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# MOLECULAR SYSTEMATICS OF THE AVIAN SCHISTOSOME GENUS *TRICHOBILHARZIA* (TREMATODA: SCHISTOSOMATIDAE) IN NORTH AMERICA

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## Abstract

*Trichobilharzia* is a genus of thread-like schistosomes with a cosmopolitan distribution in birds. Species of *Trichobilharzia* achieve notoriety as major etiological agents of cercarial dermatitis, or swimmer's itch. There are 40 species described in the literature, for which the majority lacks molecular sequence information. To better understand the phylogenetic relationships, diversity, species boundaries, host use, and geographic distribution of this genus, we surveyed 378 birds and over 10,000 snails from North America. The phylogenetic analysis was based on nuclear 18S, 28S rDNA, internal transcribed spacer region and mitochondrial cytochrome oxidase I sequence data. Specimens were recovered that could be related to 6 of the 14 described species of *Trichobilharzia* from North America (*T. physellae, T. querquedulae, T. szidati, T. stagnicolae, T. franki,* and *T. brantae*). An additional 5 lineages were found that could not be related directly to previously described species. *Trichobilharzia brantae*, transmitted by *Gyraulus parvus,* grouped outside the clade containing the recognized species of *Trichobilharzia*. A subgroup of the *Trichobilharzia* clade designated Clade Q was comprised of closely related species whose adults and eggs are similar, yet the European species use lymnaeids whereas the North American species use physids as snail hosts. This molecular phylogeny provides a useful framework to: 1) facilitate identification of worms, including those involved in dermatitis outbreaks; 2) test hypotheses about the evolution, diversification, host-parasite interactions and character evolution of *Trichobilharzia*; and 3) guide future taxonomic revision of *Trichobilharzia*.

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*Trichobilharzia*Skrjabin and Zakharov, 1920 is the most speciose genus within the Schistosomatidae Weinland 1858. The genus is comprised of 40 described species worldwide (Blair and Islam, 1983; Horák et al., 2002), of which 14 (Table I) have been described from North America. Members of this genus are reported to infect 5 orders of aquatic birds and 4 families of freshwater snails (Horák et al., 2002). Adult worms occur in the mesenteric or nasal veins of their definitive hosts, usually ducks, except in Africa where they also have been reported from other groups of aquatic birds such as grebes and ibises (Fain, 1956; Blair and Islam, 1983) and in Japan, from passerine birds (Oda, 1973). Blair and Islam (1983) and Horák et al. (2002) present the most recent reviews of this genus. The known snail intermediate hosts for most of the species are members of the basommatophoran families Lymnaeidae Rafinesque, 1815 and Physidae Fitzinger, 1833. Some species of *Trichobilharzia* also infect snails of Planorbidae Rafinesque, 1815, another basommatophoran family (Basch, 1966; Nassi, 1987; Rind, 1991), and the Pleuroceridae Fischer, 1885, a caenogastropod family (Ito, 1960a, b). The 14 described North American species of *Trichobilharzia* (Table I) are transmitted by physid snails (*T. physellae*, *T. querquedulae*, *T. adamsi, T. cameroni,* and *T. oregonensis*) or lymnaeid snails (*T. stagnicolae, T. elvae*, *T. alaskensis,* and *T. ocellata*) snails. The snail hosts of *T.*

*waubesensis*, *T. kegonsensis*, *T. burnetti*, and *T. horiconensis*, and *T. brantae* (until this study) are unknown. All of the North American species occur in anatid ducks (Anatinae, Aythyinae, Merginae), except *T. brantae*, which infects geese (Anserinae).

Cercariae of species of *Trichobilharzia* were the first to be implicated in causing cercarial dermatitis or swimmer's itch (Cort, 1928), an underappreciated and underreported condition occurring worldwide except in Antarctica (Cort, 1950; Lindblade, 1998; Larsen et al., 2004). Contemporary swimmer's itch cases are most frequently caused by *Trichobilharzia* cercariae (Loken et al., 1995; Kolářová et al., 1997; Farahnak and Essalat, 2003; Voronin and Beer, 2002; Bouree and Caumes, 2004; Sheng et al., 2004; Żbikowska, 2004; Coady et al., 2006), although several other genera of avian schistosomes can also cause swimmer's itch (Buckley, 1938; Cort, 1950; Stunkard and Hinchliffe, 1952; Chu, 1958; Tang and Tang, 1976).

Avian schistosomes, including *Trichobilharzia*, are a challenging group to identify and fully characterize due to the difficulties in obtaining intact adult specimens, the paucity of informative adult characters, the short duration of infection in birds and the difficulty of experimentally completing life cycles thereby relating the adult worms in birds to their larval stages, including cercaria, from snails. Moreover, changes over the last several decades in land use and water management have altered habitats for birds and snails, leaving no guarantee that transmission dynamics and species composition reported in the original species descriptions remain the same. Compounding this problem, morphological and behavioral features of the worms may vary depending on their age, season, age and size of the host, and whether or not worms have been collected from a primary or minor host (McMullen and Beaver, 1945; Wu, 1953; Farr and Blankemeyer, 1956; Stunkard, 1959; Combes, 1967; Bayssade-Dufour et al., 2006). In an understandable effort to identify dermatitis-causing schistosomes, some species were named based on only cercariae or on variable or difficult to locate adult morphological features (e.g. length, testes arrangement, position of the cecal reunion). The lack of clearly distinguishable features to identify *Trichobilharzia* and other avian schistosomes, including their cercariae, has impeded our understanding of the etiology and epidemiology of swimmer's itch.

The application of molecular systematics methods to this group of worms offers great promise as an initial step in resolving many of these difficulties. Molecular markers have expanded our understanding of schistosome parasites by permitting much less ambiguous identification of species or distinct genetic lineages (Morgan et al., 2003; Vilas et al., 2005; Brant and Loker, 2005; Brant et al., 2006; Štefka et al., 2009). The solid reference points provided by DNA sequence data permit differentiation of morphologically similar parasites and the linking of different life cycle stages that may have been collected decades apart (Vilas et al., 2005; Brant et al., 2006). These DNA sequences can be used to augment taxonomy and species delimitation, as corroborating evidence for existing hypotheses, or for falsifying systematic hypotheses (DeSalle et al., 2005).

Throughout this paper, we focus on a clade of schistosomes found in birds, herein designated as the BTGD clade (*sensu* Brant et al., 2006). Carmichael (1984) using morphological characters was the first to propose the phylogenetic relationships within Schistosomatidae and placed the genus *Trichobilharzia* within the BTGD clade. To date molecular phylogenetic analyses undertaken for *Trichobilharzia* include only 3 species (*T. franki*Müller and Kimmig, 1994*, T. szidati* Neuhaus, 1952, and *T. regenti* Horák et al., 1998), all believed to be primarily European in distribution (Picard and Jousson 2001; Dvořák et al., 2002; Ferté et al., 2005; Rudolfová et al., 2005, 2007; Jouet et al., 2008; Aldhoun et al., 2009). This leaves the remaining 37 putative species virtually unknown with respect to molecular markers. To expand our knowledge of the systematics of

*Trichobilharzia*, we surveyed North American birds and freshwater snails and incorporated these data into a molecular phylogenetic analysis. These results will be valuable for future taxonomic revisions of the genus and this framework will shed new light on understanding the origins, radiation, evolution and patterns of host usage of this diverse group of blood flukes. They will also assist investigators seeking more precise identification of the cercariae involved in dermatitis outbreaks, and will contribute to the eventual unraveling of this complex etiology of this common affliction.

## MATERIALS AND METHODS

### Specimen collection and examination

Birds were obtained from a variety of sources: our own hunting/collecting; hunters; or frozen carcasses provided by the State of New Mexico Department of Game and Fish or the Museum of Southwestern Biology, Division of Ornithology. The viscera and nasal tissues of freshly killed birds were examined in saline for schistosomes between 30 min to 12 hr postmortem. Frozen birds were thawed and examined immediately. The intestine was divided into thirds and scrapings were made to look for eggs. Worms were teased out and either relaxed and killed in hot water or put immediately into 95% ethanol for subsequent DNA analysis. Young of the year birds, targeted and collected by us before their flight feathers had developed, were collected in Churchill, Manitoba and Douglas Lake, Michigan as a way to guarantee that their parasites were acquired from their natal habitats.

Snails were collected by hand or wire mesh scoop and kept cool and moist until returned to the lab. Each snail was isolated individually in a 24-well tissue culture plate in artificial spring water and placed in natural light to induce cercarial shedding. If conditions allowed, snails that did not shed the first day were placed in aerated containers with lettuce and screened again 2 to 7 days later. In most cases, snails shed cercariae within 30 min after being placed in natural light. All schistosome cercariae were saved in 95% ethanol.

Adult worms were stained in Semichon's acetocarmine and mounted in Canada balsam on slides for measurements and morphological observation (Pritchard and Kruse, 1982). Specimens collected from this study were identified both by morphology (when possible) and by DNA sequence. Morphological determinations were made by comparison with the original species descriptions, and if available, with voucher specimens from the U.S. National Parasite Collection: *Trichobilharzia kegonsensis* (USNPC 044865), *Trichobilharzia horiconensis* (USNPC 044866), *Trichobilharzia burnetti* (USNPC 044867), *Trichobilharzia waubesensis* (USNPC 044868), *Trichobilharzia querquedulae* (USNPC 079068), *Trichobilharzia physellae* (USNPC 079636, 083314), and *Trichobilharzia brantae* (USNPC 047609). Voucher specimens for adults and cercariae from this study were deposited in the Division of Parasitology, Museum of Southwestern Biology, University of New Mexico, Albuquerque, New Mexico (Accession numbers: *T. brantae* male and cercariae MSB Para176, 182, 184; *T. physellae* males and cercariae MSB Para177–178; *T. stagnicolae* cercariae MSB Para179; *T. querquedulae* males and cercariae MSB Para180, 181, 183).

### Life cycle investigations

**Host verification—**In an attempt to verify host use, snails or domestic ducks were exposed experimentally to species of *Trichobilharzia*. Miracidia were hatched from eggs by rinsing and then diluting the feces of the avian host in artificial spring water in an Erlenmeyer flask. All but the neck of the flask was covered with aluminum foil, leaving just the top exposed to light, to concentrate miracidia (McMullen and Beaver, 1945). Flasks were placed in natural light and miracidia were collected within 30 min. Snails were isolated

individually in a tissue culture well plate with artificial spring water and were exposed each to 3 miracidia. The wells were examined after 1 hr to ensure no miracidia remained. Snails were screened for cercarial shedding 3–6 wk post-infection (PI). To verify that adult worms and miracidia derived from the same bird were the same species (in cases where both were collected), *cox*1 and ITS were sequenced for both. Any worms collected from experimental infections were also sequenced for *cox*1 and ITS and compared to the initial life cycle stage used in the experiment to verify it was the same species. This was done both to confirm that adults and cercariae were the same species and that all worms collected from experimental infections were identical.

**Trichobilharzia querquedulae—**In April 2004, at Bitter Lake National Wildlife Refuge, lab-reared strains of *Physa acuta* Draparnaund, 1805 n=30, and *Stagnicola elodes* (Say, 1821) n=30 and pre-screened and trematode-negative wild-caught found not to be shedding cercariae over a 3-day period *Gyraulus parvus* (Say, 1817) n=10 were exposed to miracidia. Half the snails of each species were exposed to miracidia from cinnamon teal, *Anas cyanoptera* Vieillot, 1816, and the other half to miracidia from blue winged teal, *Anas discors* L.

**Trichobilharzia physellae—**In March 2005, at Bitter Lake National Wildlife Refuge, New Mexico, lab-reared *Physa gyrina* (Say, 1821) n=30, and *S. elodes* n=30 were exposed to miracidia from either the lesser scaup, *Aythya affinis* (Eyton, 1838), or the bufflehead, *Bucephalus albeola* L. At Douglas Lake, Michigan, in August 2005, wild-caught *Stagnicola emarginata* Say, 1821 (n=10) and *Physa parkeri* Currier, 1868 (n=10), determined not to be shedding cercariae over a 3-day period, were exposed to miracidia of *T. physellae* hatched from the feces of *Mergus merganser* L. collected from the lake.

**Trichobilharzia stagnicolae—**In August 2005, individuals of the type host species, *S. emarginata*, were collected from the type locality for *T. stagnicolae* at Douglas Lake, University of Michigan Biological Station (McMullen and Beaver, 1945). Morphology of the cercariae collected was consistent with the original descriptions by Talbot (1936) and McMullen and Beaver (1945). Six domestic mallards and 6 peking ducks, all 10-days-old, were exposed to about 100 cercariae each for up to 30 min. The birds' feet were checked for cercarial dermatitis to determine if penetration had occurred. Feces were examined every other day from 1–4 wk PI. The birds were killed at either 2 and 4 wk PI and examined for worms. Ten specimens each of wild-caught *S. emarginata* (n=10) and *P. parkeri* (n=10) from Douglas Lake determined not to be shedding cercariae over a 3-day period were exposed to miracidia of *T. stagnicolae* from of the common merganser, *M. merganser*, collected from the same lake.

## Sequencing data and phylogenetic analysis

DNA was extracted from fresh or alcohol preserved worms with the DNeasy Tissue Kit (Qiagen, Valencia, California) according to manufacturer's guidelines or HotShot Lysis (Truett et al., 2000). In a few cases, multiple worms from a single host were extracted. DNA was amplified by polymerase chain reaction (Takara Ex Taq kit, Takara Biomedicals, Otsu, Japan) and sequenced using previously published primers. For 18S–28S, we used primers listed in Brant et al. (2006). For ITS we used its4, its5 (Dvořák et al., 2002), 3S (Bowles et al., 1995), and 4S (Bowles and McManus, 1993). We designed primers for *cox*1: CO1F15: 5′-TTT NTY TCT TTR GAT CAT AAG C-3′ and CO1R15: 5′-TGA GCW AYH ACA AAY CAH GTA TC-3′ and an internal sequencing primer CO1RH3R: 5′-TAA ACC TCA GGA TGC CCA AAA AA-3′. PCR products were purified with Montage Microcon columns (Millipore, Billerica, Maryland). Sequencing reactions were performed with Applied

Biosystems BigDye direct sequencing kit, version 3.1 (Applied Biosystems, Foster City, California).

