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Revealing the secret lives of cryptic species: Examining the phylogenetic relationships of echinostome parasites in North America

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ABSTRACT

The recognition of cryptic parasite species has implications for evolutionary and population-based studies of wildlife and human disease. Echinostome trematodes are a widely distributed, species-rich group of internal parasites that infect a wide array of hosts and are agents of disease in amphibians, mammals, and birds. We utilize genetic markers to understand patterns of morphology, host use, and geographic distribution among several species groups. Parasites from >150 infected host snails (*Lymnaea elodes*, *Helisoma trivolvis* and *Biomphalaria glabrata*) were sequenced at two mitochondrial genes (ND1 and CO1) and one nuclear gene (ITS) to determine whether cryptic species were present at five sites in North and South America. Phylogenetic and network analysis demonstrated the presence of five cryptic *Echinostoma* lineages, one *Hypoderaeum* lineage, and three *Echinoparyphium* lineages. Cryptic life history patterns were observed in two species groups, *Echinostoma revolutum* and *Echinostoma robustum,* which utilized both lymnaied and planorbid snail species as first intermediate hosts. Molecular evidence confirms that two species, *E. revolutum* and *E. robustum*, have cosmopolitan distributions while other species, *E. trivolvis* and *Echinoparyphium* spp., may be more geographically limited. The intra and interspecific variation detected in our study provides a genetic basis for seven species groups of echinostomes which will help accurately identify agents of disease as well as reveal cryptic aspects of trematode biology.

1. Introduction

The existence of cryptic species occurs because of morphological similarity and appears to be wide-spread among metazoan taxa and geographical regions (Pfenninger and Schwenk, 2007). Following Bickford et al. (2007), we define cryptic species as two or more distinct lineages that are classified as a single species, owing to the lack of conspicuous morphological differences. Crypsis among species is often discovered through molecular genetic, behavioral, or ecological studies of diversity. When revealed, cryptic species complexes clarify and modify our views of biodiversity and biogeography, life history, evolutionary theory, and ecological interactions (Bickford et al., 2007). For instance, elucidation of crypsis may shed light on species diversity, the process of speciation, and the dynamics of populations and demes.

Parasitic species are a large and diverse group of organisms and are no exception to the commonality of cryptic speciation (Jousson et al., 2000; Steinauer et al., 2007; Miura et al., 2005; Vilas et al., 2005). While mechanisms of speciation are similar between free-living and parasitic species, parasites differ in that speciation patterns may be directly associated with their hosts (Malenke et al., 2009), though in the case of parasites with complex life cycles, it is often less clear which host may have the most influence on parasite speciation. Several mechanisms of host-mediated structuring include host feeding strategies (Jousson et al., 2000; Criscione et al., 2002) and host immune systems (Štefka et al., 2009). The recognition of cryptic parasite species allows for an accurate assessment of parasite diversity, a biological indicator of species-richness in the free-living host community (Hudson et al., 2006). By measuring the changing abundances of parasite populations over time, we can predict when there is an increasing risk of disease (Johnson and McKenzie, 2009). Identifying cryptic species among parasites reveals the unrecognized diversity in the sources and mechanisms of human and wildlife diseases. Failing to recognize cryptic species leads to inaccurate assessments of parasite prevalence and intensity, underestimates of multi-species infections, and lost opportunities to study parasite–parasite interactions. Ultimately, resolving relationships among cryptic parasite species will help us understand the dynamics of parasite/disease dispersal, mechanisms underlying parasite speciation, and other factors involved in parasite epidemiology. Such understanding serves as the basis for intervention, treatment, and prevention of disease caused by parasites (Bickford et al., 2007; Putaporntip et al., 2009).

Echinostome trematodes (Echinostomatidae) are a diverse and ubiquitous group of parasites that are commonly used as a model system in laboratory and field investigations. In addition to infect-

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ing numerous avian and mammalian species, echinostomes are increasingly associated with disease in amphibians and may contribute to their decline (*Echinostoma revolutum*, Holland et al., 2007; *Echinostoma trivolvis*, Schotthoefer et al., 2003), especially in urban environments (Skelly et al., 2006). Establishing spatial scale of parasite distributions and host use is important in the face of emerging diseases, yet morphological similarity among species may mask differences in small-scale spatial distributions of parasites (Leung et al., 2009) and in host specificity (Detwiler and Minchella, 2009). Echinostomes are prone to misidentification due to similar morphology and the lack of isolates in molecular databases (Kostadinova et al., 2003). For instance, although echinostome species can be distinguished by the number of spines on the head collar of the larval and adults stages, some species have the same number of spines. More comprehensive sampling is needed to establish a better idea of the underlying geographical and genetic variation within and among lineages.

Echinostomes differ from other platyhelminth parasites in several ways. First, echinostomes are species-rich, for example, approximately 60 and 120 nominal species are described from just 2 of the 91 recognized echinostome genera (Kostadinova and Gibson, 2000). Yet, *E. revolutum* and *E. trivolvis* are the only two species from North America that appear in a phylogenetic framework (Sorensen et al., 1998; Olson et al., 2003). Including additional samples from North and South America may clarify the phylogenetic relationships of these echinostome species to those from other geographic regions around the world. Second, species generally have cosmopolitan distributions in which a single species is reported from Europe, Africa, Asia and Australia (Kostadinova et al., 2003). Third, members of this parasite group have a broad host range, especially in second intermediate and definitive hosts (Kanev et al., 2000). Therefore, they are an interesting group to test how host association has influenced cryptic speciation in parasites. Some cryptic species complexes have been more associated with their definitive host, while others have been more strongly associated with the co-evolution of their molluscan intermediate host (Adamson and Caira, 1994; Jousson et al., 2000). Echinostomes often use at least three successive hosts to complete their life cycle. Larval parasites are found within invertebrate intermediate hosts and adult parasites develop and reproduce sexually in vertebrate definitive hosts.

Compared to previous studies, we obtained a wider sampling of species and isolates from North America and utilized a multigene approach along with morphological, host use, and geographic data to accomplish two goals. First, we determine the level of geographic variation present within genetic clades, especially when clade members originate from different geographic locations (within and outside of North America). Second, we reveal whether there are cryptic species of echinostomes in North America. We use phylogenetic and network analyses to identify highly divergent, monophyletic lineages. Additionally, host use is often used to define parasitic species (Kostadinova and Gibson, 2000), and so we also employ comparative host use data in recognizing distinct lineages. We note that others have used a genetic distance-based measure to identify parasitic species (Vilas et al., 2005), so we calculate genetic distance between groups as a comparative measure, but do not explicitly use genetic distance as a species criterion.

