Molecular systematics of some North American species of *Diplostomum* (Digenea) based on rDNA-sequence data and comparisons with European congeners

Daniel E. Galazzo, Selvadurai Dayanandan, David J. Marcogliese, and J. Daniel McLaughlin

Abstract: The systematics of *Diplostomum* species, common intestinal parasites of piscivorous birds, has long been problematic, owing to phenotypic plasticity and the paucity of morphological features that are often subject to age- and host-induced variation. We sequenced the ITS1–5.8S–ITS2 regions of the rDNA from adult *Diplostomum huronense*, *Diplostomum indistinctum*, and *Diplostomum baeri* obtained from experimentally infected ring-bill gulls (*Larus delawarensis*) and compared them with partial ITS1 sequences from several species of *Diplostomum* in GenBank. The three North American species were distinguishable on the basis of ITS sequences. Sequences from *D. huronense* differed from those of *D. indistinctum* at 12 sites in ITS1 and 4 sites in ITS2, supporting morphological and morphometric data that indicate the two are distinct species. Sequences of *D. huronense* and *D. indistinctum* differed from those of *D. baeri* at 27 and 24 sites, respectively, in ITS1 and 15 and 12 sites, respectively, in ITS2. Phylogenetic analysis of partial ITS1 sequences revealed that the North American and European species of *Diplostomum* formed separate groups, with the former being basal to the latter. The results indicated that *D. huronense* and *D. indistinctum* from North America are distinct from *Diplostomum spathaceum* and other similar species from Europe. Furthermore, sequences from specimens identified as *D. baeri* from North America differed from those of *D. baeri* from Europe by 3.8% in ITS1 (23 sites). While morphologically similar, the two are not conspecific. Sequences of the North American species have been deposited in GenBank (AY 123042–123044).

Résumé : La systématique des espèces de *Diplostomum*, parasites intestinaux communs des oiseaux piscivores, a toujours été problématique à cause de leur plasticité phénotypique et de la rareté des caractères morphologiques qui sont souvent soumis à des variations dues à l'âge et à l'hôte. Nous avons procédé au séquençage des régions ITS1–5.8S–ITS2 du gène d'ADNr de *Diplostomum huronense*, de *Diplostomum indistinctum* et *Diplostomum baeri* adultes provenant de goélands à bec cerclé (*Larus delawarensis*) infectés expérimentalement et nous avons comparé les séquences obtenues à des séquences partielles d'ITS1 de plusieurs espèces de *Diplostomum* de la banque génétique GenBank. Les trois espèces nord-américaines se distinguent par les séquences de leurs ITS. Les séquences de *D. huronense* diffèrent de celles de *D. indistinctum* à 12 sites sur ITS1 et à 4 sites sur ITS2, ce qui corrobore les données morphologiques et morphométriques qui indiquent qu'il s'agit de deux espèces distinctes. Les séquences de *D. huronense* et de *D. indistinctum* diffèrent de celles de *D. baeri* à 27 et 24 sites sur ITS1 et à 15 et 12 sites sur ITS2, respectivement. L'analyse phylogénétique des séquences partielles D'ITS1 a révélé que les espèces nord-américaines et européennes de *Diplostomum* forment deux groupes distincts, le premier groupe étant plus ancien. Les résultats indiquent que *D. huronense* et *D. indistinctum* d'Amérique du Nord sont des espèces distinctes de *Diplostomum spathaceum* et des autres espèces européennes semblables. De plus, les séquences provenant de spécimens nord-américains identifiés comme *D. baeri* diffèrent par 3,8 % des séquences de *D. baeri* européens sur ITS1 (23 sites). Bien que morphologiquement semblables, les spécimens appartiennent à deux espèces différentes. Les séquences des espèces nord-américaines ont été déposées à la banque génétique GenBank (AY 123042–123044).

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D.E. Galazzo,[1] S. Dayanandan, and J.D. McLaughlin.[2] Department of Biology, Concordia University, 1455 de Maisonneuve Boulevard West, Montreal, QC H3G 1M8, Canada.
D.J. Marcogliese. St. Lawrence Centre, Environment Canada, 105 McGill Street, 7th Floor, Montreal, QC H2Y 2E7, Canada.

[1]Present address: Institute of Parasitology, Macdonald College, McGill University, Ste.-Anne-de-Bellevue, QC H9X 1C0, Canada.
[2]Corresponding author (e-mail: mcljd@alcor.concordia.ca).

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Introduction

Species belonging to the genus *Diplostomum* are intestinal parasites of gulls, terns, and other piscivorous birds, and infections are commonplace throughout the holarctic. Snails and fish serve as the first and second intermediate hosts, respectively. Various aspects of the taxonomy, biology, and host–parasite relations of *Diplostomum* spp. have been reviewed in detail by Chappell et al. (1994), Chappell (1995), and Niewiadomska (1996). *Diplostomum* is of particular interest as the metacercariae are pathogenic to fish. Metacercariae of many species infect the lens, affecting vision, which in turn may alter other aspects of fish behavior. Heavier infections can cause cataracts and may be fatal (Chappell et al. 1994; Chappell 1995; Niewiadomska 1996). Given the possibility that different species may vary in pathogenicity, there is a clear need for reliable methods to identify infections in fish to the species level (Chappell 1995).