Phylogenetic analyses were performed on 6 different datasets. The first dataset was comprised of combined 18S–28S sequence data to place the samples collected for this study within the larger context of the family Schistosomatidae (Snyder, 2004; Brant et al., 2006). The second dataset comprised a combined matrix of 18S–28S-partial ITS (ITS1-5.8S-ITS2)-*cox*1 regions to reconstruct the relationships within the genus with existing isolates of *Trichobilharzia* from GenBank and included a greater sampling of individuals from more localities and hosts from our collections. The third and fourth datasets included separate analyses to look at congruence between nuclear DNA (ITS1-58S-ITS2) and mtDNA (*cox*1). The ITS1-5.8S-ITS2 region was used because it was the only region that was conserved enough to align unambiguously all available schistosomes. The fifth dataset was ITS1, and included *T. franki* samples as well as samples of *Trichobilharzia* that were designated as unidentified from Europe from the studies of Picard and Jousson (2001) and Rudolfová et al., (2007). The sixth dataset was ITS2 that included *T. franki* samples from Jouet et al., (2008). The last 2 analyses were to assess the positions of all available European taxa in GenBank relative to the North American taxa.

Phylogenetic analyses using maximum parsimony (MP), maximum likelihood (ML), and Minimum evolution (ME) were carried out using PAUP* ver 4.0b10 (Swofford, 2002) and Bayesian inference (BI) using MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). jModeltest (Posada, 2008) was used to determine the best nucleotide substitution model for ML and ME analyses. In cases where the Bayesian Information Criteria (B.I.C) or Akaike Information Criteria (A.I.C) criteria selected different models, both were used in analyses and in all cases, the tree topologies were the same. The combined 18S-28S-ITS-*cox*1 was rooted with members of *Schistosoma*Weinland, 1858 since ITS sequences were available only for *Schistosoma*.

The model, GTR+I+G, from jModeltest was used for both combined datasets, as well as for the ITS1 dataset. For *cox*1, ITS2, and ITS1-5.8S-ITS2, the model TVM+I+G was selected for ML and ME analyses. For BI, a mixed model approach was implemented to account for the potential differences in evolutionary model parameters between data partitions (both genes and codon positions). Parsimony trees were reconstructed using heuristic searches, random taxon-input order and tree-bisection and reconnection (TBR) branch swapping. Optimal ME and ML trees were determined from heuristic searches (50 replicates for ME, 5 replicates for ML), random taxon-input order, and TBR. Nodal support was estimated by bootstrap (100 replicates) and was determined for the MP and ME trees using heuristic searches, each with random taxon-input order. In all BI that included *cox*1, the dataset was partitioned by codon positions. For the BI of the 18S-28S-ITS-*cox*1 dataset, there were 4 partitions defined by first 18S, 28S (Nst=6 rates=gamma ngammacat=4), second *cox*1 codon1, third *cox*1 codon2, and fourth *cox*1 codon3 (Nst=6 rates-invgamma ngammacat=4). All parameters were unlinked between partitions. For all the analyses, 4 chains were run simultaneously for $5 \times 10^5$ generations, trees sampled every 100 cycles, the first 5,000 trees with preasymptotic likelihood scores were discarded as burnin, and the retained trees were used to generate 50% majority-rule consensus trees and posterior probabilities.

## RESULTS

### Specimen collections

Results of the survey for species of *Trichobilharzia* in North American birds and snails are reported in Table II and Figure 1. Ten lineages of *Trichobilharzia* were collected and the collection localities of specimens used in the phylogenetic analyses are listed in Table III. A

total of 378 birds of 46 species were necropsied (Fig. 1, Table II), of which 92 birds were infected with *Trichobilharzia* (overall prevalence 24.5%). Nine of the 10 lineages reported were collected as adults and/or miracidia. Approximately 10,000 snails representing 4 families and 21 species were examined, of which 20 snails were infected with a species of *Trichobilharzia*, representing 7 of the 10 species (Table II). In most cases, prevalence of *Trichobilharzia* infections in snails was around 1%. However, at Glen Lake in Michigan, the prevalence of *T. stagnicolae* in *Stagnicola emarginata* was 30%. No nasal schistosomes were found in birds nor were any cercariae from snails genetically similar to the avian nasal schistosome, *T. regenti*. Three species of *Trichobilharzia* (*T. physellae, T. querquedulae,* and *T. brantae*) were identified whose adult morphology corresponded to the original species descriptions (Tables IV, V). One species collected was from cercariae with sequence data that matched GenBank sequences attributed to *T. szidati* (Table III). Adult worms in the lineage identified as *T. querquedulae* could be differentiated from *T. physellae* and *T. franki* by the position of the cecal reunion and the number of testes (Table IV). Also, adults of the lineage identified as *T. physellae* are consistently smaller and have a shorter gynaecophoric canal as compared to *T. querquedulae* (Table IV).

A fifth schistosome we identified provisionally as *T. stagnicolae* (Talbot, 1936; McMullen and Beaver, 1945) was collected as miracidia from mergansers. McMullen and Beaver (1945) described *T. stagnicolae* from an experimental infection in canaries; their study is the only description and record of *T. stagnicolae* adults. Subsequent reports of presumptive *T. stagnicolae* in the literature have been as cercariae from *S. emarginata* (Swales, 1936; McLeod, 1940; Elliott, 1942; Zischke and Zischke, 1968; Keas and Blankespoor, 1997; Leighton et al., 2000; Blankespoor et al., 2001; Coady et al., 2006). In our study, we collected miracidia from mergansers, *Mergus merganser,* from the type locality, Douglas Lake, but were unable to locate adult worms or eggs. Our assignment of the name *T. stagnicolae* to the samples we collected was justified based on the following: 1) our samples were collected from the type locality and the type snail; 2) we infected successfully the type snail with miracidia collected from the type locality; 3) our cercarial measurements were the same as those reported by Talbot (1936) and McMullen and Beaver (1945); 4) previous fecal examinations of *M. merganser* on Douglas Lake revealed eggs of *T. stagnicolae* (Blankespoor et al., 2001); and 5) our collections of cercariae from *S. emarginata* from several lakes in northern Michigan, in northern Minnesota, and New Mexico (Table III), all genetically matched what we collected at Douglas Lake, the type locality (0%–1.2% for *cox*1). The finding of a distinct genetic lineage with widespread representation strongly suggests our samples are *T. stagnicolae*.

The common morphological features used to differentiate the above mentioned 5 species from other avian schistosomes are provided in Tables IV and V. The infections we observed in mergansers with both *T. physellae* and *T. stagnicolae* may occur more frequently than commonly recognized. Not only can double infections be easily overlooked because of difficulties in finding both adult worms and eggs, but care is also required to ensure adults and eggs are both assigned to the correct species.

The 5 remaining lineages sequenced, 4 of which were represented by portions of adult worms recovered from birds, grouped with species of *Trichobilharzia*, but could not be matched definitively to any existing species description or to GenBank sequences associated with a formal species name. To facilitate our discussion of these species, we designated these specimens as *Trichobilharzia* spp. A–E (Table III). *Trichobilharzia* spp. A and B were found only in the American widgeon, *Anas americana* Gmelin, 1789, from 3 widespread U.S. localities along the Pacific flyway. *Trichobilharzia* sp. A was also found in a widgeon from New Mexico. *Trichobilharzia* sp. C was from the hooded merganser, *Lophodytes cucullatus* L., from the eastern U.S. For *Trichobilharzia* spp. A–C, only worm fragments

were collected that did not have informative morphological features. *Trichobilharzia* sp. D is represented by cercariae from a single lymnaeid snail from Manitoba. *Trichobilharzia* sp. E was collected both as a posterior fragment of an immature worm from a pintail duckling, *Anas acuta*, and as cercariae from *Stagnicola* sp., both from Manitoba. Cercariae morphology and measurements of *Trichobilharzia* sp. E are consistent with *T. elvae*, but these data alone are not sufficient for accurate species discrimination. Although sufficient adult worm material for these 5 lineages was not available for a conclusive morphological study to determine if these 5 lineages represent new or previously described species, the sequence and host-use data provided here are valuable reference points for future studies as additional specimens become available (Brant et al., 2006).

## Life cycle experiments

For each life cycle experiment (Table VI), *cox*1 and ITS sequences from experimentally obtained life cycle stages were identical with sequences from the life cycle stage used as starting material for the infection. Miracidia of each schistosome lineage were able to infect snails of only a single gastropod family, similar to results of previous experiments (Wu, 1953). The only successful infections of *T. stagnicolae* were *Stagnicola emarginata* from miracidia from *M. merganser* (Table VI). None of the domestic duck experiments resulted in adult worms.

## Phylogenetic analyses

DNA sequence data was deposited in GenBank, under Accession numbers FJ174450-FJ174576, FJ711767-68 for the 18S (1776 bp), 28S (1299 bp), *cox*1 (824 bp), and ITS (1227–1395 bp) datasets (Table III). For the phylogenetic analysis of *Trichobilharzia*, 130 new sequences of *Trichobilharzia* were analyzed along with 55 sequences from GenBank. The numbers of individuals of each species sequenced are shown in Table III. Aligned *cox*1 sequences appeared to be genuine mitochondrial sequence, rather than nuclear copies: sequences contained no stop codons, overlapping fragments contained no conflicts, base compositions were homogeneous across taxa, codon positions contained expected relative divergences (3>2>1), and highly suspect relationships were not evident.

MP, ML, ME, and BI methods produced congruent results, except for some differences in the single gene analyses. Topological differences occurred, but no conflicts received high support from bootstrapping or Bayesian posterior probabilities. The 18S–28S tree supported monophyly of the Schistosomatidae and the BTGD clade (as defined in Fig. 2). Representatives of *Trichobilharzia* included in this study did not form a monophyletic group as *T. brantae* did not group with other North American or European *Trichobilharzia*, but rather grouped with the morphologically and genetically distinct *Allobilharzia*Kolářová et al. 2006 from swans (Figs. 3–5). The overall relationships among the species of *Trichobilharzia* are shown in Figure 3. There was a basal split, albeit with low node support, between (*T. stagnicolae, T. szidati,* and *Trichobilharzia* spp. D and E) and (*T. regenti* and Clade Q). Here we identify Clade Q that includes species from North American and Europe that are both morphologically (Table IV) and genetically (Table VII) very similar (Fig. 3). Clade Q was recovered in all analyses and is comprised of *T. franki, T. querquedulae*, *T. physellae,* and *Trichobilharzia* spp. A, B, and C (Figs. 3–6, see discussion). Where known, based on our phylogenetic trees, members of Clade Q all have similar sized spindle-shaped eggs and included taxa dependent on either lymnaeid or physid snail hosts. *Trichobilharzia regenti*, a nasal-inhabiting species, was always recovered basal to Clade Q (Fig. 3).

To further explore the relationships in Clade Q and to fully utilize available sequence data, separate analyses of 4 datasets (*cox*1, ITS1-5.8S-ITS2, ITS1, and ITS2), were completed. Additional individuals of *Trichobilharzia* from our collections (Table III) plus isolates of *T.*

*franki* from GenBank were used. Many isolates of *T. franki* have been sequenced, however our analyses of these sequences revealed they did not form a monophyletic species group. Several isolates identified as *T. franki* were from either *Radix auricularia* L. snails (Ferté et al., 2005; Rudolfová et al., 2005) or *R. ovata* (Draparnaud, 1805) snails (Picard and Jousson 2001), but based on our analyses, isolates from these two snail species did not appear to be the same species.

In the separate gene analyses, the topologies recovered were generally the same with some exceptions. Only the *cox*1 tree (Fig. 4) supported *Trichobilharzia* sp. A as a clade. Unfortunately, there were no additional *cox*1 samples of *T. franki* available in GenBank to include in this analysis. In the analysis of the ITS1-5.8S-ITS2 dataset (Fig. 5), *T. franki* from *Radix auricularia* was not recovered as monophyletic (see Table III for labels). Furthermore, *Trichobilharzia* sp. C was identical to several isolates of *T. franki* (Fig. 5). The ITS1 data set (Fig. 6) included additional samples of *T. franki* from *R. ovata* and our analysis indicated that not only did the *T. franki* from *R. ovata* not form a clade, but they did not group with any of the *T. franki* from *R. auricularia* (Fig. 6). Similarly, the isolates of *T. franki* from *R. auricularia* did not group together. *Trichobilharzia* sp. B grouped with some of the *T. franki* isolates from *R. auricularia* from the two different studies (Table III, Fig. 6) of Picard and Jousson (2001) and Rudolfová et al. (2005). Isolates of *T. franki* from *R. ovata* were basal in Clade Q (Fig. 6). Additional samples and gene regions are needed to determine definitively if *T. franki* as represented by available sequences is actually more than 1 species, and if our *Trichobilharzia* spp. B and C are the same as or distinct from the European samples labeled *T. franki*. Our data are suggestive that *T. franki* also occurs in North America. *R. auricularia* is found in North America, however the snail hosts for *Trichobilharzia* spp. A–C is not yet known.

The available ITS2 sequences provided yet a different perspective (Fig. 7). In this analysis 3 unidentified species of *Trichobilharzia* (*Trichobilharzia* sp. 3 Pl10, *Trichobilharzia* sp. 3 Pl7, *Trichobilharzia* sp. EAN17) in GenBank for which only ITS2 data were available were added to our dataset (Table III). The positions of *Trichobilharzia* sp. 3 Pl10 and *Trichobilharzia* sp. 3 from *Anas penelope* L. from Poland (Rudolfová et al., 2007), were equivocal, but in the ME analysis they aligned with *Trichobilharzia* sp. D, although without support. The sample *Trichobilharzia* sp. EAN17 from *Radix peregra* (Müller, 1774) from France (Jouet et al., 2008) grouped with *Trichobilharzia* sp. E from Manitoba with strong node support, suggesting they may be conspecific.

To provide a convenient yardstick to measure the extent of sequence difference among species of *Trichobilharzia*, pairwise genetic differences were calculated and compared with values obtained for the relatively well-defined species of *Schistosoma* (Table VII). Based on such comparisons, the lineages of *Trichobilharzia* are as genetically distant from each other as are the named species within both *Trichobilharzia* and *Schistosoma* (Table VII), providing good presumptive evidence that they represent distinct species (Nolan and Cribb, 2005; Vilas et al., 2005). As determined by sequence analysis, 7 of the 10 lineages of *Trichobilharzia* were collected from snails, 4 of which corresponded to sequences that we obtained from *Trichobilharzia* adults (*T. physellae*, *T. querquedulae*, *T. brantae,* and *Trichobilharzia* sp. E). The remaining 3 taxa were from cercariae that grouped with sequence data from miracidia of *T. stagnicolae*, with sequences for *T. szidati* from Europe (Figs. 4, 5; Rudolfová et al., 2005), or that did not group with any species or clade (*Trichobilharzia* sp. D; Figs. 4, 5).