2. Materials and methods

We utilize the term, species group, to refer to a set of related lineages that are genetically divergent from other lineages. Some of these lineages correspond to already described species that have been sequenced and therefore we recognize them as such. Three lines of evidence were used to determine species groups and identify cryptic species. Initially, host and geographic information for the larval stages and adult stages was collected. Typically, there is strict host specificity to the first intermediate host (Kostadinova and Gibson, 2000). Second, morphological data consisting of the number of spines surrounding the mouth was assessed with voucher specimens. The number of spines found on adult and larval stages is one of the most common features used to characterize particular species groups (Fried, 2001; Fried and Graczyk, 2004). Third, genetic sequences were used to examine phylogenetic relationships among taxa for three genes. A better understanding of inter- and intraspecific variation for echinostomes was determined by comparing genetic distances and generating haplotype networks.

2.1. Sampling

We collected samples from five field sites in North and South America (Table 1) including Indiana, Minnesota, Wisconsin and Brazil. The most extensive sampling occurred from 2005–2008 at two Indiana sites where repeated snail intermediate host sampling was conducted for snail intermediate hosts. A limited number of field-infected hosts were collected from Minnesota (4 snails), Wisconsin (1 muskrat) and Brazil (1 snail). In addition, we received a tissue sample originally collected in the USA that was previously sequenced at two rRNA genes for a trematode phylogeny (Olson et al., 2003).

Field-collected snails were transported to the laboratory and larval parasites were first assigned to species based on host specificity and morphology as described in Detwiler and Minchella (2009). Briefly, cercarial shedding was induced in field hosts, and parasites were examined for the presence/absence of collar spines and tail flaps. To obtain adult specimens for spine counts, cercariae from field-infected first intermediate hosts were exposed to laboratory-raised second intermediate host snails (*Lymnaea elodes* or *Helisoma trivolvis*). This ensured that the metacercariae matched the genetic identify of the rediae as opposed to being the result of colonization of a different species in nature. Snails were crushed after 7 days and ~100 metacercariae were fed to individual day-old chickens. After 2 weeks, chickens were dissected and parasite specimens (either whole worm or just the anterior region) were fixed with alcohol–formalin–acetic acid (AFA), stained with Semichon's acetocarmine, and mounted in Canada balsam.

2.2. Molecular data

To confirm species identification and establish levels of genetic variation in a species group, we employed markers from two mitochondrial genes (ND1 and CO1) that were previously used to identify strains and species of echinostomes (Morgan and Blair, 1998a,b). Relationships inferred from the partial mitochondrial genes were compared to our results from the internal transcribed spacer region of ribosomal DNA (ITS). This nuclear region is often used to infer species boundaries (Nolan and Cribb, 2005), although in some cases it is not variable enough to generate resolved groups (Kostadinova et al., 2003). To efficiently sample the genetic variation detectable with the more conservative ITS marker, a subset of individuals ($n = 32$) that represented unique mitochondrial haplotypes were sequenced at the ITS for eight species groups.

DNA was isolated from a single worm or larval stage (redia) using the Puregene® DNA extraction protocol (Gentra Systems). When larvae were utilized, at least two individuals from a single infected host were sequenced for groundtruthing. It was expected that both individuals should be identical as rediae are products of asexual reproduction. Nuclear and mitochondrial genes were amplified with primers developed by Morgan and Blair (1995, 1998a). PCR amplification for both mitochondrial genes was conducted in 50 μl reactions with 2 μl of extraction supernatant, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 μm each primer, 0.2 mM of each dNTP, and 5 units of Taq DNA polymerase (Promega, Madison,

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Table 1
Morphological and host use information for echinostome samples collected for this study. Since species designations are not clear, we refer to them as species groups as opposed to species. *n* Refers to the number of adult specimens examined per species group. IN1 and IN2 represents the two locations within Indiana and are defined in the Fig. 1 legend.

Species group	Number of spines	1st intermediate host use	Collection locality
Echinostoma revolutum	37 (*n* = 10)	*Lymnaea elodes* (field and lab infected)	Indiana
		Helisoma trivolvis (field infected)	IN2: 85°28′50″ W, 41°18′12″ N
		Lymnaea elodes (field and lab infected)	IN1: 85°41′06″ W, 41°20′59″ N
Echinostoma robustum	37 (*n* = 3)	*Lymnaea elodes* (field infected)	Indiana
			IN2: 85°28′50″ W, 41°18′12″ N
		Lymnaea elodes (field infected)	Minnesota
			93°57′49"W, 44°8′46″N
		Biomphalaria glabrata (field infected)	Brazil, Minas Gerais
			16°57′40″ W, 41°21′14″ S
Echinostoma trivolvis	37 (*n* = 2)	*Helisoma trivolvis* (field infected)	Indiana
			IN2: 85°28′50″ W, 41°18′12″ N
		Lymnaea elodes (field infected)	Indiana
			IN2: 85°28′50″ W, 41°18′12″ N
		Lymnaea elodes (field infected)	Minnesota
			93°57′49″ W, 44°8′46″ N
		Unknown (muskrat definitive host)	Wisconsin
			43°52′W; 91°14′N
Hypoderaeum lineage 1	41–45 (*n* = 9)	*Lymnaea elodes* (field and lab infected)	Indiana
			IN2: 85°28′ 50″ W, 41°18′12″ N
		Lymnaea elodes (field and lab infected)	IN1: 85°41′06″ W, 41°20′59″ N
Echinoparyphium lineage 1	43 (*n* = 2)	Unknown (muskrat definitive host)	Wisconsin
			43°52′W; 91°14′N
Echinoparyphium lineage 2	46–50 (*n* = 6)	*Lymnaea elodes* (field and lab infected)	Indiana
			IN2: 85°28′50″ W, 41°18′12″ N
			IN1: 85°41′06″ W, 41°20′59″ N
Echinoparyphium lineage 3	Unknown	*Helisoma trivolvis* (field infected)	Indiana
			IN2: 85°28′50″ W, 41°18′12″ N

WI). A similar protocol was followed for ITS reactions except 0.4 μm was used for each primer, and 2.5 units of Taq DNA polymerase were added. The thermocycling protocol for the mitochondrial genes was 94 °C for 1 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 45 s, and 72 °C for 1 min; and a final extension step of 72 °C for 7 min. The ITS amplification protocol was similar except an annealing temperature of 58 °C was used. PCR products were visualized with a 1.2% agarose gel containing ethidium bromide and cleaned with the QIAquick PCR Purification Kit (Qiagen). Sequencing was conducted in both the forward and reverse directions with 25–50 ng of purified product using the BigDye terminator kit version 3.1 (Applied Biosystems) and an ABI 3130xl.

