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Avian Blood Flukes (Digenea: Schistosomatidae) In North Dakota

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AVIAN BLOOD FLUKES (DIGENEA: SCHISTOSOMATIDAE) IN NORTH DAKOTA

by

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This thesis, submitted by Susana Rios in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Susana Rios
December 1, 2014

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ABSTRACT

Avian blood flukes are digeneans belonging to the family Schistosomatidae and inhabit the blood circulatory system of birds. Their life cycle includes an intermediate snail host and a definitive bird host. They are found in representatives of several bird orders, but are most prevalent in waterfowl. North Dakota has a large number of wetlands, which provide important breeding sites for many aquatic birds and serve as stopover sites for migratory birds. The abundance and diversity of bird species that congregate in these wetlands ensures optimal conditions for parasite transmission within and among avian species. However, no study on avian blood flukes has ever been conducted in the state and little to no information is available from the surrounding states/provinces as well. The goals of this study were to investigate the diversity, host associations and distribution of avian schistosomatids in North Dakota. Phylogenetic analysis was also conducted to determine the systematic positioning of blood flukes collected in North Dakota within the Schistosomatidae.

Both the intermediate (snail) and definitive (bird) hosts were surveyed in the state. The larval (cercariae) and adult stages of the parasites were collected from their respective hosts and preserved for morphological and molecular study. Morphological study included measurements of cercariae on temporary mounts, preparing permanent total mounts of adult worms and scanning electron microscopy study of cercariae and adults. Sequences of the partial nuclear ribosomal 28s gene and partial mitochondrial cox1 gene were used in this study for species differentiation and phylogenetic analyses. Sequences obtained in this work were compared with the previously published sequences available in the GenBank database. Intraspecific and

interspecific sequence nucleotide variation was also calculated for all avian schistosomatid species collected in North Dakota. Sequences from North Dakota specimens as well as sequences from all other Schistosomatidae genera available from GenBank were included in a phylogenetic analysis of the Schistosomatidae.

This study focused on the collection of the intermediate snail host to determine the distribution of avian schistosomatid species that may complete their life cycle in the state. Snails were collected from 105 sites throughout the state between May-September of 2013 and screened for the larval stages of parasites. A total of 17,653 snails were collected from six snail genera with an overall infection prevalence of 0.8%. Seven avian schistosomatid species were collected from 4 snail genera. Numerous bird species were also examined and 11 species of avian blood flukes were collected from 22 species of birds. A total of 13 avian schistosomatid species were collected from North Dakota with five species collected from both their intermediate and definitive host. Molecular and morphological analysis also provided evidence for the existence of two *Dendritobilharzia* species in North America.

These results demonstrated high diversity of avian schistosomatids in North Dakota. *Trichobilharzia* was the most speciose genus containing 7 of the 13 avian blood fluke species collected in the state. The majority of infected snails belonged to the genera *Stagnicola* and *Lymnaea*, which were the most heavily sampled snail genera in this study. Further sampling of other snail genera including members of the families Physidae and Planorbidae may reveal the presence of additional avian schistosomatid species circulating in North Dakota. Further sampling of avian hosts, particularly passerines, in North Dakota may also reveal additional species of blood flukes and additional hosts of a widely distributed species *Gigantobilharzia huronensis*.