Despite the broad geographic and host sampling for *T. physellae* and *T. querquedulae,* which were both collected from across North America (Fig. 1; Table III), we did not find indications of geographic structuring within either species (Figs. 4, 5). With the genes used

in this study, haplotypes that were identical or that differed in only 1–2 base pairs were found between both eastern and western samples. The *T. stagnicolae* isolates collected from Minnesota, Michigan, Montana, and New Mexico, also show little evidence of geographic differentiation (Fig. 4). The collection of *T. szidati* related isolates from North America was unexpected. Based on the ITS1-5.8S-ITS2 region (Table VII), our samples of *T. szidati*, from Montana (Flathead *Stagnicola* MT) in *Lymnaea stagnalis* L., and Michigan (Blind Sucker *Lymnaea* MI) in *Stagnicola elrodi* (Baker and Henderson, 1933), were genetically very similar (0.4%) to each other, as well as to the European isolates of *T. szidati* (0.36%). These figures are within the range of variation noted for *T. szidati* from Europe based on ITS1-5.8S-ITS2 (0–1.1%). In general, the genetic differences between species pairs within continents were not less than the differences between species pairs from different continents, Europe and North America (Table VII).

## DISCUSSION

### General observations

This is the first molecular systematics study of species of *Trichobilharzia* collected from a diversity of avian and snail species collected across North America. From North America we collected 5 morphologically identifiable and genetically distinct species of *Trichobilharzia*. These species were *T. brantae, T. physellae, T. querquedulae, T. stagnicolae,* and *T. szidati,* one of which (*T. szidati*) was reported previously from Europe (Rudolfová et al., 2005). We also collected 5 additional genetically distinct lineages that group within *Trichobilharzia* that could not be associated with a named species. These results suggest that at least 10 genetically distinct lineages of *Trichobilharzia* exist in North America. How these latter 5 species relate to the remaining species of *Trichobilharzia* described from North American not found in this study remains to be determined. Nevertheless, the sequence database generated here for North American species of *Trichobilharzia* will contribute to future studies revealing the broader species diversity and the host preferences for each species.

Although an analysis that includes additional specimens of putative *Trichobilharzia* from other continents is necessary to understand the full scope of the genus, our morphology (Table V; Fig. 8) and genetic differences strongly suggests that *T. brantae* should not be included as a member of *Trichobilharzia*. This species from geese and planorbid snails did not group within the *Trichobilharzia* clade, but rather aligned with *Allobilharzia*, a genus of schistosome collected thus far only from swans. *Allobilharzia* is morphologically and genetically distinct from *Trichobilharzia*; egg shape and position of cecal reunion are the two major differences (Table V, Fig. 8; Kolářová et al., 2006;Brant, 2007). Blair and Islam (1983) also suggested that *T. brantae* did not belong in the genus *Trichobilharzia*, but rather *T. brantae* should be transferred to the genus *Jilinobilharzia*Lui and Bai, 1976. However, a morphological comparison does not support inclusion of *T. brantae* in *Jilinobilharzia* or *Allobilharzia*. Table V compares some of the major morphological differences such as; shape of the eggs, position of the seminal vesicle and the start point and length of the gynaecophoric canal.

The *cox*1 genetic difference values obtained for pairs of *Trichobilharzia* taxa outside of Clade Q (9–14%) were comparable to those obtained for congeners of other flatworm groups (Figs. 3, 6), including the confamilial *Schistosoma* (9–21%) in mammals (Vilas et al., 2005). Genetic differences among pairs of taxa within Clade Q were variable but generally low (7–9%), indicative of perhaps a more recent divergence among members of this clade (Table VII). Morphology as well as host use (definitive and intermediate) were considered relative to the molecular phylogenetic results by mapping these features onto the BTGD clade (Fig. 8). Host, morphology and DNA taken together revealed some interesting

patterns discussed below, as well as highlighting the small number of distinguishing morphological features. The molecular and host use data provided here will eventually facilitate assessment of the validity of other species descriptions, assuming the specimens in question belong to a species described previously, and will help delineate new species (Štefka et al., 2009).

## Life cycles of species of *Trichobilharzia*

Sequence markers provide an invaluable tool in connecting life cycle stages from wild hosts that have not been previously integrated into a complete life cycle through experimental infections. Given the challenges in obtaining the necessary approvals for maintenance of vertebrate animals, it may become difficult in the future to resolve life cycles through experimental infections. In our molecular survey, we found 4 sequence matches for cercariae from snails with adult worms from wild birds, thus providing strong direct inferences for the wild hosts involved in those particular life cycles. Such matches were obtained for *T. physellae*, *Trichobilharzia* sp. E, *T. szidati,* and *T. brantae*.

## Snail Host Use

Excluding *T. brantae*, all *Trichobilharzia* from Europe for which life cycles are known use lymnaeid snails, whereas species from North America use lymnaeids or physids. Members of Lymnaeidae are worldwide in distribution, with their greatest species diversity occurring in North America. Interestingly, *Lymnaea stagnalis* and *Radix auricularia,* the former a host of *T. szidati* and the latter host to *T. franki*, are not considered endemic to North America (Remigio, 2002). Most of the diversity of *Trichobilharzia* in North America was found in species of *Stagnicola* (Table III). This is in contrast to studies in Europe where most of the diversity of *Trichobilharzia* was found in species of *Radix* (e.g. Picard and Jousson, 2001; Jouet et al., 2008; Aldhoun et al., 2009). Physidae is mainly a New World family, members of which have spread secondarily to other continents (Taylor, 2003). Thus far, only North America is known to have sequence-verified members of the *Trichobilharzia* clade that use physid snails. Although physid transmitted schistosomes are known from other continents (Ostrowski de Núñez, 1978; Rudolfová and Horák, 2001; Gerard, 2004), they have not been verified as a species of *Trichobilharzia* and may be representatives of *Gigantobilharzia* Odhner, 1910, one species of which in North America is also transmitted by physids (Brackett, 1942; Najim, 1956; Daniell, 1978). In no case did we find representatives of a single species of *Trichobilharzia* in snails belonging to more than 1 family, although in some cases we found more than 1 species of a particular snail family could host the same species of *Trichobilharzia*. For example, we found *T. stagnicolae* in *Stagnicola emarginata* and *Stagnicola* sp. and *T. physellae* in *Physa parkeri* and *P. gyrina* (Table II). In other studies, *T. regenti* has been collected from both *Radix peregra* and *R. ovata* snails (Picard and Jousson, 2001; Dvořák et al., 2002; Rudolfová et al., 2006). There is also an indication that *T. franki* occurs in more than one species of *Radix*, excluding those samples of *T. franki* from Piccard and Jousson (2001) from *R. ovata* snails (Jouet et al., 2008).

Physid transmitted species of *Trichobilharzia* were found only in Clade Q (Figs. 3,6) delineated in this study. In fact, so far as is known, *Trichobilharzia* is the only avian schistosome genus to use lymnaeid snails. The planorbid transmitted *T. brantae*, *Dendritobilharzia*Skrjabin, 1920, and *Bilharziella* Looss, 1899 and the physid transmitted *Gigantobilharzia huronensis* Najim, 1950 are basal to *Trichobilharzia* within the BTGD clade, suggesting that in our results, lymnaeids are the basal hosts within species of *Trichobilharzia* (Fig. 8). Members of Clade Q (Fig. 6) are not strongly differentiated from one another on either morphological or genetic characters (Tables VI, VII), yet given that the clade includes 2 known physid transmitted species, *T. physellae* and *T. querquedulae*, it is suggested that at least 2 switches from one snail family to another occurred within Clade

Q. Also, even though the genetic distances between physid and lymnaeid transmitted species in Clade Q (Table VII) are not great, all the available specimens for each physid transmitted species cluster together with unequivocally strong support in all analyses. Taken together, these results suggest that members of Clade Q have diverged relatively recently from one another, and that switches between 2 different snail families have occurred after which the taxa occupying different snail families remained genetically distinct from one another.

## Definitive host use

In North America and Europe, all specimens of *Trichobilharzia, Allobilharzia*, and *T. brantae* for which there are molecular data, were found in avian hosts of the order Anseriformes (ducks, geese, and swans). *Allobilharzia* is known only from swans (Kolářová et al., 2006; Brant, 2007), and *T. brantae* is known only from geese (Farr and Blankemeyer, 1956; Wojcinski et al., 1987). The remaining species of *Trichobilharzia* collected for this study parasitize ducks of the Anatinae, Aythyinae, and Merginae. Species of *Trichobilharzia* from other continents have been described from other orders of birds, but thus far representatives of these species have not been available for sequencing to determine if they fall within the *Trichobilharzia* clade defined here.

Although there is not a strong pattern of definitive host specificity, some trends were identified (Fig. 8). *Trichobilharzia querquedulae* has been found only in 3 species of dabbling ducks (Table II; *Anas clypeata* L.*, A. cyanoptera,* and *A. discors*) that are each other's closest relatives (Johnson and Sorenson, 1999). In contrast, *T. physellae* utilizes mainly diving ducks (Aythyinae, mostly species of *Aythya* Boie*,* 1822) and mergansers (Merginae) as its major definitive hosts (ecological rather than phylogenetic). While these duck hosts are not each other's closest relative, they are united ecologically by their preferred feeding habitat and style (diving). Although other duck species are also infected, prevalence is very low or there were few worms, most immature (Table II). One of the principal hosts of *T. stagnicolae* is a merganser (Blankespoor and Reimink, 1988;Leighton et al., 2000;Blankespoor et al., 2001;Coady et al., 2006), corroborated by our survey. It is interesting to note that 2 of the 3 unidentified lineages in Clade Q came from *Anas americana*, the American widgeon.

## Some biogeographical remarks regarding North American *Trichobilharzia*

North American species of *Trichobilharzia* that we collected have broad geographic ranges and, at least as suggested by the markers used here, show little evidence of intraspecific genetic structure (Figs. 4–6). This is true for *T. stagnicolae,* which has been collected from Michigan, Minnesota, Montana, and New Mexico (Table III), and for specimens of *T. physellae* and *T. querquedulae* were collected from all the major avian migratory flyways, and from latitudes as distant as Alaska and Manitoba to Louisiana and Florida (Fig. 1; Table III). The latter 2 species have yet to be collected outside of North America.

Using sequence similarity as the criterion to designate species as outlined in Vilas et al. (2005), 4 avian schistosome lineages from North America have presumptive representatives in Europe (Table VII): (1) *T. szidati*, which is considered a European species (Rudolfová et al., 2005), was collected from North American snails (Table III, Fig. 5); (2) *Trichobilharzia* sp. B grouped with the European *T. franki* from *R. auricularia* (Fig. 6); (3) *Trichobilharzia* sp. E was closely aligned with *Trichobilharzia* sp. EAN17 from the snail, *Radix peregra,* collected in France (Fig. 7); and (4) although not the specific subject of this paper, specimens of *Allobilharzia visceralis* collected from the North American swans as part of this survey were indistinguishable from worms collected from swans in Iceland (see Kolářová et al., 2006; Brant, 2007). Thus, the continent of origin by no means represents an

infallible indicator for species designations either for species of *Trichobilharzia* or other genera of avian schistosomes.

## Diversification of *Trichobilharzia*

Incomplete taxon sampling and uncertainty among the basal nodes of the *Trichobilharzia* spp. radiation continue to challenge our understanding of the global diversification of this genus. The 40 named species of *Trichobilharzia* have been described from multiple locations in Europe and North America, Brazil (Leite et al., 1978), Australia (Blair and Islam, 1983; Islam, 1986; Islam and Copeman, 1986), New Zealand (Davis, 2006), China (Pao and Yung, 1957; Tang and Tang, 1976; Lui et al., 1977; Tsai et al., 1979), India (Baugh, 1963; Chauhan et al., 1973), Malaya (Basch, 1966), Japan (Ito, 1960; Yamaguti, 1971), Congo-Rwanda (Fain, 1955, 1956, 1959) and South Africa (Appleton, 1982, 1986). Most of these species were reported from ducks, geese and swans, however a few were reported from passerine birds, kingfishers, grebes and ibises (Fain, 1955, 1956; Ito, 1960; Tsai et al., 1979). Snail hosts where known, with one exception (Ito, 1960), are physid or lymnaeid snails. Reports of species of *Trichobilharzia* in North America, including this study, were all collected from ducks and/or physid or lymnaeid snails. The exception is *T. brantae*, which we now know occurs in geese and uses a planorbid snail as an intermediate host. A true global definition of *Trichobilharzia* awaits inclusion of genetically verified species from South America, Asia, and Africa.

Recent studies have shown that uncovering additional diversity among avian schistosomes is a frequent occurrence, particularly when snails are surveyed and molecular approaches are applied (Larsen et al., 2004; Brant et al., 2006; Rudolfová et al., 2007; Jouet et al., 2008; Skirnisson and Kolářová, 2008; Aldhoun et al., 2009). This suggests there is more diversity to discover with respect to *Trichobilharzia*, already considered the most speciose genus in the family. The second most speciose genus is *Schistosoma*, currently comprised of 22 species.

Perhaps what is more noteworthy is the relatively large number of distinct lineages for a parasite group that colonizes vagile, migratory definitive hosts. This is particularly so considering the overlaps in host species use and spatial and temporal sympatry among host species that regularly occurs on their breeding/wintering grounds and in other wetland habitats. The lack of host isolation coupled with the mobility of their host species would seem to weaken barriers to gene flow among the avian schistosomes. Moreover, lymnaeid and physid snails are both common, occur in large numbers, and are widely distributed, seemingly further reducing opportunities for regional diversification. The extent to which mating behavior/preferences or temporal or spatial separation within definitive hosts may disrupt gene flow and isolate species is not well known for avian schistosomes and will be excellent model systems for future investigations. Also, the acquisition of new molluscan hosts (for example, a switch from lymnaeid to physid snails as seems to have occurred in Clade Q) may also serve as a major isolating mechanism.