2.3. Phylogenetic and network analyses

Sequences from our study were combined with echinostome sequences from GenBank (8 for CO1, 36 for ND1, 21 for ITS, see Supplementary data). Sequences were aligned with ClustalW and then adjusted by eye. Each dataset was reduced to non-redundant taxa and homogeneity of base frequencies was tested with χ^2 analysis to determine whether base frequencies vary among taxa. We performed a partition homogeneity test using PAUP* 4.0b10 (Swofford, 2003) to determine whether the two mitochondrial datasets could be concatenated. Phylogenetic analysis was computed for each gene with maximum likelihood (PAUP* 4.0b10) and Bayesian approaches (MrBayes 3.12, Huelsenbeck and Ronquist, 2001). Using Modeltest, a best fit model of substitution was chosen based on Aikaike Information Criterion (Posada and Crandall, 1998). We followed the approaches of Brumfield and Edwards (2007) to construct our maximum likelihood gene trees. Briefly, a fast heuristic search (addition sequence = as-is) was performed with our initial model parameters from Modeltest. Then, using re-estimated parameters from this search, a final search was performed with 10 random-addition replicates. These same parameters were then used in a bootstrap analysis (nreps = 100) with 10 random-addition replicates. For the Bayesian analysis, model parameters were treated as uniform priors and estimated as part of the analysis. Two independent analyses were run with four heated chains for 10,000,000 generations. Samples were taken every 1000 generations and a burnin of 2500 was used. Following Morgan and Blair (1998a), *Fasciola hepatica* was used to root the mitochondrial gene trees and *Isthmiophora hortensis* rooted the ITS tree (1995).

To better characterize genetic lineages, we examined both within and between lineage variation with genetic distance and network analysis (see Figures for demarcation of lineages). The genetic divergence (uncorrected *p* distance) within and among species groups and between some subclades was calculated for each gene with MEGA version 4.1 (Kumar et al., 2008). To further understand the intraspecific variation within each of the suggested clades, haplotype networks were constructed with statistical parsimony analysis for both mitochondrial genes (TCS, version 1.6, Clement et al., 2000). The number of parasites for each haplotype is corrected for host. Since trematodes reproduce asexually within their first intermediate hosts, we only counted one individual per host if several individuals within the same host shared a haplotype.

3. Results

3.1. Host use

None of the *Echinostoma* species were host specific to a single first intermediate host species (Table 1). First, *E. revolutum* did not appear to be strictly host-specific to *L. elodes*, as it was also found in *H. trivolvis* as a first intermediate host. Most of the *E. revolutum* samples came from infected *L. elodes* snails, however, *E. revolutum* was also genetically identified from a single field-collected *H. trivolvis* snail (confirmed by sequencing four rediae with two different PCRs each). Second, *E. trivolvis* which is often described as host specific to planorbid hosts, was isolated from three field-infected *L. elodes* in addition to several *Helisoma trivolvis* hosts. In contrast to *E. revolutum*, the lack of specificity for *E. trivolvis* may be the result of a species complex. The third exception was from

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the *E. robustum* species group, where individuals were recovered from both lymnaied hosts and planorbid hosts in North and South America, respectively. All the North American *Echinoparyphium* and *Hypoderaeum* lineages in our study originated from one species of intermediate host, *L. elodes*.

3.2. Morphology

As expected, our voucher specimens from adult worms confirmed that all *Echinostoma* lineages collected for our study had 37 spines (Table 1). The number of spines varied among and within the voucher specimens of the *Echinoparyphium* and *Hypoderaeum* lineages (Table 1). Though not all larval parasites were morphologically identified in our study before genetic sequencing, we did examine the cercarial morphology of parasites from 87 field-collected snails before DNA extractions. Forty-three of the snails were thought to have *Echinostoma* infections, while 44 snails were thought to be infected with *Echinoparyphium* lineages. Approximately 14% of the parasites (6/43 hosts) that were thought to be *Echinostoma* species were misassigned to the wrong congeneric species, or even to the nongeneric *Echinoparyphium*. Nearly ~20% (9/44 hosts) of *Echinoparyphium* lineages were misidentified as *Echinostoma*, and larval stages of *Echinoparyphium* were indistinguishable 100% of the time. It was not until we observed adult specimens that we could distinguish *Echinoparyphium* and *Hypoderaeum*.

3.3. Phylogenetic and network analyses

A total of 298 ND1 sequences from individual echinostome parasites (adult or redia stage) from 187 individual hosts (intermediate host or definitive host) were collapsed into 107 unique haplotypes. Our study contributed 78 unique ND1 haplotypes, while 29 haplotypes were derived from Genbank sequences. A total of 278 CO1 sequences from individual parasites (adult or redia stage) from 164 individual hosts (intermediate or definitive host) were collapsed into 54 unique haplotypes. Our study contributed 44 unique CO1 haplotypes, compared to the 8 haplotypes derived from GenBank. In addition, 2 haplotypes from our dataset exactly matched 2 GenBank samples (AY222132/AY222246 and AF025823). The former sample was originally identified as *E. revolutum* in a recent trematode phylogeny (Olson et al., 2003) but our analysis revealed a clustering with the *E. trivolvis* species group. The ITS dataset consisted of a subset of 32 individuals that were sequenced at both mitochondrial genes in addition to 22 sequences from GenBank. From the 50 ITS sequences, a total of 33 unique haplotypes were found (15 haplotypes from our study and 18 from GenBank).

Although individuals collected for our study were assessed at all three genes, not all samples in Genbank were sequenced at all three genes. Therefore, we examined individual gene trees. As expected, the percentage of parsimony informative sites was higher in the mitochondrial genes than the nuclear gene: ND1: 49% (230/474 total bp); CO1: 29% (63/216 total bp); ITS: 13% (133/1042 total bp). Homogeneity of base frequencies across taxa was not rejected for any of the datasets ($P > 0.05$) indicating the stationarity of base frequencies. The partition homogeneity test indicated there was a significant difference in topology between the two mitochondrial genes ($P = 0.01$), therefore we did not conduct any analysis with a concatenated dataset. For both mitochondrial genes, Modeltest selected the GTR + I + G model of nucleotide substitution using the Aikaike Information Criterion (AIC). The base frequencies for ND1 were $A = 0.19$, $C = 0.26$, $G = 0.27$, $T = 0.29$, the proportion of invariable sites was 0.39, and the gamma shape parameter was 0.84. The base frequencies for CO1 were $A = 0.21$, $C = 0.07$, $G = 0.22$, $T = 0.50$, the proportion of invariable sites was 0.53, and the gamma shape parameter was 0.42. The base frequencies for ITS were $A = 0.20$, $C = 0.23$, $G = 0.28$, $T = 0.29$, the proportion of invariable sites was 0.44, and the gamma shape parameter was 0.76.