CHAPTER I

INTRODUCTION

Parasites comprise a large portion of all known species (~40%) and it is estimated that there could be 75,000 to 300,000 species parasitizing vertebrates worldwide (Dobson et al 2008). Birds can harbor many different types of parasites including lice, tapeworms, roundworms and blood flukes (Wobeser 2008). One group of avian parasites that is of particular importance because it can infect both wildlife and people are blood flukes (family Schistosomatidae). Avian blood flukes are a specialized group of parasites that inhabit the circulatory system of their avian hosts. They have been found to infect representatives of multiple bird orders but are most prevalent in waterfowl (Anatidae) (Huffman and Fried 2008). The morbidity/mortality associated with avian schistosomatid infections in wild bird populations is not well understood due to the difficulties in studying wildlife epizootiology, but the pathology caused by avian schistosomatid infection in birds is believed to be comparable with that of the much better studied mammalian schistosomes (Horak et al 2002). Although most parasitic infections do not directly result in the death of their host, they may still reduce host fecundity and affect host survivability, especially in cases where disease and environmental stressors are also present (Wobeser 2008). Recent trends in bird populations have shown a 20-25% reduction in the past 500 years (Sekercioglu et al 2004). Some of the factors attributed to the decline in bird numbers are climate change, habitat loss and fragmentation, and agricultural intensification (Both et al 2006, Newton 2004, Herkert 1994). The combination of environmental stressors with parasitism and disease can ameliorate

current declines in bird populations and highlights the importance of studying bird parasites and their associated diversity, transmission, life cycle and distribution (Lafferty and Kuris 1999).

The study of avian schistosomatids is also important for understanding the broader evolutionary history and taxonomic relationships between avian and mammalian schistosomatids within the family Schistosomatidae. Mammalian schistosomes, specifically *Schistosoma*, have been studied extensively due to the global disease impact associated with human schistosomiasis but information regarding diversity, distribution and host associations for the remaining Schistosomatidae genera is still lacking from many areas around the world.

This study provides new information on avian schistosomatid diversity and host associations by conducting a survey of avian schistosomatid hosts in North Dakota, one of the most important waterfowl production areas in the United States. North Dakota has a high number of wetlands that serve as breeding grounds and migratory stopover sites for a diverse assemblage of bird species including waterfowl, shorebirds and passerines. This diverse and dense aggregation of birds in shallow, highly productive water bodies provides ideal conditions for avian schistosomatid transmission within and between avian species.

To the best of our knowledge, this is the first study of avian schistosomatids in the state. The data presented in this thesis provide an overview of the distribution, diversity and host associations of avian schistosomatids in North Dakota as well as their systematic position and phylogenetic affinities.

Evolution and Phylogeny

The family Schistosomatidae includes blood flukes parasitic in birds and mammals. Avian schistosomatids (AS) encompass 10 genera and approximately 67 species (Fig.1). The remaining

