraction was paired with 1, or more, apparently

identical specimens from the same tissue of the same individual host that were stained and mounted on slides. All sequences, chromatograms, collection data, specimen images, and voucher accession information are available for each specimen at [www.barcodinglife.org](http://www.barcodinglife.org) in project TREMA. Seventy-seven of 106 sequences are presented here for the first time. The remainder were published by Moszczyńska et al. (2009) and Locke,

McLaughlin, and Marcogliese (2010). All sequences are deposited in GenBank (accessions FJ477181, FJ477182, FJ477188, FJ477203, HM064712–HM064717, HM064883–HM064887, HM064894–HM064901, HM064925–HM064927, HM064966–HM064969, JF769422–JF769486, and JF904528–JF904538).

Flukes were identified by comparison with specimens from the USNPC (accessions 51966, 76595, 78085, 87700, 90992, 90993, 92289, 92290, 92292) and descriptions in Hughes (1929), Bosma (1934), Olivier (1940), Byrd and Ward (1943), Chandler (1942), Chandler and Rausch (1948), Huggins (1954), Dubois (1968, 1970), Johnson (1979), and McAlpine and Burt (1998).

## RESULTS

### *Alaria mustelae*, *Alaria* spp., and *Fibricola* sp.

Bi-directional CO1 sequences were obtained from 15 specimens and in 1 direction from 5 additional specimens, from 48 *A. mustelae* assayed in total. The CO1 sequences of adults from a mink (*Neovison vison*) found dead on Fighting Island (Detroit River, Ontario) were identical, or similar to, those of mesocercariae from adult *Lithobates pipiens* collected near Montreal and *Lithobates clamitans* from New Hampshire. The mean K2P distance among sequences was 1% (range, 0–2.2%), and there was no variation among translated amino acids. The ITS sequences of 2 mesocercariae from *L. clamitans* differed by 0.46%. Bulk lot vouchers were obtained for 12 adults (USNPC 104573) but for no mesocercariae.

The most similar CO1 sequences to those of *A. mustelae* were from *Alaria* mesocercariae from Quebec and California corresponding to 2 unidentified species (Fig. 1). Interspecific divergence in CO1 sequences among the 3 *Alaria* spp. ranged from 10.8 to 13.7%. Two specimens of *Alaria* sp. 1 with identical CO1 sequences were obtained from the same *L. pipiens* collected near Montreal (see above) that was infected by *A. mustelae*. *Alaria* sp. 2 was found in *Anaxyrus boreas*, *Lithobates catesbeianus*, and *Pseudacris regilla* collected from 5 localities in California. The mean K2P distance among CO1 sequences from all specimens of *Alaria* sp. 2 was 1.5% (range, 0–3.1%), and there was a single amino acid insertion in 1 sequence. Among 8 specimens yielding bi-directional sequences of high-quality, mean intraspecific K2P was 0.9% (range, 0–1.8%), and there was no variation in translated amino acids. Identical ITS sequences were obtained from 6 specimens of *Alaria* sp. 2. Limiting the comparison to longer, overlapping sequences of better quality, net divergence in ITS between *A. mustelae* and *Alaria* sp. 2 was 3% for 749 positions.

Sequences of CO1 indicated diplostomid specimens from *L. pipiens* in Quebec and *Rana aurora* in California were the same species. Sequences from these specimens did not match those of any adults, and the species is tentatively assigned to the genus *Fibricola* based on Chandler (1942) (Fig. 1). CO1 sequences from this widely distributed species varied by mean K2P of 0.4% (range, 0–0.7%). The CO1 sequence of the specimen from Quebec varied little (0.3–1.0%) from those from California. A bulk lot voucher was obtained for the Quebec specimen (USNPC 104576).