Bayesian and maximum likelihood trees yielded similar topologies for all three genes, therefore we utilized the Bayesian trees and displayed both posterior probabilities and bootstrap proportions. The ND1, CO1, and ITS gene trees show strong support for monophyly of *Echinostoma* (Figs. 1–3). There was also strong support for a phylogenetically closer relationship between *Echinoparyphium* and *Hypoderaeum* than between either of the genera and *Echinostoma* (Figs. 1 and 3). There was concordance for all species groups among the mitochondrial gene trees and the ITS tree (Fig. 3) except for CO1 and *E. trivolvis*. Between the two mitochondrial trees, our lineages corresponded to five already named *Echinostoma* species (Figs. 1 and 2; see Kostadinova et al., 2003). From samples unique to our study, we recovered evidence for three *Echinostoma* species groups in North America and South America: *E. trivolvis*, *E. robustum*/*E. friedi*, *E. revolutum*. This is the first report of *E. robustum* for North and South America. We discovered three highly divergent *Echinoparyphium* lineages and 1 *Hypoderaeum* lineage amongst the samples from North America (Figs. 1–3). In two highly sampled sites, echinostome species diversity within a site was estimated to be 4 (IN1) and 6 (IN2) species groups. Relationships between clades were better resolved in the ND1 gene tree compared to the CO1 gene tree which was not surprising. This CO1 region appears to be saturated in echinostomes which leads to a loss of deeper phylogenetic signal (Morgan and Blair, 1998a). In addition, the taxon sampling was greater, and the sequence length was longer and more variable than the CO1 region.

Our study indicates that two *Echinostoma* species, *E. revolutum* and *E. robustum* occur in North America. *E. revolutum* lineages have now been confirmed with molecular sequencing in Europe and North America suggesting that this species is truly cosmopolitan. Network analysis indicates gene flow and population expansion within North America (Fig. 4a), but not on a global scale as the European sample did not form a connected network with North American samples. This species co-occurs with other echinostome species in North America such as *E. robustum*, which was verified with molecular sequencing for the first time from Brazil and the US (Minnesota and Indiana) in addition to its previous localities in Australia and Germany. As with *E. revolutum*, network analyses indicated geographic sub-structuring within this species group. A comparison of samples identified as *E. robustum* (U58102) and *E. friedi* (AY168937) reveals that they are found within the same monophyletic clade and thus do not qualify as distinct species according to a phylogenetic definition. Additionally, they are genetically similar (0.009 genetic divergence, ND1 compared to an average genetic divergence between species in this study of 0.169). From a morphological perspective, Toledo et al. (2000) separated the species based on the length difference in the oesophagus and type locality. However, they have other morphological similarities (37 collar spines) and host use. Our study provides the first genetic evidence for *Hypoderaeum* in the United States, which is described as a cosmopolitan genus (Jones, 2005). Other groups have been collected only from the US: *Echinostoma trivolvis*, and the newly identified genetic lineages *Ep.* lineage 1, *Ep.* lineage 2, and *Ep.* lineage 3. In addition, we verified with all three genes that the echinostome from Olson et al. (2003) was misidentified as *E. revolutum* and may be *E. trivolvis*.

All three genes suggest that there may be unrecognized species complexes in the two species groups as the intraspecific variation within groups is high for all genes (Table 2). By examining the differences according to subclades (see Fig. 1) in both *E. trivolvis* and *E. robustum*/*E. friedi*, the genetic divergence between the 2 well-supported subclades within both species was 8.1 and 9.4 (ND1),

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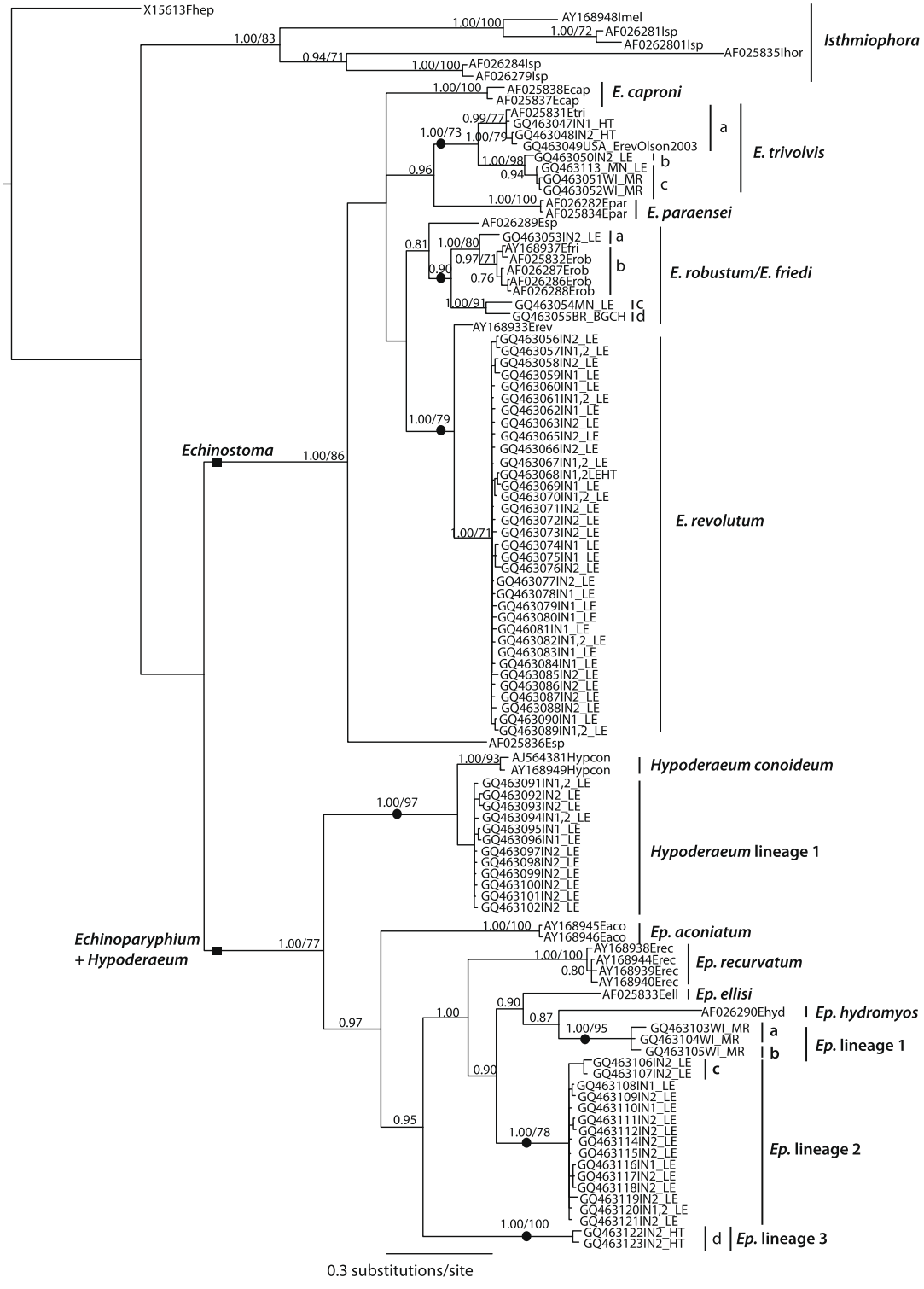