The relationships among *T. franki, T. physellae*, and *Trichobilharzia* spp. A, B, and C in Clade Q provide an interesting opportunity to address questions about gene flow, and speciation, and ultimately, diversification. There are several hypotheses, not necessarily mutually exclusive, that might explain the patterns observed in this clade that includes geographically distant, yet closely related North American and European species. It may be that (1) given the genetic and morphological similarities, these taxa are not fully differentiated as species because they have only recently diverged; (2) there may be isolation and incipient diversification among populations or species of *Trichobilharzia* that is diminished by ongoing gene flow that is maintained by the mobility of their hosts; (3) even though they are found in hosts considered mobile and that migrate long distances, the

different taxa of Clade Q actually have subtle patterns of host use, or different geographical preferences that are not yet differentiated or require more sensitive genetic markers (like microsatellites) to reveal cryptic variation (Štefka et al., 2009); (4) hybridization may have occurred (Morgan et al., 2003; Fan and Lin, 2005; Steinauer et al., 2008); (5) the equivocal positions, or low branch support of individuals like *Trichobilharzia* spp. A, B, and C might imply that there remains undiscovered diversity (missing taxa) that, if available, would clarify relationships in this clade. Future work to increase the sample size within Clade Q and selection of alternative, faster evolving, markers to estimate gene flow will help address which of these processes have been important to shaping the diversity we find. We also need to accumulate more morphological data and understand how it correlates with genetic variation, to better define the status of species such as *T. szidati* and *T. franki* in North America.

### Medical significance including cercarial dermatitis

None of the North American adult worms was found in host nasal turbinates, a location inhabited by some *Trichobilharzia* species in Europe (*T. regenti*), Australia (*T. australis*Blair and Islam, 1983, *T. arcuata* Islam, 1986), and Africa (*T. spinulata* Fain, 1955, *T. rodhaini* Fain, 1955, *T. nasicola* Fain, 1955, *T. aureliani* Fain, 1956, *T. duboisi*, Fain, 1959). This is of note from a public health perspective because the nasal-dwelling *T. regenti* migrates via both peripheral nerves and the central nervous system to reach its preferred site of infection. This species has been shown to cause anomalous behavior in both experimentally infected birds and mammals (Horák et al., 1999; Hrádková and Horák, 2002; Kouřilová et al., 2004) and has the potential to present similar consequences in humans.

Although most North American outbreaks of cercarial dermatitis are ascribed to *T. physellae* or *T. stagnicolae* (Swales, 1936; Cort, 1950; McMullen and Brackett, 1941; McLeod, 1940; Hunter, 1960; Zischke and Zischke, 1968; Leighton et al., 2000; Blankespoor et al., 2001; Coady et al., 2006), such identifications typically reflect whether the cercariae were shed from a physid or lymnaeid snail, respectively. The framework incorporating molecular markers developed here will be of immediate use in making more precise determinations. For example, although *T. physellae* was the taxon we most frequently collected from snails, at least 3 additional avian schistosome taxa from physid snails were collected, including representatives of other genera; accordingly, caution is required in ascribing physid transmitted outbreaks of dermatitis to *T. physellae* (S. Brant pers. obs.). The extent to which each of the 10 different taxa of *Trichobilharzia* noted here is actually involved in causing cercarial dermatitis in North America is an important priority for future study.

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**FIGURE 1.**

Collection localities. Refer to Table III for more details. Localities are as follows: **1** Canada: Manitoba, Churchill 58.7541 N; 93.8066 W, July/August 2007; **2** Colorado: El Paso Co. 38.827 N; 104.804 W, June 2007; **3** New Mexico: Bernalillo Co. 35.1305 N; 106.6822 W, July 2002; **4** New Mexico: Sandoval Co. 35.8485 N; 106.4907 W, July 2006; **5** Pennsylvania: Erie Co. 42.1703 N; 80.0868 W, November 2004; **6** New Mexico: Chavez Co. 33.45 N; 104.4 W, April 2005, March 2006; **7** Alaska: North Slope Borough 68.9820 N; 148.8318 W, June 2005; **8** Nevada: Churchill Co. 39.9 N; 118.817W, November 2005; **9** Michigan: Cheboygan Co. 45.581 N; 84.697 W, July, 1999, August 2005; **10** Florida; **11** Louisiana, Cameron Parish 26.661 N; 92.688 W, November 2003; **12** California: Imperial Co. 33.2988 N; 115.5875 W, November 2004; **13** New Mexico: Socorro Co. 33.7131 N 106.9579 W, April 2004; **14** Alaska: Yukon-Koyukuk Borough 65.665 N; 149.098 W, May 2005; **15** Nebraska: Nemaha Co. 40.467 N; 95.7 W, November 2004; **16** Montana: Big Fork Lake Co. 47.483 N; 114.217 W, 1999; **17** New Mexico: Taos Co. 36.8467 N; 105.3794 W, June 2004; **18** Minnesota: Itasca Co. 47.510 N; 94.185 W, July 2008; **19** Michigan: Luce

Co. 46.667 N; 85.733 W, July 1999; **20** New Mexico: Sierra Co. 32.9071 N; 107.3116 W, February 2005; **21** Pennsylvania: Crawford Co. 41.575 N; 80.212 W, November 2004.

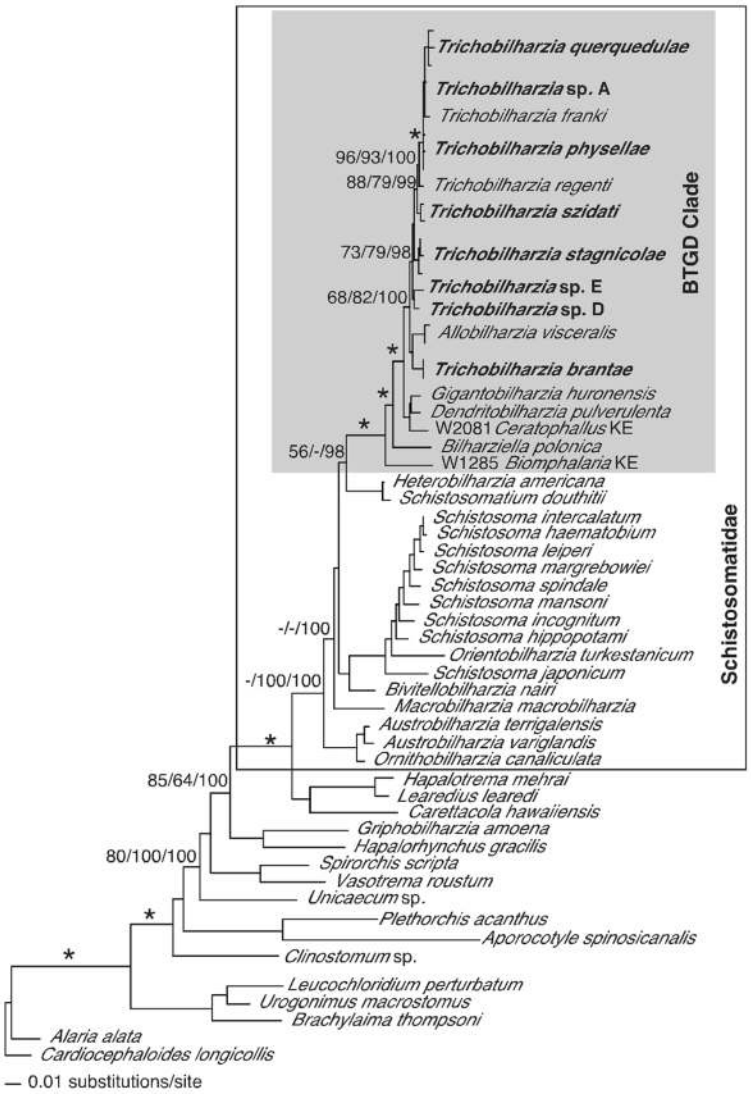


**FIGURE 2.**

Maximum likelihood tree based on 18S–28S sequences. The schistosomatids are enclosed in the box, and the BTGD clade delimited by shading. Samples in bold are those collected from this study. Within the BTGD clade, individual specimens of *Trichobilharzia* were collapsed and are labeled only by the taxon name. Node support is indicated by MP and ME bootstrap values and Bayesian posterior probabilities (PP), respectively. The "*" indicates MP and ME bootstrap values of >90 and PP of 100. The '-' indicates no significant node support. Branch support is designated only for the major clades.

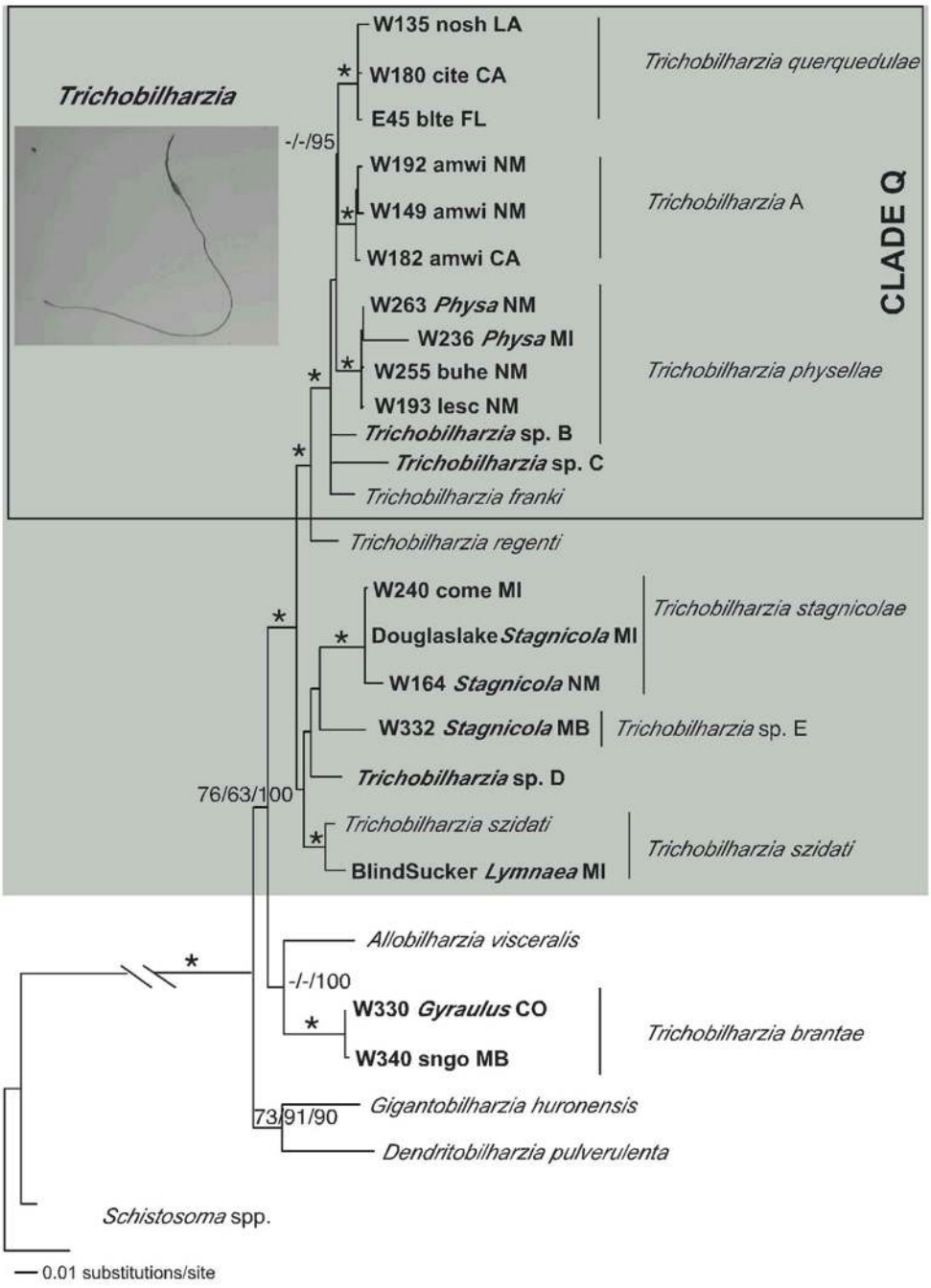


**FIGURE 3.**

Maximum likelihood tree based on 18S-28S-*cox*1-ITS sequences. *Trichobilharzia* is highlighted. Samples in bold are those collected from this study. Node support is indicated by MP and ME bootstrap values and Bayesian PP, respectively. The '*' indicates MP and ME bootstrap values of >90 and PP of 100. The '-' indicates no significant node support. Image is of *Trichobilharzia physellae* (USNPC# 079636).

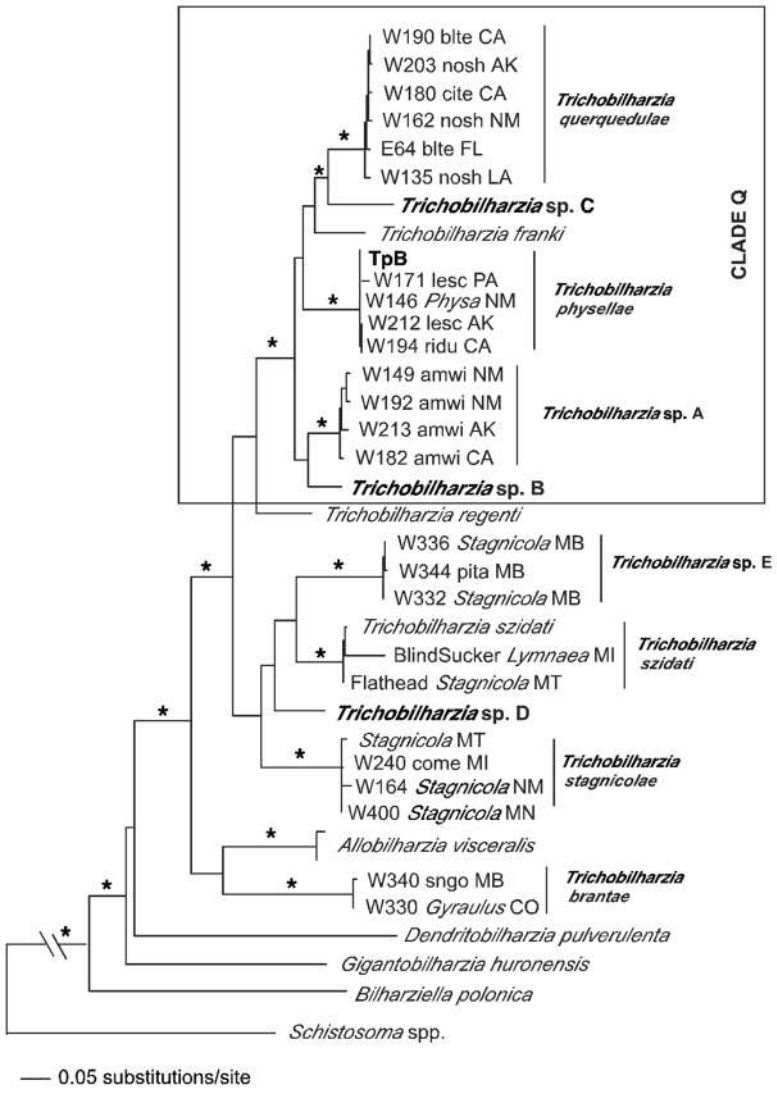


**FIGURE 4.**

Maximum likelihood tree based on *cox*1 sequences. For the *Trichobilharzia querquedulae* clade only some of the worms sequenced are represented as many differed by only one base pair. The "*" indicates node support of >95% bootstrap for MP and ME and >98 Bayesian PP. The '-' indicates no significant node support. Outgroup species of *Schistosoma* were collapsed. For convenience, the following taxa were trimmed from the tree, but were fully supported in the clade: W137blteLA, W156blteNM, W148.1citeNM, W148.2citeNM, W155.3citeNM, W158noshNM, W162noshNM, W183noshCA, SDS1006noshNE, E45blteFL (Table III). The same was done for *T. physellae,* except in one case there were identical haplotypes: **TpB** = W171lescPA, W193lescNM, W255buheNM, W263*Physa*NM. Otherwise, the following with only 1–2 bp differences were removed W211olsqAK, W193lescNM, W236*Physa*MI, W230.1comeMI, and W256lescNM.