Identification of species within *Diplostomum* is difficult at all stages in the life cycle. This is due in part to the phenotypic plasticity of the organisms themselves, the paucity of morphological features in certain life-cycle stages, age- and host-induced variation, artefacts produced during fixation, and the extensive overlap in morphological characteristics that occurs among species (Chappell et al. 1994; Chappell 1995; Gibson 1996; Niewiadomska 1996; Niewiadomska and Laskowski 2002). Although cercariae appear to be the least difficult to identify to the species level (Niewiadomska 1996; Niewiadomska and Laskowski 2002), identification of metacercariae and adults, which are much more frequently encountered, is more difficult. Keys and monographs produced to identify the metacercariae (e.g., Sudarikov 1971; Shigin 1976, 1986) have not been satisfactory and disagreement exists over identifications that have been based on metacercarial morphology (Niewiadomska 1996). At present, the most reliable way to identify the metacercariae is to raise them to adulthood in day-old chicks (Field et al. 1994) or, preferably, in the natural definitive host. The logistics involved in maintaining birds and the fact that fish samples are often frozen for later study usually precludes this approach, and species level identification of the adults presents its own difficulties. The validity of several nominal species has been questioned (Niewiadomska 1996) and much of the published data may be based on misidentified specimens (Chappell et al. 1994; Niewiadomska 1996).

DNA-based approaches provide an independent method of distinguishing between species when morphological criteria are equivocal or are subject to variation (McManus and Bowles 1996). Sequences of the internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal RNA gene (rDNA), in particular, have been used successfully to distinguish between adults of closely related species within several genera of digeneans (e.g., Luton et al. 1992; Morgan and Blair 1995; Blair et al. 1997; León-Règagnon et al. 1999; Bartoli et al. 2000; Jousson and Bartoli 2000, 2001; Tkach et al. 2000; Bell et al. 2001; Niewiadomska and Laskowski 2002 (who used partial ITS1 sequences to examine the phylogeny of six species of *Diplostomum* from Europe)), among many others. Because DNA remains constant throughout ontogeny, ITS sequences have proven useful in the identification of various life-history stages as well (e.g., Cribb et al. 1998; Jousson et al. 1998, 1999; Anderson 1999; Jousson and Bartoli 2000, 2002; Overstreet et al. 2002). The availability of molecular markers would be particularly valuable for flukes like *Diplostomum* spp., where identification of larval stages rather than of adults is often required.

In previous studies (Marcogliese et al. 2001*a*, 2001*b*), we reported three species of *Diplostomum*, viz. *D. huronense* (La Rue, 1927), *D. indistinctum* (Guberlet, 1923), and *D. baeri* Dubois, 1937, from laboratory-raised gulls fed the eyes of infected fish caught in the St. Lawrence River near Montreal, Canada. *Diplostomum huronense* and *D. indistinctum* belong to a group of species that are similar morphologically to *Diplostomum spathaceum* (Rud., 1819) and a number of other species that occur commonly throughout Europe and the former USSR. Dubois (1970) considered the North American forms (*D. huronense* and *D. indistinctum*) sufficiently similar to *D. spathaceum* to include them, and *Diplostomum murrayense* (Johnston and Cleland, 1938) from Australia, as a subspecies of it. Similarly, Dubois (1970) recognized two subspecies of *D. baeri*, one from North America and the other from Europe. As in previous studies (Marcogliese et al. 2001*a*, 2001*b*), we have followed Sudarikov (1971), who recognized *D. huronense* and *D. indistinctum* as independent species, and Shigin (1977), who, along with several other European authors, did not recognize subspecies of *D. baeri*.

In the present study, we report genetic information based on the nuclear rDNA sequences from *D. huronense*, *D. indistinctum*, and *D. baeri* that can be used to corroborate the morphological data used to distinguish them at the species level. Using these sequences and those obtained by Niewiadomska and Laskowski (2002), we reconstructed the phylogeny of *Diplostomum*, to evaluate the relationship between the North American and European species. On the more applied side, resolution of the relationships between the nominal species of *Diplostomum* will permit development of DNA-based techniques that could be used for species-level identification of larval stages, particularly metacercariae, in fish.

Materials and methods

Experimental material and identifications

Eggs of ring-bill gulls (*Larus delawarensis*) were collected from local colonies near Montreal and hatched in the laboratory; the chicks were raised in the waterfowl facility at Concordia University. All housing, rearing, and experimental procedures conformed to guidelines set forth by the Canadian Council on Animal Care.

Fish were captured at various sites in the St. Lawrence River near Montreal with a large beach seine, as described by Marcogliese et al. (2001*b*). Metacercariae of *D. indistinctum* and *D. huronense* were obtained from the lenses of white suckers, *Catostomus commersoni* (Lacépède), and silver redhorse, *Moxostoma anisurum* (Rafinesque). The eyes were opened in 0.85% saline to remove the lenses and examined under a dissecting microscope for metacercariae. Infected lenses were rinsed in 8-cm Tyler sieves (No. 40; mesh size 425 μm), to ensure that only metacercariae infecting the lens were used. The intact lenses were fed to gull chicks using pipettes. Metacercariae of *D. baeri* were obtained from yellow perch, *Perca flavescens* (Mitchill). Eyes were removed from the fish and opened in 0.85% saline. The metacercariae

of *D. baeri* occur in the humor rather than in the lens. After dissecting the eye, the lens was removed and examined under a dissecting microscope, to determine whether it was infected. To ensure that no accidental contamination with lens forms occurred, only metacercariae from fish with uninfected lenses were used. The metacercariae were stored briefly in 0.85% saline and then fed by pipette to gull chicks. Metacercariae belonging to an unidentified species of *Tylodelphys* were also present in the vitreous humor of *P. flavescens*. These were frozen for molecular analysis. Birds that received metacercariae in intact lenses developed heavy infections; those that received unprotected metacercariae (*D. baeri*) did not.

Gulls were necropsied 10–22 days post exposure. The intestine was opened in lukewarm tap water, shaken vigorously to dislodge the flukes, and the intestinal contents and the mucosa were examined under low-power magnification for flukes. The flukes were cleaned in lukewarm tap water and given a final rinse in distilled water before processing. The majority of specimens treated in this way died in an extended position.