### *Hysteromorpha triloba*

Bi-directional CO1 sequences were obtained from 23 specimens and in 1 direction from 4 additional specimens, from 48 *H. triloba* assayed in total. The CO1 sequences of adults from a double-crested cormorant (*Phalacrocorax auritus*) found dead on Snake Island, Lake Erie, in 2008 were identical or similar to those of metacercariae from *Notropis hudsonius*, *Notemigonus crysoleucas*,

*Ictalurus nebulosus*, and *Catostomus commersonii* (Fig. 1). These fish were caught between 2006 and 2009 at 8 localities in the St. Lawrence and Ottawa rivers (Quebec, Ontario) and Feely Lake, Nova Scotia. Identical ITS sequences were obtained from 4 specimens sequenced for CO1, namely, a metacercaria from *N. hudsonius* and from 3 metacercariae from 3 *C. commersonii*, all from the Ottawa and St. Lawrence rivers. The mean K2P distance among the 27 CO1 sequences was 1.5% (range, 0–4.6%) and similar values were obtained if only the best-quality, overlapping regions of bi-directional sequences were analyzed. Two specimens had identical CO1 sequences that were relatively divergent from those of all other specimens (K2P = ~4.0%; see upper branch within *H. triloba* cluster in Fig. 1), among which there was less variation (mean K2P = 1.0%; range, 0–2.4%). This pair of divergent CO1 haplotypes occurred in 1 of 3 metacercariae sequenced from the same individual *C. commersonii* from Nova Scotia and in the only metacercaria sequenced from a *N. crysoleucas* from the Ottawa River. Sequences of CO1 from the other 2 metacercariae from the same individual *C. commersonii* from Nova Scotia were not particularly divergent from other specimens (mean K2P divergence, 1.1%; range, 0.3–2.4%). There were no differences in the translated amino acid sequences among the 23 bi-directional CO1 sequences, including the 2 specimens with divergent CO1 haplotypes.

Other than a thymine insertion in the ITS1 of 1 sequence, there were no differences in the 1,107-bp alignment of ITS1-5.8S-ITS2 from 4 metacercariae from different fish collected from different localities in the St. Lawrence River.

True morphological vouchers for sequences were obtained from 3 adult *H. triloba* (USNPC 104570). Bulk lot vouchers were obtained for 4 adults (USNPC 104569) and 5 metacercariae of this species (USNPC 104571–104572).

### *Apharyngostrigea cornu* and *Apharyngostrigea pipientis*

Bi-directional CO1 sequences were obtained from 6 specimens and in 1 direction from 8 additional specimens of *A. cornu*. The CO1 sequences from adults from a heron (*Ardea herodias*) collected in 2006 at Ile aux Herons, Lake St. Louis, in the St. Lawrence River, were identical or similar to those of metacercariae from *Notemigonus crysoleucas*, *Pimephales notatus*, and *C. commersonii* from several localities in the St. Lawrence River. The mean intraspecific K2P distance among the 14 CO1 sequences was 0.5% (range, 0–1.6%). There was no variation in translated amino acid sequences. ITS sequence was obtained from a metacercaria from *C. commersonii*.

True vouchers were obtained for 2 adults (USNPC 104574) but not for metacercariae of *A. cornu*. Bulk lot vouchers were obtained for all adults (USNPC 104574). No vouchers were obtained for metacercariae, which were the tetracotyle type.

*Apharyngostrigea cornu* differed from another species in our database by 7.6% in CO1 and 0.5% in ITS sequences (Fig. 1). These metacercariae were obtained from *L. pipiens* from Quebec and are tentatively identified as *A. pipientis*, based on overall morphology, lack of a pharynx, encystment characteristics, infection site, host, and sequence similarity to *A. cornu* (bulk lot voucher 104575 has been deposited in the USNPC). Mean intraspecific K2P distance among CO1 sequences from 5 *A. pipientis* was 1.0% (range, 0.3–1.6%), and there was no variation in translated amino acid sequences. There were no differences

among ITS sequences from 3 of the same specimens of *A. pipientis* from which CO1 data were obtained.

## DISCUSSION

Using molecular markers, we were able to link morphologically indistinct larval stages of 3 species of digeneans with known adults in surveys of naturally infected hosts from across North America. This provided new information on the geographic distribution and second intermediate host spectra of all 3 identified species. Similar information was obtained for several related species of larval digeneans that could not be identified to species.