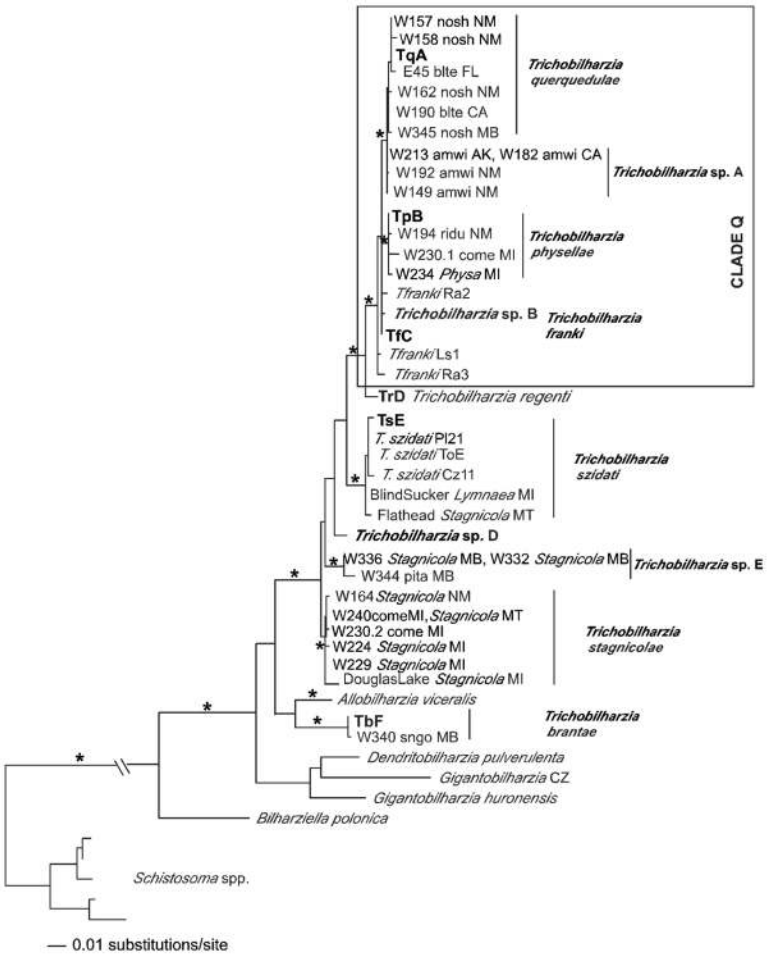


**FIGURE 5.**

Maximum likelihood tree based on ITS sequences. The following labels apply to samples with identical haplotypes: *Trichobilharzia querquedulae* **TqA**= W135blteLA, W137blteLA, W156blteNM, W148.1citeNM, W155.3citeNM, W180citeCA, W203noshAK, W183noshCA, SDS1006noshNE. *Trichobilharzia physellae* **TpB** = W146*Physa*NM, W263*Physa*NM, W171lescPA, W212lescAK, W249cabaNV, W255buheNM. *Trichobilharzia franki* **TfC** = *Trichobilharzia* sp. C, *T. franki* Ra1, and *T. franki* RSFO1. All haplotypes of *T. regenti* downloaded from GenBank were identical; **TrD** = *T. regenti* Cz79, *T. regenti* Cz31, *T. regenti* Pl27, *T. regenti* Pl20, *T. regenti* Pl17, *T. regenti* Pl14. *Trichobilharzia szidati* **TsE** = *T. szidati* Tsz, *T. szidati* Ls5, *T. szidati* ToA. *Trichobilharzia brantae* **TbF** = W346*Gyraulus*MB, W331*Gyraulus*CO, W330*Gyraulus*CO. Isolates of *T. franki* are from *R. ovata* (ov) and *R. auricularia* (Ra) snails (one sample is from *Lymnaea stagnalis* = Ls). The "*" indicates node support of >95% bootstrap for MP and ME and >98 Bayesian PP. The '-' indicates no significant node support.

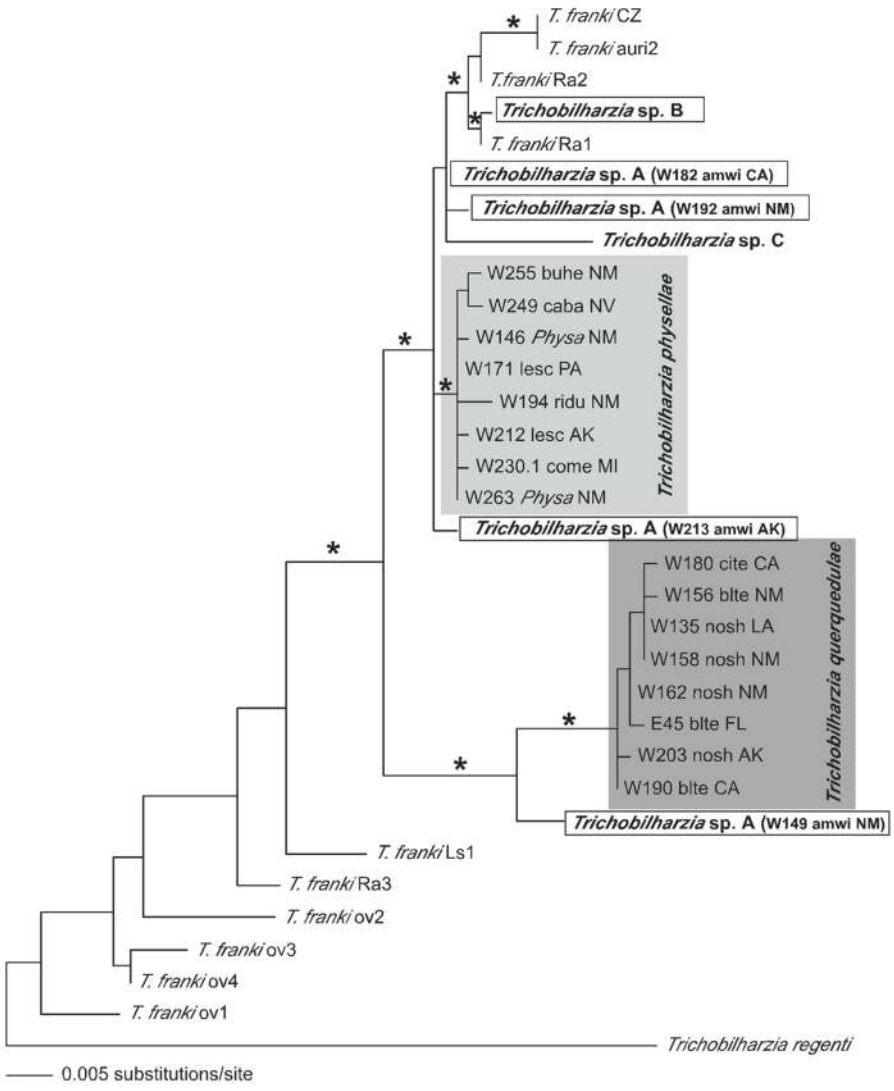


**FIGURE 6.**

Maximum likelihood tree based on ITS1 sequences of an expanded Clade Q. *Trichobilharzia* A is boxed to show its variable position in the tree and paraphyly of *T. franki*. Isolates of *T. franki* are from *R. ovata* (ov) and *R. auricularia* (Ra) snails (one sample is from *Lymnaea stagnalis* = Ls). See Table III for label descriptions. The "*" indicates node support of >95% bootstrap for MP and ME and >98 Bayesian PP. The '-' indicates no significant node support.

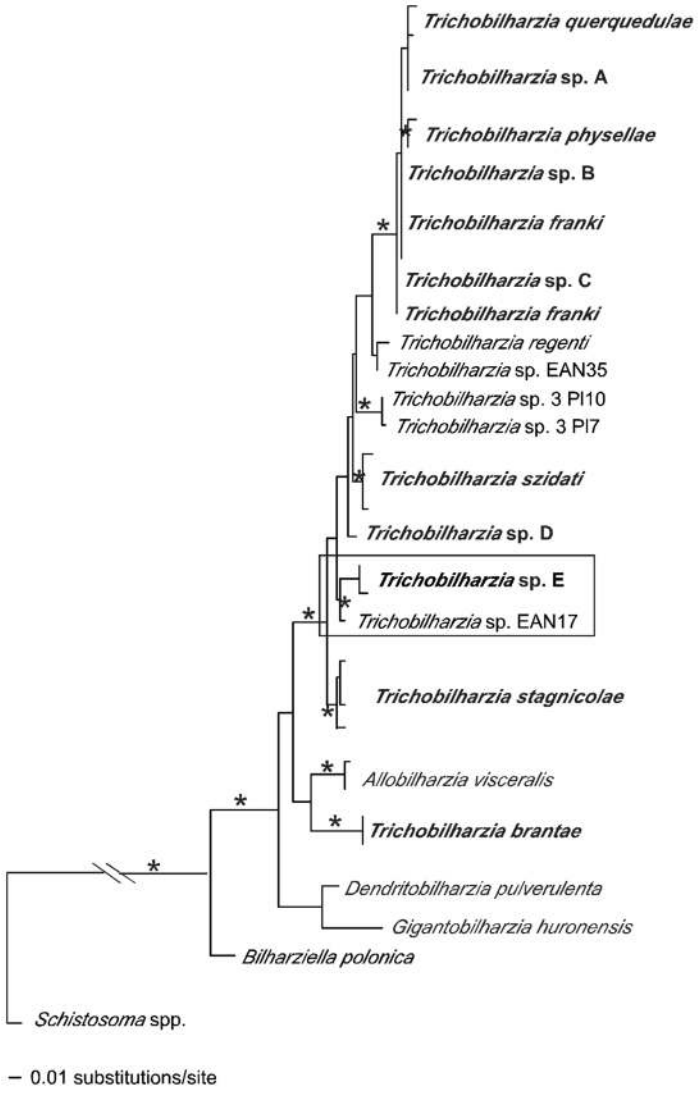


**FIGURE 7.**

Maximum likelihood tree based on ITS2 sequences showing the positions of the unidentified avian schistosome isolates from GenBank. The boxed clade highlights the relationship between the samples from North America and France. Bolded samples indicate those from this study. See Table III for label descriptions. The "*" indicates node support of >95% bootstrap for MP and ME and >98 Bayesian PP. The '-' indicates no significant node support.

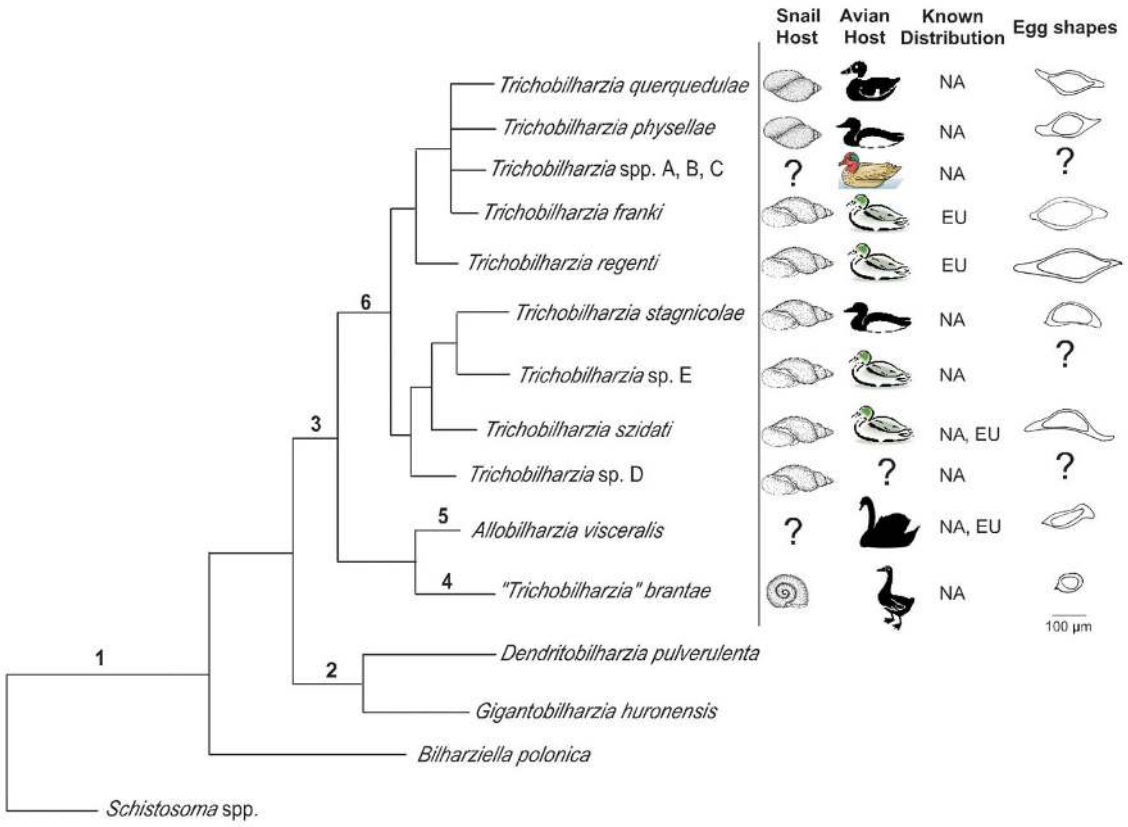


**FIGURE 8.**

Summary tree based on 28S depicting comparative features (hosts, distribution, and egg morphology) for North American and European avian schistosomes. Morphological features listed for well-supported nodes. **1**– reduced sexual dimorphism, males and females flattened or thread-like, gynaecophoric canal absent or weakly developed or short (not extending to posterior), testes numerous; **2** – Absence of ventral sucker, absent or weakly developed oral sucker, uterus with numerous eggs, eggs ovoid; **3** – Well developed oral and ventral suckers, uterus usually with single egg, seminal vesicle between gynaecophoric canal and ventral sucker; **4** – cecal reunion at or anterior to seminal vesicle, >400 testes, gynaecophoric canal terminates well anterior to first testes; **5** – cecal reunion posterior to gynaecophoric canal, >400 testes, gynaecophoric canal terminates well anterior to first testes; **6** – Position of the cecal reunion overlaps the position of the seminal vesicle, gynaecophoric canal terminates at first testes, cercariae large with eyespots.