Fig. 1. Phylogenetic relationships of echinostomes based on a partial ND1 gene inferred from Bayesian (support values) and maximum likelihood (bootstrap values) and rooted with 1 outgroup species, *Fasciola hepatica*. Support values greater than 0.70 or 70 are shown near the node. The squares denote the genera and the lines signify each genetic group. The circles denote the species or lineages to which our sample contributed. Samples from GenBank are indicated with their accession number and an abbreviation (first letter of genus and first 3 letters of species). To create continuity among studies, species designations follow Kostadinova et al. (2003). Samples from the current study are labeled with their GenBank accession number followed by an abbreviation with collection locality (State/Country): BR = Brazil; IN1 = Shock Lake, IN; IN2 = Pond A, IN; MN = Minnesota, WI = Wisconsin. The remainder of the name indicates the intermediate host and/or definitive host. Snail intermediate host: LE = *Lymnaea elodes*, HT = *Helisoma trivolvis*. BG = *Biomphalaria glabrata*. Definitive host: MR = muskrat. The letters a–d indicate subnetworks within species groups.

5.9 and 1.5 (CO1), and 0.3 and 0.2 (ITS) which are within the ranges of difference found between established species (e.g. *E. revolutum* vs *E. robustum*; Table 2). The network analysis shows that each subclade for each species group represented an unconnected subnetwork (not shown). There were three unconnected subnetworks within the *E. trivolvis* species group that consisted of eight unique haplotypes from twelve individuals (a–c; Fig. 1). A total of eight *E. robustum* haplotypes (from 14 total sequences) formed four uncon-

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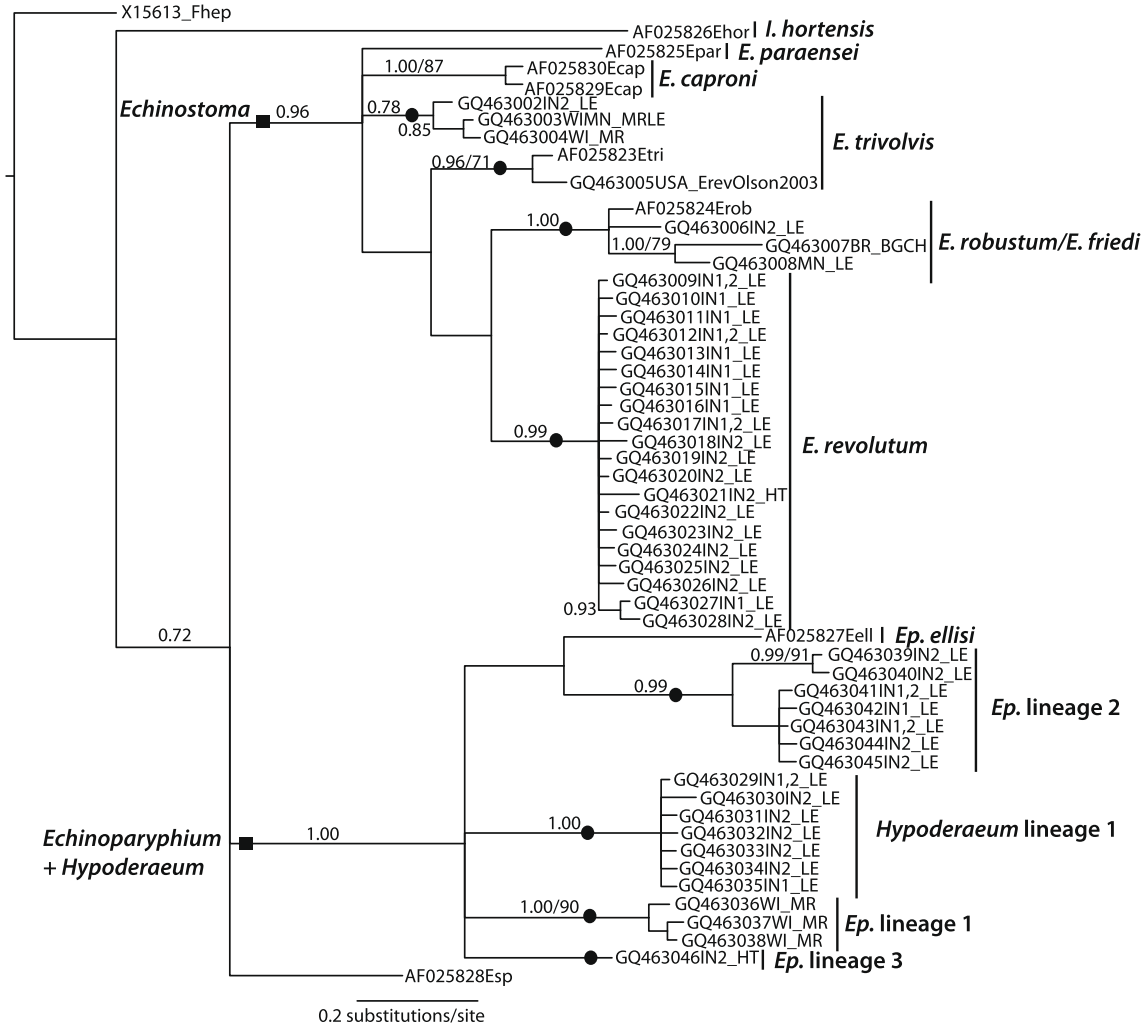

Fig. 2. Phylogenetic relationships of echinostomes based on a partial CO1 gene inferred from Bayesian (support values) and maximum likelihood (bootstrap values) and rooted with 1 outgroup species, *Fasciola hepatica*. Support values greater than 0.70 or 70 are shown near the node. The squares denote the genera and the lines signify each genetic group. The circles denote the species or lineages to which our sample contributed. Samples from GenBank are indicated with their accession number and an abbreviation (first letter of genus and first 3 letters of species). To create continuity among studies, species designations follow Kostadinova et al. (2003). Samples from the current study are denoted with their GenBank accession number and their collection locality (State/Country): BR = Brazil; IN1 = Shock Lake, IN; IN2 = Pond A, IN; MN = Minnesota, WI = Wisconsin. The remainder of the name indicates the intermediate host and/or definitive host. Snail intermediate host: LE = *Lymnaea elodes*, HT = *Helisoma trivolvis* BG = *Biomphalaria glabrata*. Definitive host: MR = muskrat.

nected subnetworks, which corresponded to *E. robustum/E. friedi* subclades a–d (Fig. 1). The network analysis confirms the species groups shown in the phylogenetic analysis and suggests even further subdivision with four unconnected subnetworks (ND1 gene) within the *Echinoparyphium* lineages (see Fig. 1, letters a–d).