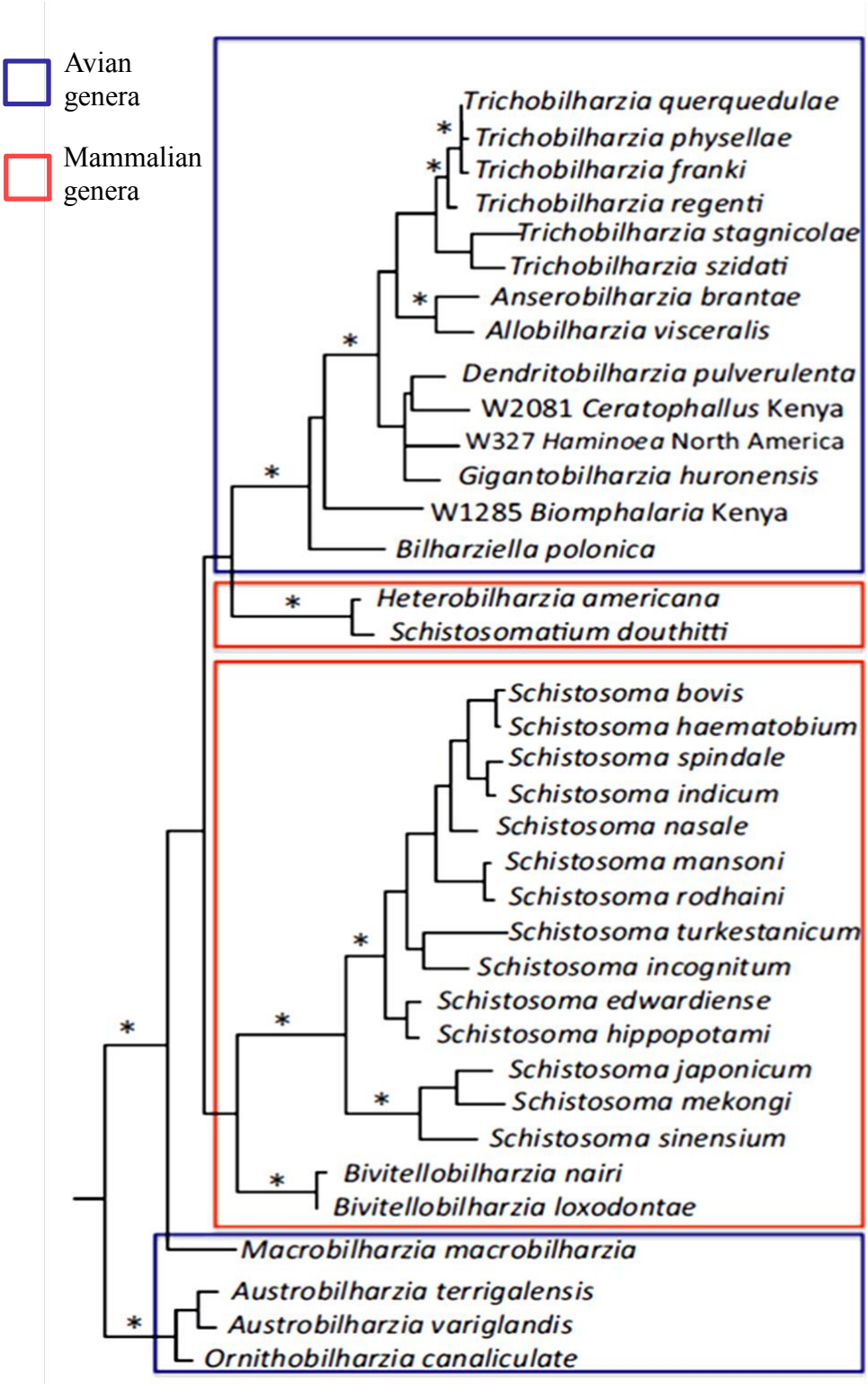

Figure 1. Phylogenetic tree of Schistosomatidae taxa based on Bayesian analysis of nuclear 28s rDNA (1200bp). Nine identified avian schistosomatid genera (blue boxes) and 4 mammalian genera (red boxes) are included. Three unresolved taxa are also included and are represented by the snail host and location they were collected from. Asterisks denote significant posterior probabilities (>0.95). Figure taken from Brant and Loker 2013

4 genera consist of 30 species of mammalian blood flukes, of which *Schistosoma* is the most well-known and best studied (Brant and Loker 2013). A phylogenetic study by Snyder (2004), proposed that the Schistosomatidae arose from blood flukes within their sister taxon, the Spirorchidae. The hypothesis is that a marine turtle blood fluke successfully colonized marine birds and then transitioned to the freshwater life cycle using freshwater avian and snail species (Snyder 2004). The basal position of the genera *Austrobilharzia* and *Ornithobilharzia* (both of which have a marine life cycle) within the Schistosomatidae lends support to this hypothesis as well as the close relationship of marine spirorchid blood flukes to the Schistosomatidae (Snyder 2004).

Morphology

Avian schistosomatids exhibit morphological characteristics typical of most digeneans but also have features unique to their group. They are acoelomates with a dorsoventrally flattened, unsegmented body (Bush et al 2001). Most possess an oral sucker at their anterior end and a ventrally positioned acetabulum that aids in attachment (Fig. 2). They have a syncytial tegument (outer body covering), which functions in nutrient absorption and protection from environmental conditions during free-living and parasitic life stages. What is unusual about this group is that they are dioecious (separate sexes), whereas most digeneans are hermaphroditic (Khalil 2002). This has resulted in varying degrees of sexual dimorphism (Fig. 3) for most AS genera (Loker and Brant 2006). Body shape of adult flukes can also vary in shape from filiform to lanceolate as well as in size from a 1.7 mm long in *Bilharziella* to 57 mm long in *Macrobilharzia* (Baugh 1963, Fain 1955b).