Metacercariae of at least two species of *Diplostomum* present in the lenses of local fish may infect gulls (Marcogliese et al. 2001*a*, 2001*b*). Specimens were divided into two lots: one for molecular and one for morphological identification. The forebodies of specimens to be used for molecular work were severed from the hindbodies with a small scalpel. The sections of each fluke were placed in separate, identically numbered, 1.5-mL Eppendorf tubes. The forebody was frozen at –20°C for molecular work and the hindbody was fixed in hot 70% ethanol for identification. This provided an accurate method of identifying each specimen and a permanent morphological record for each sequence obtained. Additional specimens were frozen intact. Fixed specimens and the hindbodies of the specimens used for the DNA analysis were stained in acetocarmine for taxonomic study. They were compared with type specimens of *D. huronense* (USNM 060230 (from the U.S. National Museum of Natural History, Washington, D.C.)) and *D. indistinctum* (USNM 060444) and with additional specimens of *D. indistinctum* (CMNPA 1980-0226 (from the Canadian Museum of Nature, Ottawa, Ont.); Dick and Rosen 1981) and *D. baeri* (CMNPA 1980-0225; Dick and Rosen 1981; and CMNPA 1987-1591, 1987-1593, and 1987-1595; Shostak et al. 1987) from Canada.

DNA extraction, amplification, and sequencing

DNA was extracted from the forebodies of individual specimens of *Diplostomum* and from the metacercariae of *Tylodelphys* sp., using the Qiagen DNeasy Extraction Kit™ following the manufacturer's protocols, and stored at –20°C until needed. A portion of the rDNA that included the complete ITS1–5.8S–ITS2 region was amplified by PCR on a PTC-100 Programmable Thermo Controller (MJ Research Inc., Watertown, Mass.). The PCR primers designated D1 (5′-AGG AAT TCC TGG TAA GTG CAA G-3′) and D2 (5′-CGT TAC TGA GGG AAT CCT GGT-3′) were complementary to positions near the 3′ and 5′ ends of the 18S and 28S regions, respectively. D1 was based on primer 18b of Hillis and Dixon (1991) but was modified slightly according to sequence data for the 18S region in species of *Echinostoma* listed in GenBank. D2 was identical to primer 28u of Hillis and Dixon (1991). The DNA amplification reactions contained 5 μL PCR buffer (4.0 mM $MgCl_2$), 1 μL dNTPs (10 mM), 25 pmol of each primer, 2.5 U Taq polymerase, and 10 μL genomic DNA, in a total volume of 50 μL.

Following the initial denaturing step (94°C for 2 min), the cycling protocol was as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min; with a final extension at 72°C for 5 min. Samples were then held at 4°C. The product was run through electrophoresis using a 1% agarose gel containing ethidium bromide (0.5 μg/mL) and visualized under UV light.

PCR products were purified using the QIAquick PCR Purification Kit™ (Qiagen) following the manufacturer's protocols. Sequencing was performed at the Concordia University Centre for Structural and Functional Genomics, using the CEQ 2000XL DNA Analysis System (Beckman). Primers BD1 (5′-GTC GTA ACA AGG TTT CCG TA-3′) and BD2 (5′-TAT GCT TAA ATT CAG CGG GT-3′) of Luton et al. (1992) were used as the forward and reverse sequencing primers, respectively.

Sequence analysis

Sequence quality was assessed using CEQ software (Beckman, Fullerton, Calif.) and edited manually as needed. Sequences were assembled using the Contig Assembly Program (http://tagc.univ-mrs.fr/bioinfo/sequtil/options/cap.html) and aligned using Clustal X software with default settings. The boundaries between the 18S (5′) and ITS1 regions, the ITS2 and 28S (3′) regions, and the limits of the 5.8S region were determined by comparison with those reported for other digenean species in GenBank. Voucher specimens, including those used in the illustrations and the posterior ends of the specimens of *D. indistinctum* and *D. huronese*, whose sequences are shown in Fig. 4, have been deposited in the parasite collection of CMN under the following numbers: *D. baeri*, CMNPA 2001-0002; *D. huronense*, CMNPA 2001-0003, and *D. indistinctum*, CMNPA 2002-0004. The DNA sequences of each species were submitted to GenBank and are listed under the following accession numbers: *D. baeri*, AY123042; *D. indistinctum*, AY123043; and *D. huronense*, AY123044.