Within three species groups, there were closely related haplotypes (Fig. 4a–c). Allelic sharing occurred among collection sites and thus infers gene flow at local spatial scales for *E. revolutum, Hypoderaeum* lineage 1, and *Ep.* lineage 2, (Fig. 4a–c). Each species' network contains at least one star-like structure.

4. Discussion

This study reveals several cryptic species of echinostomes with similar ecological niches. All species were collected from freshwater ponds and lakes and mostly used the same intermediate host species (*L. elodes* or *H. trivolvis*). Using morphology, host use, and genetic data, we found a single *Hypoderaeum* lineage and three highly divergent *Echinoparyphium* lineages in North America, and we extend the known ranges of several species. We also find that by sampling a wide array of individuals within a species, there can be cryptic life history patterns and morphology that may further complicate species identifications, and studies of both parasite–host and parasite–parasite interactions.

Higher levels of intraspecific variation may indicate the presence of cryptic species. Vilas et al. (2005) suggest that greater than a 5% difference with mitochondrial markers and ~1% difference with the ITS marker indicates separate species for trematode parasites. The intraspecific variation within two of the *Echinostoma* species groups was greater than 5%, and the interspecific variation between some of the subclades was also greater than 5% (Table 2). By sampling a large number of individuals, we found cryptic species in three established species groups of *Echinostoma*: *E. revolutum*, *E. robustum*, and *E. trivolvis*. The first two groups, *E. revolutum* and *E. robustum* have a history of being confused with each other. Sorensen et al. (1998) uncovered variation in the ITS region between echinostomes believed to be *E. revolutum*. Morgan and Blair (1998b) identified some genetic lineages as *E. revolutum* which were later suggested to be *E. robustum* by Kostadinova et al. (2003). Several studies have since suggested that the intraspecific variation was high because two different cryptic species were com-

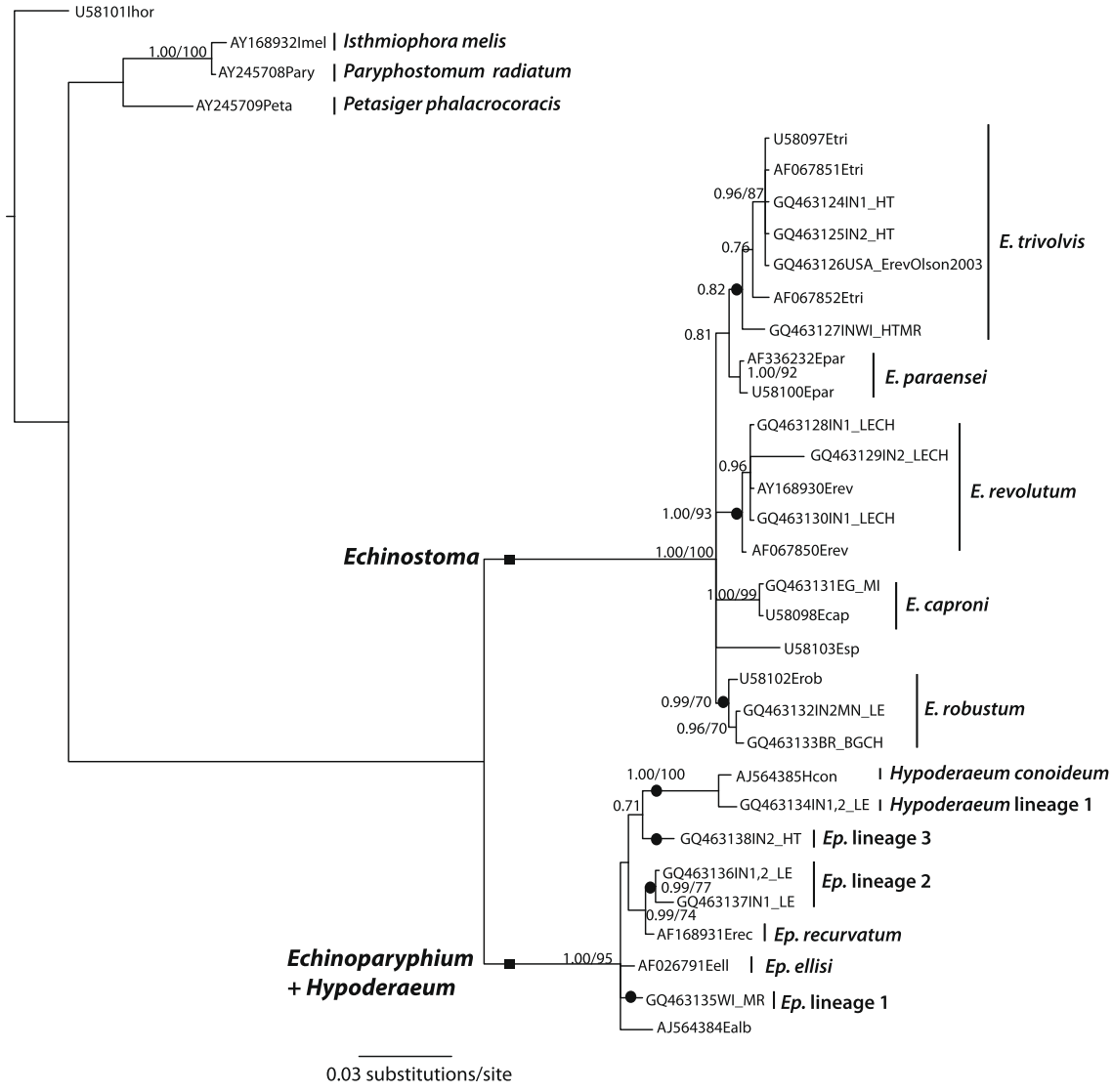

Fig. 3. Phylogenetic relationships of echinostomes based on the ITS gene inferred from Bayesian (support values) and maximum likelihood (bootstrap values) and rooted with two outgroup species, *Isthmiophora hortensis*, *Isthmiophora melis*. Support values greater than 0.70 or 70 are shown near the node. The squares denote the main genera and the lines signify each genetic group. The circles denote the species or lineages to which our sample contributed. Samples from GenBank are indicated with their accession number and an abbreviation (first letter of genus and first 3 letters of species). Samples from the current study have an associated GenBank number and abbreviation for the collection locality (State/Country): BR = Brazil; EG = Egypt lab strain from B. Fried; IN1 = Shock Lake, IN; IN2 = Pond A, IN; MN = Minnesota, WI = Wisconsin. The remainder of the name indicates the intermediate host and/or definitive host. Snail intermediate host: LE = *Lymnaea elodes*, HT = *Helisoma trivolvis* BG = *Biomphalaria glabrata*. Definitive host: CH = chicken, MR = muskrat, MI = mouse.

pared (Kostadinova et al., 2003; Nolan and Cribb, 2005). Our larger dataset allows us to confirm that two species were compared, but also report for the first time that both species, *E. revolutum* and *E. robustum* occur in North America. The single sample from South America also extends the range of *E. robustum*, although with further sampling this may prove to be a different species.