*Apharyngostrigea cornu* is commonly encountered in surveys of ardeids (Navarro et al., 2005), but little is known of the spectrum of its second intermediate hosts. Metacercariae occur in cyprinid fishes in the Volga River (Dubois, 1968) and in Iraq (Salih et al., 1988), but there are no data from the New World. With the exception of an introduced species, *Cyprinus carpio*, the second intermediate hosts of *A. cornu* listed by Dubois (1968) and Salih et al. (1988) do not occur in the New World, yet the parasite is common in North American ardeids (Byrd and Ward, 1943; Flowers et al., 2004). This species was previously unknown in *N. crysoleucas*, *P. notatus*, and *C. commersonii*; the latter species is the first record in a catostomid host. These specimens were among numerous morphologically similar tetracotyle metacercariae sequenced from a large and diverse sample of sympatric fishes (Locke, McLaughlin, and Marcogliese, 2010). Taken together, the present study and previous records suggest metacercariae of *A. cornu* are specific to cyprinids and may occasionally infect catostomids (Cypriniformes) and that this specificity is stable across its global distribution (see gamma specificity of Krasnov et al., 2011).

There are few studies of the life cycles of other species of *Apharyngostrigea* (Dubois, 1968). In Argentina, Ostrowski de Núñez (1989) experimentally verified 2 fish species (Poeciliidae, Cichlidae) as competent hosts of *Apharyngostrigea simplex* metacercariae. It is tempting to speculate that unidentified *Apharyngostrigea* in Mexican cichlids (Vidal-Martínez et al., 2001) may be the same species. However, Ukoli (1967) suggested that *A. simplex* and *Apharyngostrigea serpentia* are transmitted to definitive hosts by amphibians. Finally, in contrast to *A. cornu* that has been almost exclusively reported from definitive hosts, *A. pipientis* is nearly always encountered in second intermediate hosts (Ranidae), including in our study area (Olivier, 1940; McAlpine and Burt, 1998; King et al., 2007). We are aware of only 1 record of adults recorded in naturally infected hosts (Sepúlveda et al., 1999). The molecular data provided here will be useful for further studies of the life cycles of *A. cornu*, *A. pipientis*, and other species of *Apharyngostrigea*. Our results also support the notion that metacercariae of *A. cornu* and *A. pipientis* are specific to cypriniform fishes and ranid frogs, respectively, across a large geographic scale.

Both nuclear and mitochondrial markers differed between *A. mustelae* and *Alaria* sp. 2, but there is another way to interpret this observation. Genetic structure may arise in geographically distant populations of single species, particularly in those with low dispersal potential (Irwin, 2002; Kuo and Avise, 2005). Because their definitive hosts are terrestrial mammals, species of *Alaria* are probably less widely dispersed than diplostomoids that mature in more vagile avian hosts. Thus, it could be argued that the CO1 and ITS divergence between *Alaria* spp. specimens collected in Quebec and New Hampshire and those from California reflects the spatial

separation of samples within 1 species (*A. mustelae*) rather than divergence between 2 (*A. mustelae* and *Alaria* sp. 2). However, in this case the levels of genetic divergence (~10% in CO1, 3% in ITS) are high even for fragmented populations of a single, low-dispersal species (Meyer and Paulay, 2005). More generally, if sequence divergence levels within species sampled on large spatial scales overlap with divergence levels between species, this could pose a problem for the sequence-based identification approach used here. In this context, the species tentatively identified as *Fibricola* sp. provides a useful counter example. Despite the large spatial scale at which this species was sampled, there was little divergence in CO1 sequences in material from Quebec and California. By analogy, this suggests that speciation, not isolation by distance, is the mechanism responsible for the genetic variability among specimens of *A. mustelae* and *Alaria* sp. 2.