Phylogenetic analysis

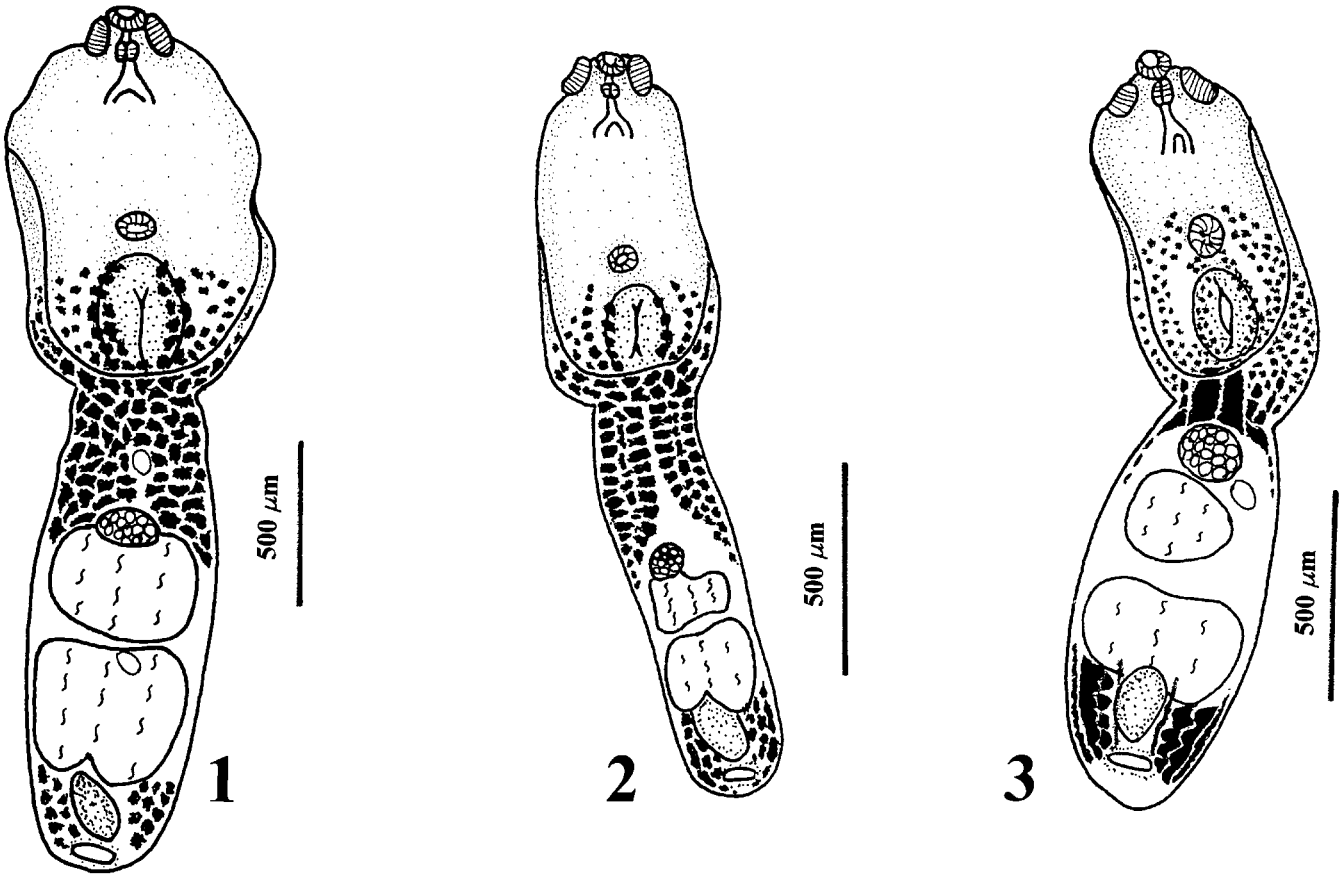
Phylogenetic analyses were conducted on the aligned partial nucleotide sequences of ITS1 from the North American and European species of *Diplostomum* using PAUP* 4.0 (Swofford 2002). *Tylodelphys* sp., which is considered to be the genus immediately ancestral to *Diplostomum* (Shoop 1989), was used as an outgroup. Parsimony analysis was performed using the exhaustive-search option with default settings of the program. Robustness of the branches was evaluated using bootstrap analysis with 10 000 replicates. Only genetic data was used in the phylogenetic analyses.

Results

Identifications

The mean and range of the body-size measurements, those of various organs, and the pertinent ratios and distances (expressed as percentages) of *D. huronense* (Fig. 1), *D. indistinctum* (Fig. 2), and *D. baeri* (Fig. 3) are presented in Table 1. The data from each species were compared with previous de-

Figs. 1–3. *Diplostomum* species from experimentally infected ring-bill gulls. Fig. 1. *Diplostomum huronense*. Fig. 2. *Diplostomum indistinctum*. Fig. 3. *Diplostomum baeri*. The band of vitellaria situated dorsally to the testes has been omitted.



scriptions of *D. huronense* (La Rue 1927; Dubois 1970), *D. indistinctum* (Guberlet 1923; Dubois 1970; Dick and Rosen 1981), and *D. baeri* (Dubois 1970; Dick and Rosen 1981; Shostak et al. 1987; Hoglund and Thulin 1992). Our specimens of *D. huronense* and *D. indistinctum* were virtually identical to the type specimens and corresponded closely to the descriptions in La Rue (1927) and Guberlet (1923), respectively. The range measurements of both species overlapped extensively with those of earlier descriptions. However, the minimum and maximum measurements in the present material were generally less than those previously recorded, particularly for those specimens described by Dubois (1970). Measurements of *D. baeri* corresponded closely to those from a large sample obtained by Shostak et al. (1987) from two species of experimentally infected gulls and with samples from experimentally and naturally infected gulls from Sweden (Hoglund and Thulin 1992), although the mean dimensions in our specimens tended to be larger than those in the Swedish ones.

The rDNA PCR products from five *D. huronense*, nine *D. indistinctum*, and five *D. baeri* specimens were sequenced. For each species, specimens came from gulls that had been infected with metacercariae obtained from two to four different localities and (or) from 2 different years. We obtained complete sequences for three, nine, and four specimens of *D. huronense*, *D. indistinctum*, and *D. baeri*, respectively. These were used for intraspecific comparisons and each sample included specimens that originated from two to four different sources. The length of the ITS1 region differed slightly among species and consisted of 603, 607, and 604 nucleotides in *D. huronense*, *D. indistinctum*, and *D. baeri*, respectively. The number of nucleotides in the 5.8S region (160) was identical in the three species. The ITS2 regions of *D. huronense* and *D. indistinctum* consisted of 298 nucleotides, whereas 297 were present in that of *D. baeri*. In contrast with a number of studies on digeneans, no repeat sequences were found in the ITS regions of the three species examined.

No intraspecific differences were found in the ITS regions of *D. baeri* and only a few (1–9 including gaps) were present in the other species. The positions reported for these correspond to those in the consensus sequences presented in Fig. 4. One specimen of *D. huronense* had a G–A transition at position 467 in ITS1. Six of the nine specimens originally identified as *D. indistinctum* had identical ITS sequences. Three specimens differed at three sites in the ITS1 region; each had A–C transversions at positions 574 and 612 and a G–A transition at position 599. One specimen differed further with a four-base deletion (positions 155–158), a C–T transition at position 307 in ITS1, and a G–A transition at site 845 in the ITS2 region. These three sequences were obtained from specimens that had been frozen intact and, as they could only be identified tentatively to the species level, they were not included in the interspecific comparisons.