It is clear from our study that the sample tentatively identified as *E. friedi* in Kostadinova et al. (2003) is genetically very similar to *E. robustum*. Toledo et al. (2000) first described *E. friedi* but only included morphological evidence for their comparisons to other species. Few morphological differences were observed between *E. robustum* and *E. friedi*. They focused mostly on a single character and found that the oesophagus was longer in *E. robustum* than *E. friedi*. Further genetic sampling would help to resolve the relationship between these two named species. For the third species group *E. trivolvis*, we find high levels of intraspecific variation suggestive of multiple species. In addition, we also show with evidence from three genes that misidentification between *E. revolutum* and *E. trivolvis* is possible and that the echinostome from Olson et al. (2003) may have been *E. trivolvis* not *E. revolutum*. Thus, the parasite species identified in Holland et al. (2007) was probably misidentified because of the comparison to the previous study and should be considered *E. trivolvis*.

Two species of *Echinoparyphium* have been described from North America, *Ep. flexum* (45 spines) and *Ep. rubrum* (43 spines), though it is unclear whether we encountered them in our study. First, there are no genetic samples for either of these species in GenBank. Second, the spine count range for the two species groups in this study matched only one of the previously described species, *E. rubrum* (43 spines), which has been described as infecting only physid snails as first intermediate hosts (Kanev et al., 1998). None of the larval parasites in our study were sampled from physid intermediate hosts, although the intermediate host for the muskrat-derived parasites is unknown. Given our current results, we recognize at least three lineages within North America from the genus *Echinoparyphium*, which may be considered species upon

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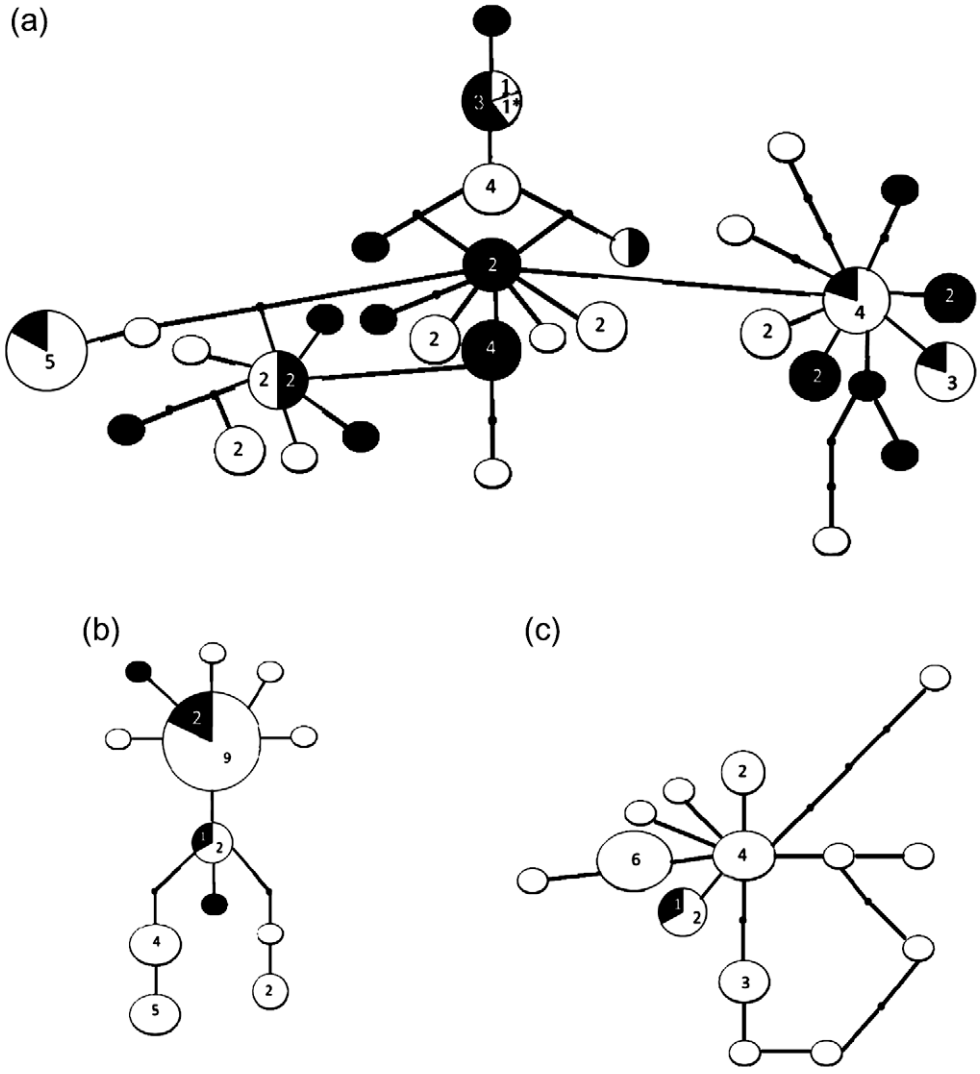

Fig. 4. Haplotype networks (ND1) for three lineages of echinostomes (a) *Echinostoma revolutum* (66 individuals sampled), (b) *Hypoderaeum* lineage 1 (32 individuals sampled), (c) *Echinoparyphium* lineage 2 (26 individuals sampled). Each line represents a single mutational step with black circles indicating inferred haplotypes. The size of the circles reflects the number of individuals in the haplotype. The colors within the circles represent individuals from different geographic locations. White = individuals from Pond A, IN; black = individuals from Shock Lake, IN. The asterick indicates an *E. revolutum* infection within a *Helisoma trivolvis* snail.

further investigation. All three lines of evidence suggest that we encountered *Hypoderaeum* in our sampling. The first intermediate hosts were *Lymnaea* and the spine size and number matched previous descriptions (see Jones, 2005). Genetic evidence indicates that samples were phylogenetically closely related to *Hypoderaeum conoideum*.