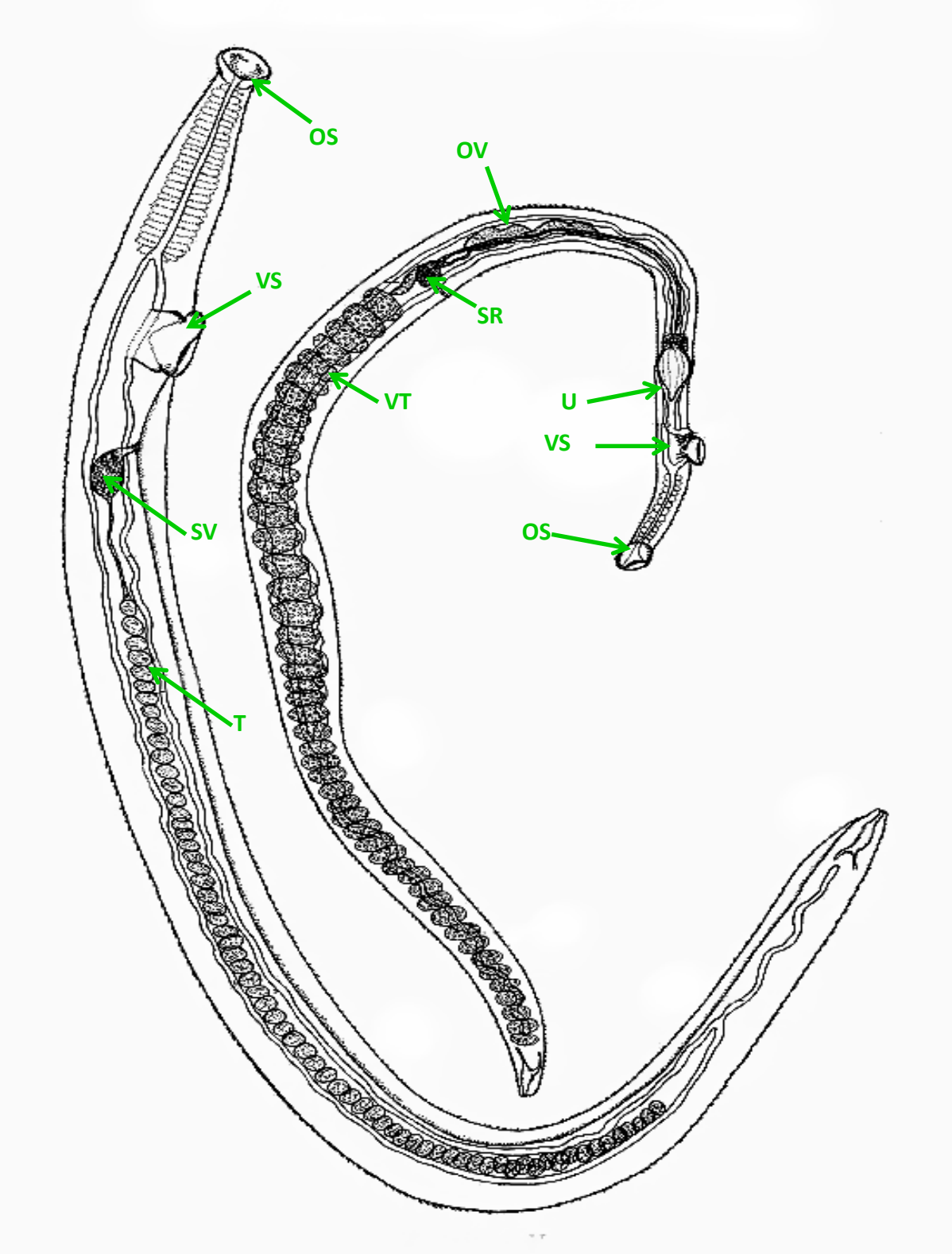

Figure 2. Male (left) and female (right) avian schistosomatid, *Ornithobilharzia* sp. OS, oral sucker; SR, seminal receptacle; SV, seminal vesicle; T, testes; U, uterus (with egg); VS, ventral sucker (acetabulum); VT, vitellaria. Figure taken from Faust 1924 (modified).