The complete alignment of the ITS1–5.8S–ITS2 regions for the three species consisted of 1065 bp (607 bp in ITS1, 160 bp in 5.8S, and 298 bp in ITS2; Fig. 4). There were 46

Table 1. Morphometrics of *Diplostomum huronense* (*n* = 14), *Diplostomum indistinctum* (*n* = 10), and *Diplostomum baeri* (*n* = 10), from experimentally infected ring-bill gulls (*Larus delawarensis*).

	D. huronense		*D. indistinctum*		*D. baeri*	
Variable	Mean	Range	Mean	Range	Mean	Range
Length (mm)	2.24	1.63–2.99	2.01	1.79–2.38	1.87	1.66–2.11
Forebody length (mm)	1.02	0.81–1.28	0.82	0.74–0.89	0.93	0.81–1.02
Forebody width (mm)	0.54	0.37–0.71	0.47	0.38–0.62	0.40	0.37–0.42
Hindbody length (mm)	1.21	0.77–1.97	1.18	0.95–1.58	0.95	0.81–1.10
Hindbody width (mm)	0.43	0.13–0.62	0.27	0.21–0.34	0.38	0.32–0.43
Oral sucker length	61	48–72	61	55–74	77	48–84
Oral sucker width	72	50–100	74	64–88	93	84–108
Prepharynx length	5	0–12	9	1–16	6	1–12
Pharynx length	57	45–74	62	57–72	62	55–72
Pharynx width	56	48–84	60	52–64	62	57–72
Esophagus length	28	12–54	25	14–36	24	6–42
Acetabulum length	70	50–86	75	62–91	93	84–108
Acetabulum width	81	48–108	80	72–84	94	84–105
Tribocytic organ length	240	120–312	244	192–283	240	192–300
Tribocytic organ width	182	96–288	221	192–261	173	144–211
Ovary length	121	87–177	102	86–124	118	96–144
Ovary width	133	96–172	103	96–124	131	96–144
Anterior testis length	248	148–384	195	168–240	234	168–365
Anterior testis width	363	252–484	229	204–280	286	288–360
Posterior testis length	333	204–564	251	228–268	294	192–444
Posterior testis width	345	206–540	240	204–288	353	240–444
Egg width	102	96–108	101	96–108	—	—
Egg width	61	52–69	71	69–72	63	60–67
Hindbody/forebody	1.19	0.71–2.03	1.43	1.07–1.82	1.01	0.80–1.09
Acetabulum/forebody*	48	42–54	46	30–52	46	38–56
Tribocytic organ/forebody*	62	53–69	56	51–61	59	50–68
Ovary/hindbody 1*	25	12–34	32	27–38	7	2–16
Ovary/hindbody 2*	35	27–41	41	36–46	20	16–27
Anterior testis/hindbody 1*	33	21–39	41	36–46	17	11–21
Anterior testis/hindbody 2*	51	46–55	56	52–60	40	35–45
Posterior testis/hindbody 2*	81	75–88	82	79–86	68	61–72

Note: Each sample contained individuals from the host from which the sequenced specimens were obtained. All measurements are reported in micrometres unless indicated otherwise. Values with asterisks (*) are percentages indicating the position of the anterior (1) or posterior (2) border of the respective structure relative to the anterior edge of the forebody or hindbody of the specimen.

variable sites, including gaps, over the entire alignment. Thirty variable sites (4.9%) were present in ITS1 and another 16 sites (5.3%) were present in ITS2. Pairwise comparisons of the entire ITS region revealed that *D. huronense* and *D. indistinctum* were more similar to each other (a 1.8% difference overall) than either was to *D. baeri* (4.6 and 3.9%, respectively). *Diplostomum huronense* differed from *D. indistinctum* at 12 (1.9%) sites in ITS1 and four (1.3%) sites in ITS2, whereas *D. huronense* and *D. indistinctum* differed from *D. baeri* at 27 (4.4%) and 24 (3.9%) sites, respectively, in ITS1 and 15 (5%) and 12 (4%) sites, respectively, in ITS2.