Physidae, Lymnaeidae, Planorbidae, teal, diving ducks, *Anas americana* most ducks, swans, geese. Eggs scaled to relative sizes.

## Table I

List of the North American species of *Trichobilharzia* and their definitive and/or intermediate hosts from the type descriptions.

| Species | | Intermediate host | Definitive host | Type Locality |
|---|---|---|---|---|
| *Trichobilharzia adamsi* | Edwards & Jansch, 1955 | *Physa gyrina*† | * Exp: peking duck | Canada |
| *Trichobilharzia alaskensis* | Harkema, 1960 | *Lymnaea stagnalis*† | Exp: peking duck | Alaska |
| *Trichobilharzia brantae* | Farr & Blankemeyer, 1956 | unknown | *Branta canadensis*† | Virginia |
| *Trichobilharzia burnetti* | Brackett, 1942 | unknown | *Aythya collaris*† | Wisconsin |
| *Trichobilharzia cameroni* | Wu, 1953 | *Physa gyrina*† | Exp: canary, pigeon, domestic duck | Canada |
| *Trichobilharzia elvae* | (Miller, 1923) Talbot, 1936 | *Lymnaea stagnalis*† | Exp: peking & black duck | Michigan |
| *Trichobilharzia horiconensis* | Brackett, 1942 | unknown | *Anas americana*† | Wisconsin |
| *Trichobilharzia kegonsensis* | Brackett, 1942 | unknown | *Aythya valisinera*† | Wisconsin |
| *Trichobilharzia ocellata* | (La Valette, 1855) Brumpt, 1931 | *Lymnaea stagnalis*† | Exp: domestic duck | Germany |
| *Trichobilharzia oregonensis* | MacFarlane & Macy 1946 | *Physa gyrina*† | Exp: peking duck | Oregon |
| *Trichobilharzia physellae* | (Talbot, 1936) McMullen & Beaver, 1945 | *Physa parkeri*† | Exp: domestic duck | Michigan |
| *Trichobilharzia querquedulae* | McLeod, 1937 | | *Anas discors*† | Canada |
| *Trichobilharzia stagnicolae* | (Talbot, 1936) McMullen & Beaver, 1945 | *Stagnicola emarginata*† | Exp: canary | Michigan |
| *Trichobilharzia waubesensis* | Brackett, 1942 | unknown | *Anas americana*† | Wisconsin |

*Exp=experimental exposure;

†Type host.

**Table II**

List of the hosts examined harboring species of *Trichobilharzia*. Localities are labeled with the U.S. state abbreviation, except for Manitoba, Canada (MB). Schistosomes were found in hosts and localities in bold. See text for further description.

| North America Avian Host | Number examined | Viscera positive | Nasals examined | Species of *Trichobilharzia* | Locality |
|---|---|---|---|---|---|
| *Larus delawarensis* | 5 | 0 | 0 | - | LA, CA |
| *Larus delawarensis* | 10 | 0 | 0 | - | CA |
| *Larus californicus* | 1 | 0 | 0 | - | CA |
| *Larus fuscus* | 1 | 0 | 0 | - | LA |
| *Sterna maxima* | 1 | 0 | 0 | - | LA |
| *Anhinga anhinga* | 10 | 0 | 0 | - | FL, LA |
| *Phalacrocorax auritus* | 2 | 0 | 0 | - | LA |
| *Pluvialis dominicus* | 1 | 0 | 0 | - | LA |
| *Egretta tricolor* | 1 | 0 | 0/1 | - | LA |
| *Egretta thula* | 1 | 0 | 0/1 | - | LA |
| *Plegadis chihi* | 6 | 0 | 0 | - | LA |
| *Eudocimus albus* | 12 | 0 | 0/5 | - | LA |
| *Aramus guarauna* | 1 | 0 | 0 | - | FL |
| *Podilymbus podiceps* | 2 | 0 | 0 | - | NM |
| *Gavia immer* | 1 | 0 | 0 | - | NM |
| *Xanthocephalus xanthocephalus* | 1 | 0 | 0 | - | NM |
| ***Aythya affinis*** | 28 | 11 | 0/7 | *T. physellae* | **AK**, CA, LA, **NM**, **PA** |
| *Aythya americana* | 4 | 0 | 0 | - | CA, LA, NM |
| ***Aythya collaris*** | 5 | 1 | 0 | *T. physellae* | CA, LA, **NM** |
| *Aythya marila* | 9 | 0 | 0 | | AK, PA, MB |
| ***Aythya valisineria*** | 5 | 1 | 0/3 | *T. physellae* | NM, **NV** |
| ***Anas acuta*** | 23 | 1 | 0/1 | *Trichobilharzia* sp. E | AK, CA, LA, NV, NM, **MB** |
| ***Anas americana*** | 23 | 8 | 0/1 | *Trichobilharzia* spp. A and B | **AK, CA, NM** |
| ***Anas carolinensis*** | 41 | 4 | 0/7 | *Trichobilharzia physellae* | **AK**, CA, LA, **NM**, PA |
| ***Anas clypeata*** | 22 | 20 | 0/13 | *T. querquedulae* | **AK, CA, LA, NE, NM, MB** |
| ***Anas cyanoptera*** | 12 | 11 | 0/2 | *T. querquedulae* | **CA, NM** |
| ***Anas discors*** | 20 | 20 | 0/4 | *T. querquedulae* | **CA, FL, LA, NM, PA** |
| *Anas fulvigula* | 5 | 0 | 0/3 | - | LA |

| North America Avian Host | Number examined | Viscera positive | Nasals examined | Species of *Trichobilharzia* | Locality |
|---|---|---|---|---|---|
| ***Anas platyrhynchos*** | 12 | 2 | 0/3 | *T. physellae* | AK, LA, MI, **PA**, |
| *Anas rubripes* | 3 | 0 | 0 | - | PA |
| ***Anas strepera*** | 27 | 1 | 0/5 | *T. physellae* | LA, **NM**, PA |
| ***Aix sponsa*** | 4 | 1 | 0/1 | *T. physellae* | LA, **NM**, PA |
| ***Bucephala albeola*** | 8 | 1 | 0 | *T. physellae* | **NM**, NV, **PA** |
| *Bucephala clangula* | 3 | 0 | 0 | - | CA, NM |
| ***Clangula hyemalis*** | 9 | 1 | 0 | *T. physellae* | **AK** |
| *Histrionicus histrionicus* | 1 | 0 | 0 | - | AK |
| ***Lophodytes cucullatus*** | 5 | 1 | 0 | *Trichobilharzia* sp. C | LA, **PA** |
| ***Mergus merganser*** | 6 | 3 | 0/1 | *T. stagnicolae* + *T. physellae* | **MI** |
| *Mergus serrator* | 3 | 0 | 0 | - | CA, NM |
| *Oxyura jamaicensis* | 6 | 0 | 0/2 | - | CA, NM, NV |
| *Melanitta fusca* | 5 | 0 | 0 | - | AK, MB |
| *Melanitta perspicillata* | 3 | 0 | 0 | - | AK |
| *Somateria mollissima* | 1 | 0 | 0/1 | - | MB |
| *Cygnus columbianus* | 13 | 0 | 0/5 | * | NV, NM |
| ***Chen caerulescens*** | 9 | 2 | 0/7 | *Trichobilharzia brantae* | LA, **NM**, **MB** |
| ***Branta canadensis*** | 7 | 3 | 0/5 | *T. brantae* | **NM**, NV, **MB** |
| **Snail Hosts** | | | | | |
| **Physidae** | | | | | |
| ***Physa gyrina*** | | | | *T. physellae* | CA, MT, MN, **NE**, **NM**, NV, MB |
| ***Physa acuta*** | | | | *T. querquedulae* | experimental in NM |
| ***Physa parkeri*** | | | | *T. physellae* | **MI**, MN |
| *Aplexa sp.* | | | | - | MB |
| **Lymnaeidae** | | | | | |
| ***Stagnicola emarginata*** | | | | *T. stagnicolae* | **MI, MN** |
| ***Stagnicola elrodi*** | | | | *T. szidati* | **MT**, MN, MB |
| *Stagnicola elodes* | | | | - | MN, NM |
| ***Stagnicola sp.*** | | | | *Trichobilharzia* sp. D | **MB** |
| ***Stagnicola sp.*** | | | | *Trichobilharzia* sp. E | **MB** |
| *Radix auricularia* | | | | - | NM |



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| North America Avian Host | Number examined | Viscera positive | Nasals examined | Species of *Trichobilharzia* | Locality |
|---|---|---|---|---|---|
| *Fossaria* sp. | | | | - | NM |
| ***Lymnaea stagnalis*** | | | | *T. szidati* | AK, **MI**, MT, MN, MB |
| *Bulimnaea megasoma* | | | | - | MN |
| **Planorbidae** | | | | | |
| ***Gyraulus parvus*** | | | | *T. brantae* | **CO**, MT, MN, **MB** |
| *Planorbula armigera* | | | | - | MN |
| *Promenetus exacuous* | | | | - | MN |
| *Pecosorbis kansasensis* | | | | - | NM |
| *Helisoma* trivolvus | | | | - | LA, NM, MI, MN |
| *Helisoma anceps* | | | | - | MI, MN |

*This host had *Allobilharzia visceralis* reported in Brant, 2007. The '+' indicates co-infection in all three hosts examined.

**Table III**

The host and locality origin of the specimens used in this study. Numbered localities were collected for this study and relate to Fig. 1.

| | | | | GenBank Accession numbers | | | | |
|---|---|---|---|---|---|---|---|---|
| **Schistosome Taxa** | **Host** | **Life cycle stage** | **Locality** | **18S** | **28S** | **ITS** | **CO1** | **Reference** |
| ***Trichobilharzia brantae*** | | | | | | | | |
| W340 sngo MB | *Chen caerulescens* | A* | 1 Canada | FJ174451 | FJ174467 | FJ174533 | FJ174482 | This paper |
| W346 *Gyraulus* MB | *Gyraulus parvus* | C | 1 Canada | | | FJ174534 | | This paper |
| W330 *Gyraulus* CO | *Gyraulus parvus* | C | 2 Colorado | FJ174450 | FJ174466 | FJ174532 | FJ174484 | This paper |
| W331 *Gyraulus* CO | *Gyraulus parvus* | C | 2 Colorado | | | FJ174531 | | This paper |
| | | | | | | | | This paper |
| ***Trichobilharzia franki*** | *Radix* sp. | C | Germany | FJ711767 | FJ711768 | | FJ174530 | This paper |
| *T. franki* Cz | *Radix auricularia* | C | Czech Republic | | | AF356845 | | Dvořák et al., 2002 |
| *T. franki* Ra1 | *Radix auricularia* | C | Czech Republic | | | AY713969 | | Rudolfová et al., 2005 |
| *T. franki* Ra2 | *Radix auricularia* | C | Poland | | | AY713964 | | Rudolfová et al., 2005 |
| *T. franki* Ra3 | *Radix auricularia* | C | Poland | | | AY713966 | | Rudolfová et al., 2005 |
| *T. franki* RSFO1 | *Radix auricularia* | C | France | | | AY795572 | | Ferte et al., 2005 |
| *T. franki* Ls1 | *Lymnaea stagnalis* | C | Czech Republic | | | AY713973 | | Rudolfová et al., 2007 |
| *T. franki* auri1 | *Radix auricularia* | C | Switzerland | | | AJ312041 | | Picard & Jousson, 2001 |
| *T. franki* auri2 | *Radix auricularia* | C | Switzerland | | | AJ312042 | | Picard & Jousson, 2001 |
| *T. franki* ov1 | *Radix ovata* | C | Switzerland | | | AJ312043 | | Picard & Jousson, 2001 |
| *T. franki* ov2 | *Radix ovata* | C | Switzerland | | | AJ312044 | | Picard & Jousson, 2001 |
| *T. franki* ov3 | *Radix ovata* | C | Switzerland | | | AJ312045 | | Picard & Jousson, 2001 |
| *T. franki* ov4 | *Radix ovata* | C | Switzerland | | | AJ312046 | | Picard & Jousson, 2001 |
| ***Trichobilharzia physellae*** | | | | | | | | |
| W146 *Physa* NM | *Physa gyrina* | C | 3 New Mexico | | | FJ174568 | FJ174513 | This paper |
| W263*Physa*NM | *Physa gyrina* | C | 4 New Mexico | | | FJ174562 | FJ174523 | This paper |
| W171 lesc PA | *Aythya affinis* | A | 5 Pennsylvania | | | FJ174564 | FJ174515 | This paper |
| W193 lesc NM | *Aythya affinis* | A | 6 New Mexico | FJ174457 | FJ174473 | | FJ174518 | This paper |
| W212 lesc AK | *Aythya affinis* | A | 7 Alaska | | | FJ174563 | FJ174512 | This paper |
| W256 lesc NM | *Aythya affinis* | A | 6 New Mexico | | | FJ174575 | FJ174522 | This paper |
| W194 ridu NM | *Aythya collaris* | A | 6 New Mexico | | | FJ174566 | FJ174517 | This paper |