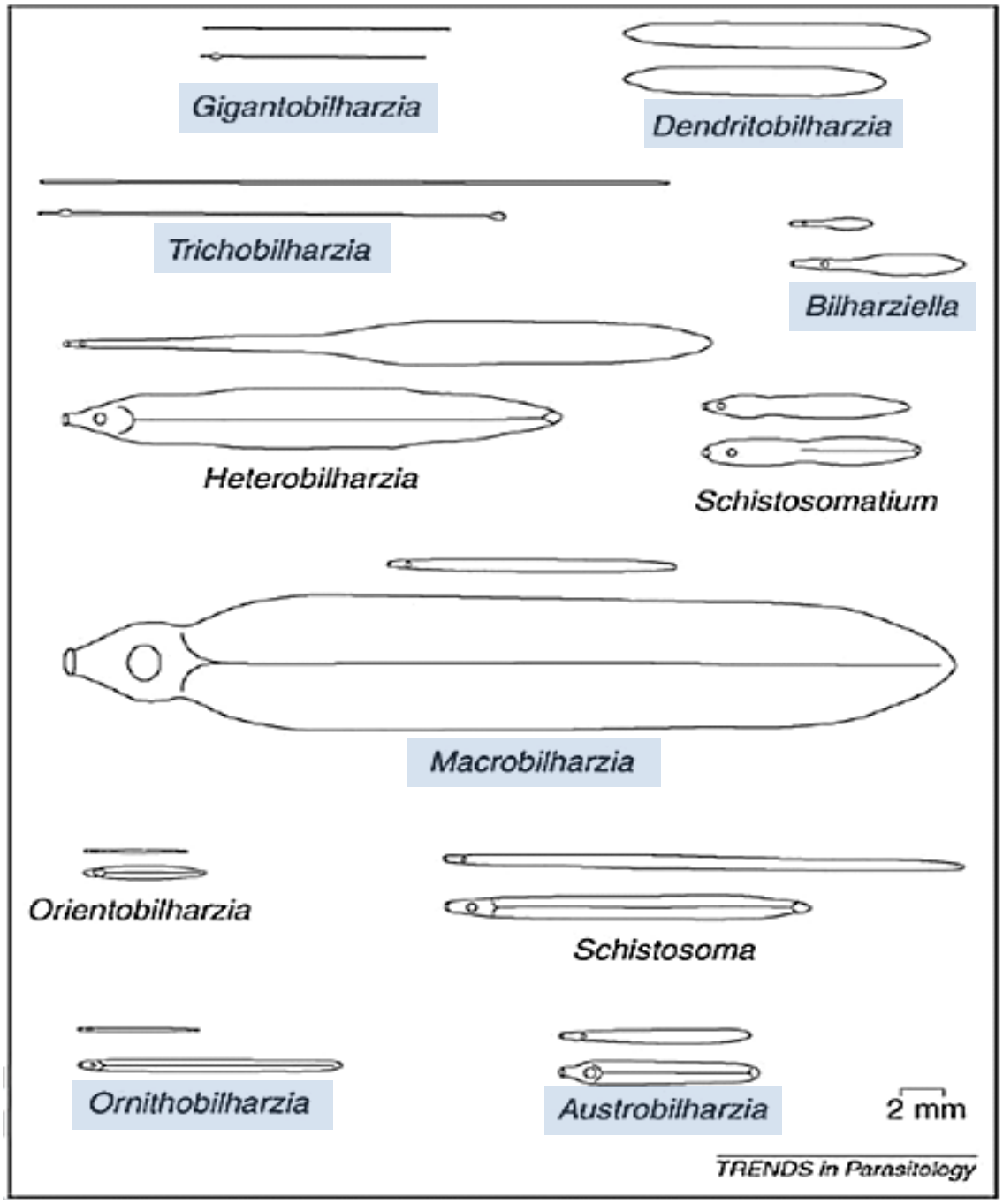

Figure 3: Variation in size and dimorphism between Schistosomatidae genera (Loker and Brant 2006). Females are on the top and males are on the bottom of each pair. The avian genera are highlighted.