*Alaria mustelae* was already known to infect all host species in which we recorded it (Bosma, 1934) but had not previously been reported in frogs in Quebec. Bosma (1934) and Johnson (1979) documented natural infections of *A. mustelae* in ranids through the identification of experimentally obtained adults. In hylid hosts, in contrast, records of *A. mustelae* are based on identifications of mesocercariae or cercariae (Yoder and Coggins 1996, 2007; Johnson et al., 1999). In our experience, distinguishing species of *Alaria* spp. mesocercariae (following Johnson, 1970) is difficult. It is notable that we recorded *Alaria* sp. 2 in the same region and the same hylid host species in which Johnson et al. (1999) reported *A. mustelae*, i.e., *Pseudacris (Hyla) regilla*, from California. Thus, molecular confirmation of *A. mustelae* in hylids is desirable. Finally, we found individual *L. pipiens* was infected by both *A. mustelae* and *Alaria* sp. 1. Similar reports by Hofer and Johnson (1970) and Johnson (1979) indicate concomitant infections by multiple species of *Alaria* are probably common in this host.

Molecular methods of discriminating species are also relevant to human health risks posed by *Alaria* spp. Several cases of alariosis (species unknown) resulting in illness or death have been documented in North America, and treatment varies for different trematodiasis (Möhl et al., 2009; Fried and Abruzzi, 2010).

*Hysteromorpha triloba* is cosmopolitan and the only species in this genus known in North America (Dubois, 1968). Because no closely related species occurs locally, it may be expected that metacercariae of *H. triloba*, a relatively large fluke, would be easily identified based on its morphology. However, there was no previous report of *H. triloba* in Canadian fish (Margolis and Arthur, 1979; McDonald and Margolis, 1995). Thus, the molecular link with the adult forms from *P. auritus* was useful in confirming the first Canadian records of *H. triloba* in fishes from Nova Scotia, Quebec, and Ontario. *Notropis hudsonius* is a new host record. In subsequent work, we have determined metacercariae of *H. triloba* are common in Canadian localities, occurring in 37 of 106 *N. crysoleucas* (St. Lawrence, Ottawa, and Richelieu rivers), 6 of 6 *I. nebulosus* (St. Lawrence and Ottawa rivers), and 12 of 27 *C. commersonii* (St. Lawrence and Ottawa rivers; Feely Lake, Nova Scotia). Quantitative data are unavailable for *N. hudsonius*. The lack of previous records of *H. triloba* in Canadian fishes may reflect the large fluctuations in populations of its definitive host *P. auritus* over the past century (Weseloh et al., 1995).

Both experimental infections and molecular surveys have certain advantages as methods of elucidating trematode life cycles

(Poulin and Keeney, 2007). One strength of the molecular approach is amply demonstrated by considering the vast scope of work necessary to reproduce the results presented here (3 parasite species, 3 definitive host taxa, 12 second intermediate host taxa, and a large geographic scale) using experimental infections. Molecular surveys provide an accurate reflection of host-parasite associations that are common in nature, whereas the use of non-natural hosts in experimental studies may reduce their relevance to natural systems. Molecular surveys can distinguish congeneric species of meta- or mesocercariae in individual hosts, a situation that is probably common in naturally infected animals but difficult to deal with in experimental infections (Johnson, 1979; Galazzo et al., 2002; Locke, McLaughlin, and Marcogliese, 2010; present study). Ideally, molecular and experimental methods can be used in tandem to mutually enhance one another (Galazzo et al., 2002; Overstreet et al., 2002).

It is our hope that the data presented here will stimulate other researchers to obtain CO1 sequences from the barcode region in these, and other, digeneans. Studies using both types of data indicate that CO1 resolves closely related species of digeneans as effectively, or better than, more commonly used ribosomal markers (Bell et al., 2001; Overstreet et al., 2002; Miura et al., 2005; Vilas et al., 2005; Detwiler et al., 2010; Locke et al., 2010; Locke, McLaughlin, and Marcogliese, 2010; Razo-Mendivil et al., 2010; Caffara et al., 2011; Rosas-Valdez et al., 2011). In addition to clarifying species boundaries, a library of sequences from a standardized region of CO1 will be a valuable resource for better understanding of the life cycles and intermediate and definitive host spectra of digeneans across large spatial scales and a broad variety of host taxa.

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