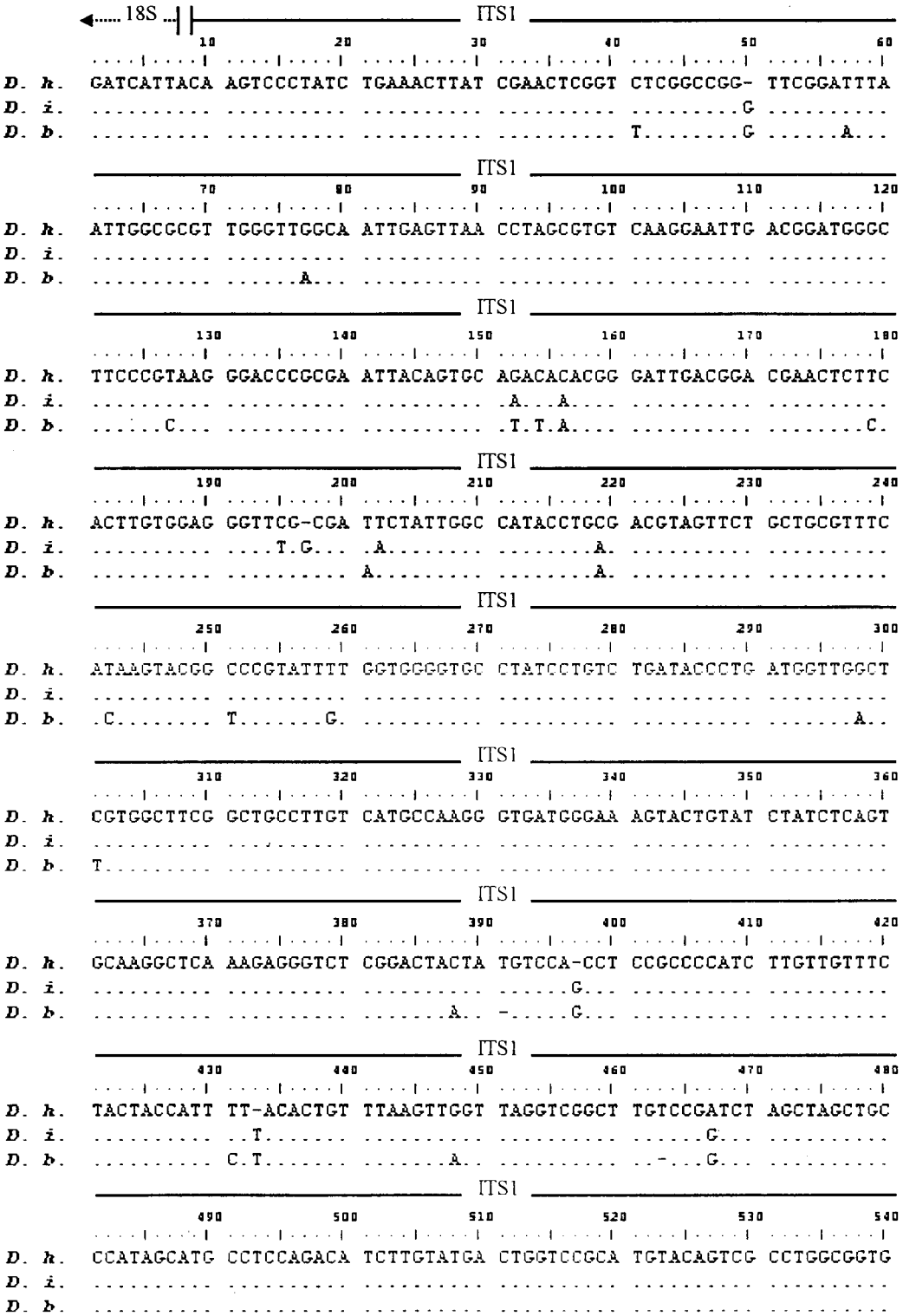
The number of nucleotide differences in the partial ITS1 sequences (578 nucleotides) of North American and European species (Niewiadomska and Laskowski 2002) are presented in Table 2. The North American and European species differed by between 11 and 38 nucleotides (2.1–7.0%).

The parsimony analysis produced two equally parsimonious trees, each with a length of 89 steps, and with a consistency index (CI) of 0.774, a retention index (RI) of 0.773, and a rescaled consistency index (RC) of 0.596. The two topologies were similar, except for the relationship between *D. huronense* and *D. baeri* from North America. The two species either clustered together or *D. huronense* occupied a position immediately basal to *D. baeri*. However, clustering of *D. huronense* with *D. baeri* was not supported by bootstrap analysis (bootstrap consensus value of <50%). The most parsimonious tree with bootstrap consensus values of >50% at all branches is given in Fig. 5. The North American species, including *D. baeri*, were basal to the monophyletic clade of European species of *Diplostomum* that included the European form of *D. baeri*.

Discussion

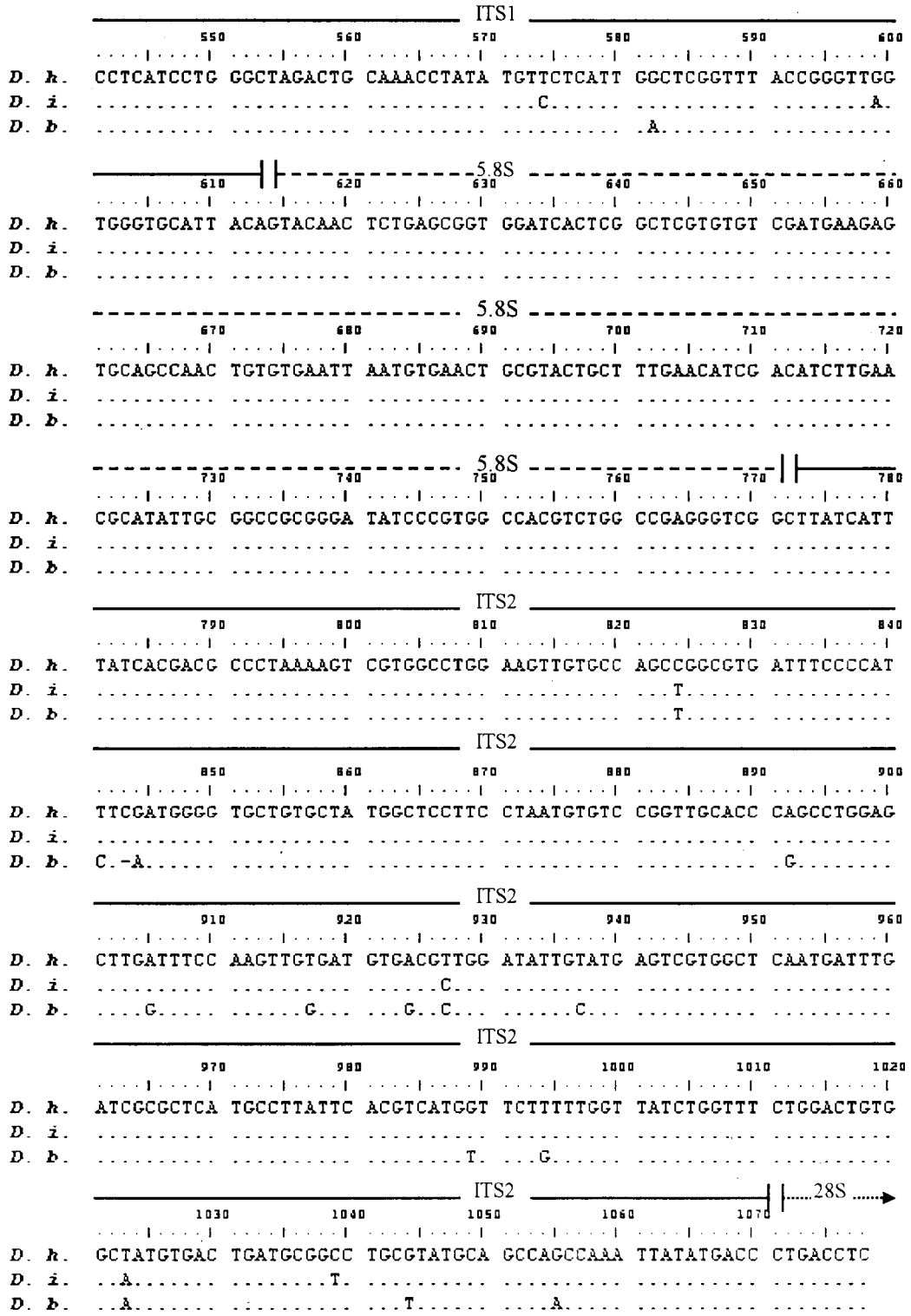
Diplostomum huronense, *D. indistinctum*, and *D. baeri* can be distinguished on the basis of their ITS regions. The molecular data support the morphological criteria currently used to distinguish *D. huronense* and *D. indistinctum*, the most distinctive of which is the shape of the anterior testis. Although subject to some variation (see Dubois 1970), it