Life Cycle

Avian schistosomatids have a two-host life cycle that includes an intermediate snail host and a definitive avian host (Fig. 4). The typical digenean life cycle usually includes a second intermediate host (three-host life cycle) but this additional step is lost AS because cercariae can directly penetrate the host and cause infection (Rollinson and Simpson 1987). In the three-host life cycle, metacercariae encyst within a secondary intermediate host, (fish, amphibian, crustacean or vegetation) which must then be ingested by the definitive host for infection to occur (Galaktionov and Dobrovolskij 2003). There are free-living and parasitic stages as well as both sexual and asexual reproduction throughout AS development (Horak and Kolarova 2011). There are also two types of migratory routes AS can utilize within the definitive host (Horak et al. 2002). One of them uses the blood circulatory system (typical of visceral species) whereas the other is through the nervous tissues (typical of nasal forms). It should be noted that only a few species of the genus *Trichobilharzia* are nasal schistosomatids, while most other schistosomatid genera are visceral forms.

The life cycle of schistosomatids requires an aquatic environment and begins when miracidia hatch from eggs and commence their search for an intermediate snail host. Once they locate a suitable snail, they penetrate its tissue and develop into mother sporocysts. The mother sporocysts then undergo asexual reproduction by producing clusters of germinal cells that develop into daughter sporocysts. The daughter sporocysts migrate to the snail's digestive organs and produce the next stage, the cercariae. After the cercariae develop, they are shed in cycles dependent on multiple abiotic factors (Galaktionov and Dobrovolskij, 2003). Two of the best known abiotic factors to affect cyclical cercarial shedding are photoperiod and temperature (Rollinson and Simpson 1987).