| Schistosome Taxa | Host | Life cycle stage | Locality | GenBank Accession numbers 18S | 28S | ITS | CO1 | Reference |
|---|---|---|---|---|---|---|---|---|
| W249 caba NV | *Aythya valisineria* | A | 8 Nevada | | | FJ174565 | | This paper |
| W255 buhe NM | *Bucephala albeola* | A | 6 New Mexico | FJ174458 | FJ174474 | FJ174561 | FJ174514 | This paper |
| W211 olsq AK | *Clangula hyemalis* | A | 7 Alaska | | | | FJ174516 | This paper |
| W230.1 come MI | *Mergus merganser* | M | 9 Michigan | | | FJ174567 | FJ174521 | This paper |
| W234 come MI | *Mergus merganser* | M | 9 Michigan | | | FJ174569 | FJ174519 | This paper |
| W236 *Physa* MI | *Physa parkeri* | C | 9 Michigan | FJ174459 | FJ174475 | | FJ174520 | This paper |
| ***Trichobilharzia querquedulae*** | | | | | | | | |
| E45 blte FL | *Anas discors* | A | 10 Florida | FJ174453 | FJ174469 | FJ174555 | FJ174510 | This paper |
| E64 blte FL | *Anas discors* | A | 10 Florida | | | | FJ174511 | This paper |
| W137 blte LA | *Anas discors* | A | 11 Louisana | FJ174452 | FJ174468 | FJ174558 | FJ174498 | This paper |
| W156 blte NM | *Anas discors* | A | 6 New Mexico | | | FJ174554 | FJ174502 | This paper |
| W190 blte CA | *Anas discors* | A | 12 California | | | FJ174550 | FJ174507 | This paper |
| W148.1 cite NM‡ | *Anas cyanoptera* | A | 13 New Mexico | | | FJ174559 | FJ174499 | This paper |
| W148.2 cite NM | *Anas cyanoptera* | A | 13 New Mexico | | | | FJ174500 | This paper |
| W155.3 cite NM | *Anas cyanoptera* | A | 13 New Mexico | | | FJ174553 | FJ174501 | This paper |
| W180 cite CA | *Anas cyanoptera* | A | 12 California | FJ174454 | FJ174470 | FJ174556 | FJ174505 | This paper |
| W135 nosh LA | *Anas clypeata* | A | 11 Louisana | | | FJ174557 | FJ174497 | This paper |
| W203 nosh AK | *Anas clypeata* | A | 14 Alaska | | | FJ174552 | FJ174508 | This paper |
| W158 nosh NM | *Anas clypeata* | A | 6 New Mexico | | | FJ174549 | FJ174503 | This paper |
| W162 nosh NM | *Anas clypeata* | A | 6 New Mexico | | | FJ174551 | FJ174504 | This paper |
| W183 nosh CA | *Anas clypeata* | A | 12 California | | | FJ174560 | FJ174506 | This paper |
| SDS1006 nosh NE | *Anas clypeata* | A | 15 Nebraska | | | FJ174548 | | This paper |
| W345 nosh MB | *Anas clypeata* | A | 1 Canada | | | FJ174547 | FJ174509 | This paper |
| ***Trichobilharzia regenti*** | *Radix peregra* | C | Czech Republic | AY157219 | AY157245 | | AY157190 | Lockyer et al., 2003 |
| *T. regenti* Pl14 | *Anas clypeata* | M | Poland | | | EF094533 | | Rudolfová et al., 2006 |
| *T. regenti* Pl17 | *Aythya fuligula* | M | Poland | | | EF094534 | | Rudolfová et al., 2006 |
| *T. regenti* Pl20 | *Anas platyrhynchus* | M | Poland | | | EF094535 | | Rudolfová et al., 2006 |
| *T. regenti* Pl27 | *Anas platyrhynchus* | M | Poland | | | EF094537 | | Rudolfová et al., 2006 |
| *T. regenti* Cz31 | *Anas platyrhynchus* | M | Poland | | | EF094538 | | Rudolfová et al., 2006 |
| *T. regenti* Cz79 | *Anas clypeata* | M | Czech Republic | | | EF094540 | | Rudolfová et al., 2006 |

| | | | | GenBank Accession numbers | | | | |
|---|---|---|---|---|---|---|---|---|
| **Schistosome Taxa** | **Host** | **Life cycle stage** | **Locality** | **18S** | **28S** | **ITS** | **CO1** | **Reference** |
| *T. regenti* ad1 | *Anas platyrhynchus* | A | Switzerland | | | AJ312049 | | Picard & Jousson, 2001 |
| *T. regenti* ov1 | *Radix ovata* | C | Switzerland | | | AJ312047 | | Picard & Jousson, 2001 |
| *T. regenti* ov2 | *Radix ovata* | C | Switzerland | | | AJ312048 | | Picard & Jousson, 2001 |
| ***Trichobilharzia stagnicolae*** | | | | | | | | |
| W240 come MI | *Mergus merganser* | M | 9 Michigan | FJ174462 | FJ174478 | FJ174544 | FJ174490 | This paper |
| W230.2 come MI | *Mergus merganser* | M | 9 Michigan | | | FJ174545 | FJ174493 | This paper |
| *Stagnicola* MT | *Stagnicola* sp. | C | 16 Montana | | | FJ174541 | FJ174488 | This paper |
| DouglasLake *Stagnicola* MI | *Stagnicola emarginata* | C | 9 Michigan | FJ174463 | FJ174479 | FJ174546 | FJ174489 | This paper |
| W164 *Stagnicola* NM | *Stagnicola* sp. | C | 17 New Mexico | FJ174461 | FJ174477 | FJ174540 | FJ174492 | This paper |
| W224 *Stagnicola* MI | *Stagnicola emarginata* | C | 9 Michigan | | | FJ174542 | FJ174494 | This paper |
| W229 *Stagnicola* MI | *Stagnicola emarginata* | C | 9 Michigan | | | FJ174543 | FJ174491 | This paper |
| W400 *Stagnicola* MN | *Stagnicola emarginata* | C | 18 Minnesota | | | | | |
| ***Trichobilharzia szidati*** | *Lymnaea stagnalis* | C | Czech Republic | | | AF263828 | AY157191 | Dvořák et al., 2002 |
| | | | | AY157219 | AY157245 | | | Lockyer et al., 2003 |
| Blind Sucker *Lymnaea* MI | *Lymnaea stagnalis* | C | 19 Michigan | FJ174460 | FJ174476 | FJ174538 | FJ174496 | This paper |
| Flathead *Stagnicola* MT | *Stagnicola elrodi* | C | 16 Montana | | | FJ174539 | FJ174495 | This paper |
| *T. szidati* Ls5 | *Lymnaea stagnalis* | C | Poland | | | AY713967 | | Rudolfová et al., 2006 |
| *T. szidati* ToA | *Lymnaea stagnalis* | C | Netherlands | | | AY713970 | | Rudolfová et al., 2005 |
| *T. szidati* ToE | *Lymnaea stagnalis* | C | Germany | | | AY713971 | | Rudolfová et al., 2005 |
| *T. szidati* Tsz | *Lymnaea stagnalis* | C | Czech Republic | | | AY713972 | | Rudolfová et al., 2005 |
| *T. szidati* Pl21 | *Anas platyrhynchos* | M | Poland | | | EF094536 | | Rudolfová et al., 2006 |
| *T. szidati* Cz11 | *Anas platyrhynchos* | A | Czech Republic | | | EF094541 | | Rudolfová et al., 2006 |
| **Unspecified species of *Trichobilharzia*** | | | | | | | | |
| *Trichobilharzia* sp. 3 Pl10 | *Anas penelope* | M | Poland | | | EF094531 | | Aldhoun et al., 2009 |
| *Trichobilharzia* sp. 3 Pl7 | *Anas penelope* | M | Poland | | | EF094532 | | Aldhoun et al., 2009 |
| *Trichobilharzia* sp. EAN17 | *Radix peregra* | C | France | | | EU413971 | | Jouet et al., 2008 |
| *Trichobilharzia* sp. EAN35 | *Radix peregra* | C | France | | | EU413974 | | Jouet et al., 2008 |
| ***Trichobilharzia* sp. A** | | | | | | | | |
| W149 amwi NM | *Anas americana* | A | 13 New Mexico | FJ174456 | FJ174472 | FJ174574 | FJ174524 | This paper |
| W182 amwi CA | *Anas americana* | A | 12 California | | | FJ174573 | FJ174525 | This paper |



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| Schistosome Taxa | Host | Life cycle stage | Locality | GenBank Accession numbers 18S | 28S | ITS | CO1 | Reference |
|---|---|---|---|---|---|---|---|---|
| W192 amwi NM | *Anas americana* | A | 20 New Mexico | FJ174455 | FJ174471 | FJ174572 | FJ174526 | This paper |
| W213 amwi AK | *Anas americana* | A | 7 Alaska | | | FJ174570 | FJ174527 | This paper |
| ***Trichobilharzia* sp. B** | | | | | | | | |
| W205 amwi AK | *Anas americana* | A | 14 Alaska | | | FJ174571 | FJ174528 | This paper |
| ***Trichobilharzia* sp. C** | | | | | | | | |
| W173 home PA | *Lophodytes cucullatus* | A | 21 Pennsylvania | | | FJ174576 | FJ174529 | This paper |
| ***Trichobilharzia* sp. D** | | | | | | | | |
| W376 *Stagnicola* MB | *Stagnicola* sp. | C | 1 Canada | FJ174465 | FJ174481 | FJ174537 | FJ174485 | This paper |
| ***Trichobilharzia* sp. E** | | | | | | | | |
| W332 *Stagnicola* MB | *Stagnicola* sp. | C | 1 Canada | FJ174464 | FJ174480 | | FJ174483 | This paper |
| W336 *Stagnicola* MB | *Stagnicola* sp. | C | 1 Canada | | | FJ174535 | FJ174486 | This paper |
| W344 pita MB | *Anas acuta* | A | 1 Canada | | | FJ174536 | FJ174487 | This paper |
| **Other schistosomatids** | | | | | | | | |
| *Ornithobilharzia canaliculata* | *Larus delawarensis* | A | U.S.A. | AY157222 | AY157248 | | | Lockyer et al., 2003 |
| *Austrobilharzia variglandis* | *Larus delawarensis* | A | U.S.A. | AY157224 | AY157250 | | AY157196 | Lockyer et al., 2003 |
| *Austrobilharzia terrigalensis* | *Batillaria australis* | C | Australia | AY157223 | AY157249 | | AY157195 | Lockyer et al., 2003 |
| *Macrobilharzia macrobilharzia* | *Anhinga anhinga* | A | U.S.A. | AY829260 | AY858885 | | | Brant et al., 2006 |
| *Bivitellobilharzia nairi* | *Elephas maximus* | A | Sri Lanka | AY829261 | AY858888 | | AY829249 | Brant et al., 2006 |
| *Schistosoma japonicum* | †*Mus musculus* | A | Tanzania | AY157226 | AY157607 | | | Lockyer et al., 2003 |
| *Orientobilharzia turkestanicum* | *Ovis aries* | A | Iran | AF442499 | AY157254 | | | Lockyer et al., 2003 |
| *Schistosoma hippopotami* | *Bulinus truncatus* | C | Uganda | | | AY197343 | | Morgan et al., 2003 |
| *Schistosoma incognitum* | *Bandicota indica* | A | Thailand | AY157229 | AY157255 | | | Lockyer et al., 2003 |
| *Schistosome spindale* | †*Mus musculus* | A | Sri Lanka | Z11979 | | | | Johnston et al., 1993 |
| | | | | | AY157257 | | | Lockyer et al., 2003 |
| *Schistosoma margrebowiei* | †*Mus musculus* | A | Zambia | AY157233 | AY157260 | | | Lockyer et al., 2003 |
| *Schistosoma leiperi* | †*Mesocricetus auratus* | A | South Africa | AY157234 | AY157261 | | | Lockyer et al., 2003 |
| *Schistosoma haematobium* | †*Mesocricetus auratus* | A | Mali | Z11976 | AY157263 | | | Lockyer et al., 2003 |
| *Schistosoma intercalatum* | †*Mus musculus* | A | Sao Tome | AY157235 | AY157262 | | | Lockyer et al., 2003 |
| *Schistosomatium douthitii* | †*Mesocricetus auratus* | A | U.S.A. | AY157221 | AY157247 | | | Lockyer et al., 2003 |

| | | | | GenBank Accession numbers | | | | |
|---|---|---|---|---|---|---|---|---|
| **Schistosome Taxa** | **Host** | **Life cycle stage** | **Locality** | **18S** | **28S** | **ITS** | **CO1** | **Reference** |
| *Heterobilharzia americana* | †*Mesocricetus auratus* | A | U.S.A. | AY157220 | AY157246 | | | Lockyer et al., 2003 |
| W1285 *Biomphalaria KE* | *Biomphalaria sudanica* | C | Kenya | AY829258 | AY858886 | | | Brant et al., 2006 |
| *Bilharziella polonica* | *Anas platyrhynchus* | A | Ukraine, Czech Republic | AY157214 | AY157240 | EF094539 | AY157186 | Lockyer et al., 2003<br>Rudolfová et al., 2006 |
| W2081 *Ceratophallus KE* | *Ceratophallus sp.* | C | Kenya | AY829259 | AY858887 | | | Brant et al., 2006 |
| *Dendritobilharzia pulverulenta* | *Gallus, Mergus* | A | U.S.A. | AY157215 | AY157241 | | AY157187 | Lockyer et al., 2003 |
| | | | | | | EF071988 | | Brant, 2007 |
| *Gigantobilharzia huronensis* | *Agelaius phoeniceus* | A | U.S.A. | AY157216 | AY157242 | | AY157188 | Lockyer et al., 2003 |
| *Gigantobilharzia huronensis* | *Agelaius phoeniceus* | A | U.S.A. | | | EF071987 | | Brant, 2007 |
| *Allobilharzia visceralis* | *Cygnus cygnus* | A | Iceland | | | DQ067561 | | Kolářová et al., 2006 |
| *Allobilharzia visceralis* | *Cygnus columbianus* | A | U.S.A. | EF114220 | EF114222 | EF071989 | EF114219 | Brant, 2007 |
| *Allobilharzia visceralis* | *Cygnus columbianus* | A | U.S.A. | EF114221 | EF114223 | EF071991 | EF114224 | Brant, 2007 |
| **Outgroups** | | | | | | | | |
| *Cardiocephaloides longicollis* | *Larus ridibundus* | | Ukraine | AY222089 | AY222171 | | | Olson et al., 2003 |
| *Alaria alata* | *Nyctereutes procyonoides* | | Ukraine | AY222091 | AF184263 | | | Olson et al., 2003 |
| *Brachylaima thompsoni* | *Blarina brevicauda* | | U.S.A. | AY222085 | | | | Olson et al., 2003 |
| | | | | | AF184262 | | | Tkach et al., 2001 |
| *Urogonimus macrostomus* | *Anas platyrhynchus* | | Ukraine | AY222086 | AY222168 | | | Olson et al., 2003 |
| *Leucochloridium perturbatum* | *Turdus merula* | | Czech Republic | AY222087 | AY222169 | | | Olson et al., 2003 |
| *Clinostomum sp. USA* | *Rana catesbeiana* | | U.S.A. | AY222095 | AY222095 | | | Olson et al., 2003 |
| *Aporocotyle spinosicanalis* | *Merluccius merluccius* | | United Kingdom | AJ287477 | | | | Cribb et al., 2001 |
| | | | | | AY222177 | | | Olson et al., 2003 |
| *Plethorchis acanthus* | *Mugil cephalus* | | Australia | AY222096 | AY222178 | | | Olson et al., 2003 |
| *Unicaecum sp.* | *Trachemys scripta* | | U.S.A. | AY604719 | AY604711 | | | Snyder, 2004 |
| *Vasotrema robustum* | *Apalone spinifera* | | U.S.A. | AY829257 | AY858883 | | | Brant et al., 2006 |
| *Spirorchis scripta* | *Chrysemys picta marginata* | | U.S.A. | AY829256 | AY858882 | | | Brant et al., 2006 |
| *Hapalorhynchus gracilis* | *Chelydra serpentina* | | U.S.A. | AY604718 | AY604710 | | | Snyder, 2004 |
| *Griphobilharzia amoena* | *Crocodylus johnstoni* | | Australia | AY899915 | AY899914 | | | Brant et al., 2006 |
| *Carettacola hawaiiensis* | *Chelonia mydas* | | U.S.A. | AY604717 | AY604709 | | | Snyder, 2004 |
| *Learedius learedi* | *Chelonia mydas* | | U.S.A. | AY604715 | AY604707 | | | Snyder, 2004 |
| *Hapalotrema mehrai* | *Chelonia mydas* | | U.S.A. | AY604716 | AY604708 | | | Snyder, 2004 |