Fig. 4. Aligned sequences of the ITS1, 5.8S, and ITS2 regions of *D. huronense (D. h.), D. indistinctum* (*D. i.*), and *D. baeri* (*D. b.*). A dot (•) indicates that the nucleotide at that point is identical to the one in the top sequence and a hyphen (-) indicates a gap in the alignment.



is symmetrical, or nearly so, in *D. huronense*, whereas in *D. indistinctum*, the larger lobe is approximately twice as long as the other (Figs. 1 and 2). Interspecific differences over the combined ITS regions ranged from 1.7 to 4.4%, well within the ranges reported in studies on several other digeneans (e.g., Luton et al. 1992; Morgan and Blair 1995 (excluding *Echinostoma hortense*); Jousson and Bartoli 2000; Bell et al. 2001). The amount of interspecific variation in ITS1

Fig. 4 (*concluded*).



alone was comparable with that in the partial ITS1 sequences of six species of *Diplostomum* reported by Niewiadomska and Laskowski (2002).

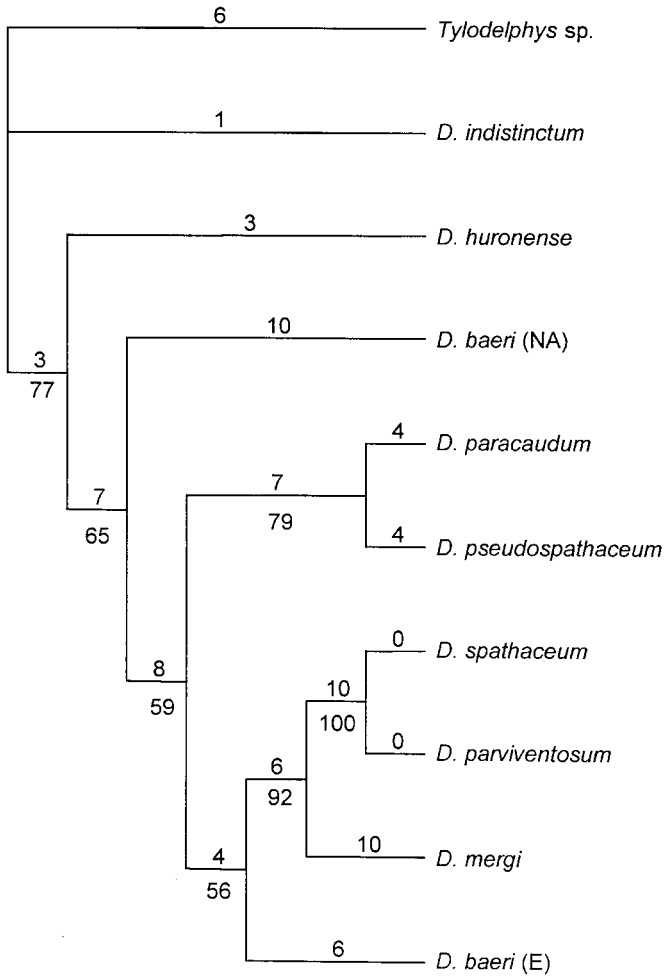
As observed in other digeneans, the ITS1 region in *Diplostomum* was more variable than the ITS2 region (e.g., Morgan and Blair 1995; Tkach et al. 2000; Bell et al. 2001). Despite the high level of intrageneric conservation, however, sufficient variation has been found within ITS2 to differentiate between species of *Fasciola*, *Schistosoma*, *Paragonimus*, *Indodidymozoon*, and *Ichthyocotylurus* (Adlard et al. 1993; Després et al. 1995; Blair et al. 1997; Anderson and Barker 1998; Bell et al. 2001). The three species of *Diplostomum* studied here could also be distinguished on the basis of their ITS2 sequences, although *D. huronense* and *D. indistinctum*

Table 2. Comparison of the number of differences (nucleotides and gaps) in the pairwise alignments of the partial ITS1 sequences of North American (present study) and European (Niewiadomska and Laskowski 2002) species of *Diplostomum*.

Species	*D. huronense*	*D. indistinctum*	*D. baeri*
D. indistinctum	11	—	—
D. baeri (NA)	27	23	—
D. baeri (E)	31	25	23
D. paracaudum	20	15	27
D. pseudospathaceum	15	13	27
D. spathaceum	36	29	32
D. parviventosum	36	29	30
D. mergi	36	34	38

Note: *Diplostomum baeri* (NA) and *D. baeri* (E) refer to the North American and European isolates, respectively.