All species were similar morphologically at the larval stage, but substantial morphological differences were observable among some species at other points in the life cycle. For example, *Hypoderaeum* lineage 1 adults were significantly larger than *Ep. lineage 2* adults, respectively ($n = 10$ adults from *Gallus gallus* laboratory definitive host for each species: 7.41 ± 0.15 mm vs 3.06 ± 0.13 mm, t-value = 21.95, $df = 18$, $P < 0.0001$). Future studies concerning the genetics, host specificity, phenotypic plasticity, and geographic range of the *Echinoparyphium* and *Hypoderaeum* lineages will clarify their relationship.

The *Echinostoma* and *Echinoparyphium* species display cryptic life history patterns. Often because parasites look similar, investigators rely on host use information to help them identify species (Poulin and Keeney, 2008). There is both field and laboratory evidence to suggest that trematode species utilize a single first intermediate host species. However, by examining a wide array of individuals within a species and also within populations (individuals collected from the same wetland), we have found that three species (*E. revolutum, E. robustum*, and *E. trivolvis*) infect at least two first intermediate hosts from two different molluscan groups (lymnaied vs planorbid snails). Morgan and Blair (1998b) had previously suggested this was the case with Australian *E. robustum* (originally identified as *E. revolutum*, but see Kostadinova et al., 2003). This level of host use variation among closely related species emphasizes the need for caution when conducting ecological studies with echinostomes. Further, proper identification may reveal subtle differences in host use between the cryptic species which has important ecological implications. For instance, Detwiler and Minchella (2009) found that an *Echinostoma* species and an *Echinoparyphium* species (now identified as *Hypoderaeum* lineage 1 in this study) that have similar larval morphology and use the same first intermediate host had different preferences for second intermediate hosts. It is also important to consider that different genetic strains/haplotypes within a species may have different patterns of host use as illustrated by this work.

This study confirms that some echinostome species have broad, cosmopolitan distributions. Both *E. revolutum* and *E. robustum* appear to be cosmopolitan parasites, which are likely transported

Table 2
Within and between group genetic distances for echinostomes found in North America and South America. The number of non-redundant haplotypes within each group is shown in the parentheses. A dash indicates only 1 haplotype per group.

	ND1	CO1	ITS
Within species groups			
Echinostoma			
Within *E. revolutum*	0.012 (35)	0.015 (20)	0.006 (5)
Within *E. trivolvis*	0.052 (8)	0.048 (6)	0.004 (7)
Within *E. robustum/E. friedi*	0.054 (8)	0.045 (4)	0.002 (3)
Hypoderaeum			
Within Hypoderaeum lineage 1	0.007 (12)	0.011 (7)	–
Echinoparyphium			
Within *Ep.* lineage 1	0.026 (3)	0.009 (3)	–
Within *Ep.* lineage 2	0.014 (15)	0.028 (7)	0.004 (2)
Within *Ep.* lineage 3	0.019 (2)	–	–
Between species groups			
Echinostoma			
E. robustum (a and b) vs *E. robustum* (c and d)	0.094	0.015	0.002
E. revolutum vs *E. robustum* (a and b)	0.113	0.063	0.01
E. revolutum vs *E. robustum* (c and d)	0.124	0.078	0.01
E. trivolvis (a) vs *E. trivolvis* (b and c)	0.081	0.059	0.003
E. sp. 3 vs *E. trivolvis* (a)	0.236	0.139	0.083
E. sp. 3 vs *E. trivolvis* (b and c)	0.234	0.142	0.081
Hypoderaeum			
Hypoderaeum lineage 1 vs *Ep.* lineage 2	0.197	0.123	0.023
Hypoderaeum lineage 1 *vs Ep.* lineage 1	0.209	0.118	0.026
Hypoderaeum lineage 1 vs *Ep.* lineage 3	0.217	0.103	0.025
Echinoparyphium			
Ep. lineage 2 vs *Ep.* lineage 1	0.157	0.101	0.011
Ep. lineage 2 vs *Ep.* lineage 3	0.186	0.106	0.016
Ep. lineage 1 vs *Ep.* lineage 3	0.181	0.105	0.014

by their most vagile definitive host, birds. Mammals have also been shown to serve as hosts for these species. The similarity in distribution, host use, and morphology for the latter two species makes them interesting to study from a speciation perspective. Sibling echinostome species have demonstrated strict specificity for different first intermediate hosts (McCarthy, 1990), which may suggest that molluscan hosts have contributed significantly to the speciation process of these echinostome species. The two species in our study not only utilize the same first intermediate host, but also likely use similar definitive hosts. This overlap in host use suggests that they compete to colonize, and probably co-occur within first and second intermediate hosts, and definitive hosts. The mechanisms involved in reinforcement are difficult to speculate upon considering the amount of spatial overlap that occurs between these species. Even in terms of their definitive hosts they infect similar species and similar locations within the host (lower gastro-intestinal (GI) tract). The muskrat host in our study was infected by *E. trivolvis* and *Ep.* lineage 1, though they were spatially segregated within the GI tract. Using different locations within the host may serve to reduce competition amongst the species, although with higher intensity infections these worms may overlap (Holmes, 1961). An alternative, but not necessarily mutually exclusive hypothesis has suggested that site selection within a host increases the chances of reproduction for a species (Rhode, 1979). It may ultimately be shown that within the echinostomes, different species groups or lineages evolve according to different processes. The cosmopolitan nature of these echinostomes contrasts with others like *E. trivolvis*, *Ep.* lineage 1, *Ep.* lineage 2, and *Ep.* lineage 3 (only found in North America) which appear to be more restricted in their geographic distributions. Many echinostome species are infective to both mammals and birds (migratory and non-migratory), which exhibit different degrees of movement. The role of host movement in parasite distributions will be better understood when we can accurately identify each echinostome species and characterize the array of hosts utilized by each.

Echinostomes have been used to address aspects of parasitism and host–parasite interactions, yet the phylogenetic relationships within and between species remain unclear. The species diversity in combination with their cryptic morphology and life history makes ecological and evolutionary questions difficult. However, these characteristics also make them a valuable model system. This group may allow an assessment of the processes that have led to and maintain species, and how much hybridization occurs between trematodes that overlap within the definitive host. The ubiquitous presence of echinostomes and their ability to use a wide array of hosts also makes them important for conservation efforts. Amphibians are experiencing stress from many aspects of their environment including trematode parasites. Recent studies have focused on particular trematodes such as *Riberioa*, but the role of echinostomes is becoming increasingly recognized (Johnson and McKenzie, 2009). It will be essential for researchers to identify what species and even how many species may be affecting amphibian populations. Further, because echinostomes utilize bird hosts that may travel long distances, it is important for researchers to know if a given echinostome species typically occurs in the area, or may be an invasive species and therefore interact differently with the local hosts and environment. The genetic variation both within and between species provided by our dataset offers the best index yet for positively identifying echinostomes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.01.004.

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