[*] A=adults M=miracidia C=cercariae;

[†] experimental;

[‡] W148.1 and W148.2 are worms from the same host individual

**Table IV**

Morphological comparisons useful in diagnosing both adults and cercariae. Measurements in micrometers unless otherwise indicated.

| | T. querquedulae | T. querquedulae | T. physellae | T. physellae | T. franki |
|---|---|---|---|---|---|
| **Reference** | this paper (average) | McLeod, 1937 | this paper (average) | McMullen & Beaver 1945 | Müller & Kimmig 1994 |
| **Adults** | n=3 | | n=3 | | |
| length of males | 4.8 mm | 3.7 mm | 2.6 mm | 1.3–7.5 mm | 3.2–4.0 mm |
| VS - OS* | 417 | 274–375 | 320 | 160–340 | 485–530 |
| VS - GC | 400 | NA† | 440 | NA | 495–550(522) |
| length SV | 325 | NA | 400 | NA | |
| length GC | 225 | 375 | 186 | 100–190 | 212–291(246) |
| cecal reunion | not seen | between SV & GC | not seen | between VS & SV | between SV & GC |
| testes size | 18–23 | NA | 25–30 | 28–32 | 95–106 |
| number of testes | >200 | 210–240 | >100 | 96–160 | 41–64 |
| egg shape | spindle with spine | spindle with spine | spindle with spine | spindle with spine | spindle with spine |
| eggs in utero | - | 140 × 30 | - | 170 × 65 | 206 × 69 |
| eggs in feces | 150 × 35 | - | 180 × 70 | - | - |
| **Cercariae** | | | | | |
| snail host | *Physa gyrina* exp. | *P. gyrina* | *P. gyrina* | *P. parkeri, P. gyrina* | *Radix auricularia* |
| | n=5 | | n=5 | | |
| length body | 327 | - | 270 | 265 | 307 |
| length tail | 410 | - | 352 | 374 | 419 |
| length furcae | 221–224 | - | 188 | 196 | 234 |
| ratio: body:furcae | 0.68 | | 0.7 | 0.74 | 0.76 |
| ratio: body:tail | 1.25 | - | 1.3 | 1.41 | 1.36 |

*VS=ventral sucker, OS=oral sucker, GC=gynaecophoric canal, SV=seminal vesicle, exp=experimental infection.

†NA=not available.

## Table V

Morphological comparisons of the key differentiating features among closely related genera of avian schistosomes.

| | Trichobliharzia brantae | Allobilharzia | Trichobilharzia | Jilinobilharzia |
|---|---|---|---|---|
| References | Farr & Blankenmeyer, 1956<br>This study | Kolářová et al., 2006<br>Brant, 2007 | Skrjabin & Zakharov, 1920<br>Blair & Islam, 1983 | Lui & Bai, 1976 |
| Total length males | 33.5 mm | 65 mm | 2.2–12 mm | 3.6–4.6 mm |
| Cecal reunion | at or anterior to the seminal vesicle | posterior to gynaecophoric canal | variable but within range of the seminal vesicle | middle gynaecophoric canal, posterior to seminal vesicle |
| Position of the seminal vesicle | between ventral sucker and gynaecophoric canal | between ventral sucker and gynaecophoric canal | between ventral sucker and gynaecophoric canal | in gynaecophoric canal |
| Start of the gynaecophoric canal | posterior to seminal vesicle | posterior to seminal vesicle | posterior to seminal vesicle | posterior to ventral sucker |
| End of the gynaecophoric canal | well before start of testes | well before start of testes | at start of testes | at start of testes |
| Testes | 585 | >400 | 57–240 | 83–132 |
| Average egg shape | ovoid with spine | long nonsymmetrical with spine | spindle with spine | spindle with spine |
| Cercaria flame cells | 5+1 | unknown | 6+1 | unknown |

**Table VI**

Results of experimental infections of birds and snails with species of *Trichobilharzia*.
Hosts from which worms were used for exposures are on the left column; hosts exposed are along the top of the table.

| Schistosome Taxa | *Stagniola emarginata* | *Stagnicola elodes* | *Physa parkeri* | *Physa gyrina* | *Physa acuta* | *Gyraulus parvus* | *Peking duck* | *Domestic mallard* |
|---|---|---|---|---|---|---|---|---|
| ***Trichobilharzia stagnicolae*** | | | | | | | | |
| ex. *Mergus merganser* | 9/10 | - | 0/10 | - | - | - | - | - |
| ex. *Stagnicola emarginata* | - | - | - | - | - | - | 0/6 | 0/6 |
| ***Trichobilharzia physellae*** | | | | | | | | |
| ex. *Aythya affinis* | - | 0/15 | - | 10/15 | - | - | - | - |
| ex. *Bucephalus albeola* | - | 0/15 | - | 8/15 | - | - | - | - |
| ex. *Mergus merganser* | 0/10 | - | 8/10 | | | | | |
| ***Trichobilharzia querquedulae*** | | | | | | | | |
| ex. *Anas cyanoptera* | - | 0/15 | - | - | 12/15 | 0/5 | - | - |
| ex. *Anas discors* | - | 0/15 | - | - | 11/15 | 0/5 | - | - |

## Table VII

Genetic differences comparing ITS1, CO1 and ITS1-5.8S-ITS2 among schistosomes.

| Taxa | ITS1* | *cox1** | ITS1-5.8S-ITS2 |
|---|---|---|---|
| **Within *Schistosoma*** | | | |
| *S. japonicum* - *S. malayensis* | | 16.40% | |
| *S. japonicum* - *S. mekongi* | | 15.80% | |
| *S. malayensis* - *S. mekongi* | 4.60% | 9.4% | |
| *S. mansoni* - *S. rodhaini* | 1.10% | 13.30% | - |
| *S. haematobium* - *S. mattheei* | 6.60% | 16.20% | 1.40% |
| *S. haematobium* - *S. intercalatum* | 0 | 11.60% | 0.50% |
| *S. hippopotami* - *S. edwardiense* | 4.80% | 21.40% | |
| **Avian genera** | | | |
| *Allobilharzia* - *T. brantae* | - | 14.30% | 2.70% |
| *Trichobilharzia* - *T. brantae* | - | 14.50% | 6.40% |
| *Allobilharzia* - *Trichobilharzia* | - | 14.80% | 5.50% |
| **Within *Trichobilharzia*** | | | |
| *T. stagnicolae* - *T. physellae* | - | 13.40% | 2.00% |
| *T. stagnicolae* - *T. regenti* | - | 12.7% | 1.90% |
| *T. stagnicolae* - *T. querquedulae* | - | 12.00% | 2.00% |
| *T. stagnicolae* - *T. szidati* | - | 11.20% | 1.80% |
| *T. stagnicolae* - *Trichobilharzia* sp. A | - | 11.40% | 2.00% |
| *T. stagnicolae* - *Trichobilharzia* sp. B | - | 12.50% | 1.80% |
| *T. stagnicolae* - *Trichobilharzia* sp. C | - | 12.50% | 1.80% |
| *T. stagnicolae* - *Trichobilharzia* sp. D | - | 10.70% | |
| *T. stagnicolae* - *Trichobilharzia* sp. E | - | 11.60% | 1.70% |
| *T. szidati* -*T. physellae* | - | 11.70% | 3.00% |
| *T. szidati* -*T. regenti* | - | 11.50% | 2.50% |
| *T. szidati* - *T. querquedulae* | - | 10.70% | 2.00% |
| *T. szidati* - Blindsucker *Lymnaea* MT | | 4.70% | 0.36% |
| *T. szidati* - Flathead *Stagnicola* MI | | 0.48% | 0.40% |
| *T. szidati* - *Trichobilharzia* sp. A | - | 11.00% | 2.80% |
| *T. szidati* - *Trichobilharzia* sp. B | - | 10.60% | 2.70% |
| *T. szidati* - *Trichobilharzia* sp. C | - | 11.50% | 2.70% |
| *T. szidati* - *Trichobilharzia* sp. D | - | 9.80% | |
| *T. szidati* - *Trichobilharzia* sp. E | - | 10.30% | 2.60% |
| *T. regenti* -*T. querquedulae* | - | 11.50% | 1.80% |
| *T. regenti* -*T. physellae* | - | 10.60% | 1.70% |
| *T. regenti* - *Trichobilharzia* sp. A | - | 10.50% | 1.50% |
| *T. regenti* - *Trichobilharzia* sp. B | - | 9.10% | 1.40% |
| *T. regenti* - *Trichobilharzia* sp. C | - | 11.10% | 1.20% |
| *T. regenti* - *Trichobilharzia* sp. D | - | 10.8% | |
| *T. regenti* - *Trichobilharzia* sp. E | - | 12.00% | 2.30% |

| Taxa | ITS1* | *cox*1* | ITS1-5.8S-ITS2 |
|---|---|---|---|
| *Trichobilharzia* sp. D -*Trichobilharzia* sp. A | - | 11.10% | |
| *Trichobilharzia* sp. D - *Trichobilharzia* sp. C | - | 12.10% | |
| *Trichobilharzia* sp. D - *Trichobilharzia* sp. E | - | 11.10% | |
| *Trichobilharzia* sp. D - *T. querquedulae* | - | 11.30% | |
| *Trichobilharzia* sp. D - *T. physellae* | - | 12.30% | |
| *Trichobilharzia* sp. E - *Trichobilharzia* sp. A | - | 13.10% | 3.20% |
| *Trichobilharzia* sp. E - *Trichobilharzia* sp. B | - | 12.30% | 2.70% |
| *Trichobilharzia* sp. E - *T. querquedulae* | - | 12.10% | 3.70% |
| *Trichobilharzia* sp. E - *T. physellae* | - | 12.80% | 3.50% |
| Within *T. stagnicolae* | - | 0.70% | 0.17% |
| Within *T. szidati* | - | 3.30% | 0.35% |
| Within *T. regenti* | - | - | 0.00% |
| Within *T. physelllae* | 0.23% | 0.80% | 0.22% |
| Within *T. querquedulae* | 0.18% | 0.82% | 0.40% |
| Within *T. franki* from *R. auricularia* | 0.70% | - | 0.20% |
| Within *T. franki* from *R. ovata* | 0.52–2.8% | - | 0.50% |
| Within *Trichobilharzia* sp. A | 0.41% | | 0.10% |
| Within *Trichobilharzia* sp. E | - | 0.50% | 0.60% |
| **Clade Q** | | | |
| *T. querquedulae* - *T. physellae* | 3.00% | 8.60% | 0.88% |
| *T. querquedulae* - *Trichobilharzia* sp. A | 3.10% | 9.00% | 0.32% |
| *T. querquedulae* - *Trichobilharzia* sp. B | 3.20% | 8.10% | 0.70% |
| *T. querquedulae* - *Trichobilharzia* sp. C | 3.80% | 8.50% | 0.50% |
| *T. querquedulae* - *T. franki* from *R. auricularia* | 3.1–3.4% | 8.10% | 0.64% |
| *T. querquedulae* - *T. franki* from *R. ovata* | 3.4–5.3% | - | 1.00% |
| *T. physellae* - *Trichobilharzia* sp. A | 0.60% | 9.30% | 0.76% |
| *T. physellae* - *Trichobilharzia* sp. B | 0.95% | 8.30% | 0.50% |
| *T. physellae* - *Trichobilharzia* sp. C | 3.00% | 9.40% | 0.50% |
| *T. physellae* - *T. franki* from *R. auricularia* | 0.82–1.3% | 9.10% | 0.60% |
| *T. physellae* - *T. franki* from *R. ovata* | 2.5–3.6% | - | 0.87% |
| *Trichobilharzia* sp. A - *Trichobilharzia* sp. B | 0.70% | 6.80% | 0.40% |
| *Trichobilharzia* sp. A - *Trichobilharzia* sp. C | 1.70% | 8.80% | 0.30% |
| *Trichobilharzia* sp. B - *Trichobilharzia* sp. C | 1.80% | 8.80% | 0.13% |
| *Trichobilharzia* sp. A - *T. franki* from *R. auricularia* | 0.60% | 8.90% | 0.40% |
| *Trichobilharzia* sp. A - *T. franki* from *R. ovata* | 3.10% | - | 0.70% |
| *Trichobilharzia* sp. B - *T. franki* from *R. auricularia* | 0.12-0-36% | 8.30% | 0.20% |
| *Trichobilharzia* sp. B - *T. franki* from *R. ovata* | 4.00% | - | 0.50% |
| *Trichobilharzia* sp. C -*T. franki* from *R. auricularia* | 2.30% | 8.60% | 0.12% |
| *Trichobilharzia* sp. C - *T. franki* from *R. ovata* | 4.60% | - | 0.40% |

*Values for ITS1 and *cox*1 in *Schistosoma* are taken from Vilas et al., 2005.