Fig. 5. The most parsimonious tree (length = 89 steps, CI = 0.775, RI = 0.773, and RC = 0.569) with bootstrap consensus values >50% at all branches for North American and European species of *Diplostomum* based on partial sequences of the ITS1 region of the rDNA. An exhaustive search was performed with default values of PAUP* 4.0. *Tylodelphys* sp. was used as an outgroup. Branch length (above the line) and percentage of bootstrap values based on 10 000 replicates (below the line) are given at each branch.



differed at only four sites. Bell et al. (2001) also observed a high level of intrageneric conservation in four species of *Ichthyocotylurus* (Strigeidae) they studied. In three instances, the species differed at a single site. In both *Icthyocotylurus* and *Diplostomum*, the independent status of each species suggested by the ITS2 sequences was supported by additional genetic and (or) morphological data.

Available evidence suggests that digeneans with identical ITS2 regions are capable of exchanging genes and producing viable hybrids (Blair et al. 1997), and nominal species of *Echinostoma* (Morgan and Blair 1995) and *Paragonimus* (Blair et al. 1997) with identical ITS2 sequences have been considered conspecific. However, exceptions exist. Some otherwise distinct species schistosomes have identical ITS2 regions (see Blair et al. 1997; Anderson and Barker 1998), and Agatsuma et al. (2000) have provided molecular evidence that *Fasciola hepatica* L., 1758 and *Fasciola gigantica* Cobbold, 1855, whose ITS2 regions differ (Adlard et al. 1993), may hybridize, which further complicates the issue. While our results are consistent with those of Adlard et al. (1993) and Anderson and Barker (1998), who suggest that the ITS2 may be sufficiently variable to permit discrimination at the species level, it is evident that this may not be the case for all digeneans.

Little intraspecific variation occurs in the ITS regions of digeneans, owing to the homogenizing effects of concerted evolution (Morgan and Blair 1995). Point substitutions occur but the level of intraspecific variation is insignificant (Jousson et al. 1999). None was detected in sequences of *D. baeri* and only a single substitution was observed in the ITS1 region of *D. huronense*. The sequences of six specimens identified as *D. indistinctum* were identical, but three others, which had also been tentatively identified as *D. indistinctum*, differed at either three or eight sites in the ITS1 region and one had a single substitution in the ITS2. The substitution in the ITS2 region is noteworthy, because intraspecific differences in this region are rare in digeneans (Adlard et al. 1993; Blair et al. 1997; Anderson and Barker 1998; Bell et al. 2001). Nevertheless, substitutions occasionally occur (Adlard et al. 1993). Analysis of mitochondrial DNA from these nine specimens is ongoing, in an attempt to determine whether the difference noted represents natural variation or whether these isolates represent a separate species.

The ITS1 sequences obtained from the North American species (603–607 nucleotides) were longer than those re-

ported by Niewiadomska and Laskowski (2002) (576–580 nucleotides). The sequences from the European species were incomplete and terminated 23 nucleotides short of the 5.8S region. If this and a four-nucleotide difference at the 5′ end of the ITS1 region are taken into account, the range in the number of nucleotides present in the ITS1 region of the European and North American species is identical. As no repeat sequences have been found in *Diplostomum* to date, the range in the number of nucleotides in the ITS1 region appears to be representative for *Diplostomum* spp. in general.

All three of the North American species can be distinguished from each of the European species on the basis of their partial ITS1 sequences. However, the data also produced some unexpected results. First, the sequences of *D. huronense* and *D. indistinctum* differed from those of *D. pseudospathceum* Niewiadomska, 1984 and *D. spathaceum* to a greater extent than was expected on the basis of the similarity of their adult morphology. As all these species infect gulls, the adult flukes would be subjected to similar selective pressures and a high degree of morphological similarity could be expected. It would appear that the constraints on phenotypic variability in *Diplostomum* are greater than those on genetic variability, and least in the adult stages. Second, the differences in the partial ITS1 sequences from isolates identified as *D. baeri* from each continent was unexpected. Morphologically, adult *D. baeri* in our study were similar to those described elsewhere from North America and Europe, but the sequence data clearly indicate that the two are not conspecific.

The phylogenetic analysis, based on the partial ITS1 region, supported the relationships among the European species studied by Niewiadomska and Laskowski (2002); however, we found much stronger support for the inclusion of *D. baeri* in the clade that consisted of *Diplostomum mergi*, *D. spathaceum*, and *D. parviventosum* than reported in the previous study. The analysis also indicated that the North American and European species represent divergent groups within *Diplostomum*. The presence of genetically distinct groups of North American and European species is consistent with a recent suggestion that species of *Bolbophorus* (also a strigeid of aquatic birds) may have diverged separately in North America and Eurasia (Overstreet et al. 2002). Compared with Eurasia, however, relatively little work has been done on *Diplostomum* in North America. Neither the diversity nor the distribution of *Diplostomum* species within North America is known with any accuracy, and resolution of this issue is confounded by taxonomic problems. Although a few records of *D. huronense* and *D. indistinctum* exist, most North American authors have identified metacercariae from the lenses of fish as *D. spathaceum* or as *Diplostomum* sp. (Hoffman 1967, 1999; Margolis and Arthur 1979; McDonald and Margolis 1995). Adult flukes from the few studies on gulls that exist have also been identified as *D. spathaceum* (e.g., Threlfall 1968; Vermeer 1969). Our results suggest that the validity of many identifications from North America, particularly those based on metacercariae, is uncertain, and questions regarding the divergence and geographical distribution of species within *Diplostomum* remain open.

Although Niewiadomska and Laskowski (2002) found no differences in the partial ITS1 sequences of *D. spathaceum* and *D. parviventosum* Dubois, 1932 (which are otherwise distinguishable), the sequence data from the ITS regions of the other species, SDS–PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) studies (Faulkner 1989) and RAPD (random amplified polymorphic DNA) studies (Laskowski 1996; Niewiadomska and Laskowski 2002) indicate that sufficient genetic variability exists to permit resolution of the systematics within this morphologically conserved genus. Such data will be valuable in the development of genetically based diagnostic procedures for species-level identification, particularly of larval stages in fish, and to address broader questions regarding the evolution and geographic distribution of species within *Diplostomum*.

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