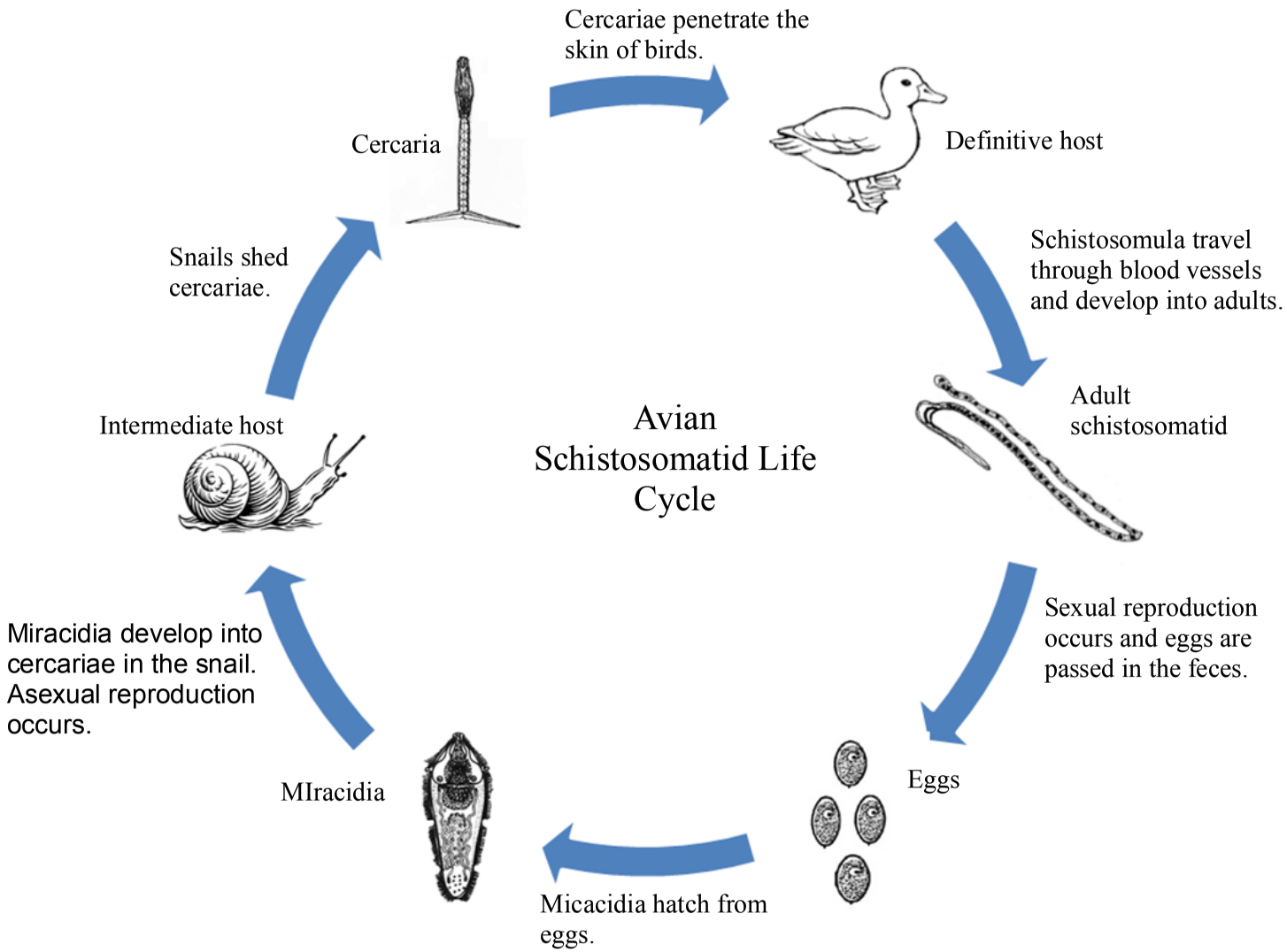

Figure 4. Life cycle of avian schistosomatids

Once cercariae leave the snail, they are free-swimming and rely solely on their glycogen reserves for energy until they can locate their final avian host. AS cercariae can display different swimming patterns depending on the species, with some found closer to the surface and others in deeper water (Rollinson and Simpson 1987). The swimming behavior and cyclical shedding of cercariae are closely tied to the behavior and activity of their intended definitive host and affects their potential to infect humans. Certain stimuli have been shown to increase swimming activity in cercariae. These stimuli are often indicative of a potential host and include water turbulence, the presence of shadows, and detection of chemicals (ceramides, cholesterol and fatty acids) emitted from the host (Rollinson and Simpson 1987, Haas 2001, Horak and Kolarova 2005,). When cercariae encounter these stimuli they exhibit continuous swimming with lateral movements introduced to increase their probability of finding a host (Rollinson and Simpson 1987). If a host is not encountered, cercariae resume their standard swimming pattern of upward swimming and passive sinking.

When a definitive host is located, cercariae will attempt to penetrate the epidermis of the potential host. After cercariae enter the epidermis, they undergo a physiological change and transform into schistosomula. The schistosomula migrate through the epidermis until they locate and penetrate a capillary vessel. Once inside the host's vascular system, the schistosomula travel to the lungs and then to the hepatic portal system where they finally develop into adult flukes. After the adult flukes mature and mate, they travel to the mesenteric veins where they deposit their eggs into the mesenteric venules of the intestine (Platt and Brooks 1997). *Dendritobilharzia* is an exception to this case and instead utilizes the host’s arterial system. The eggs then pass through the walls of the blood vessels, the intestinal wall and into the lumen where they can be excreted with the host’s feces back into the environment. When an influx of water enters the

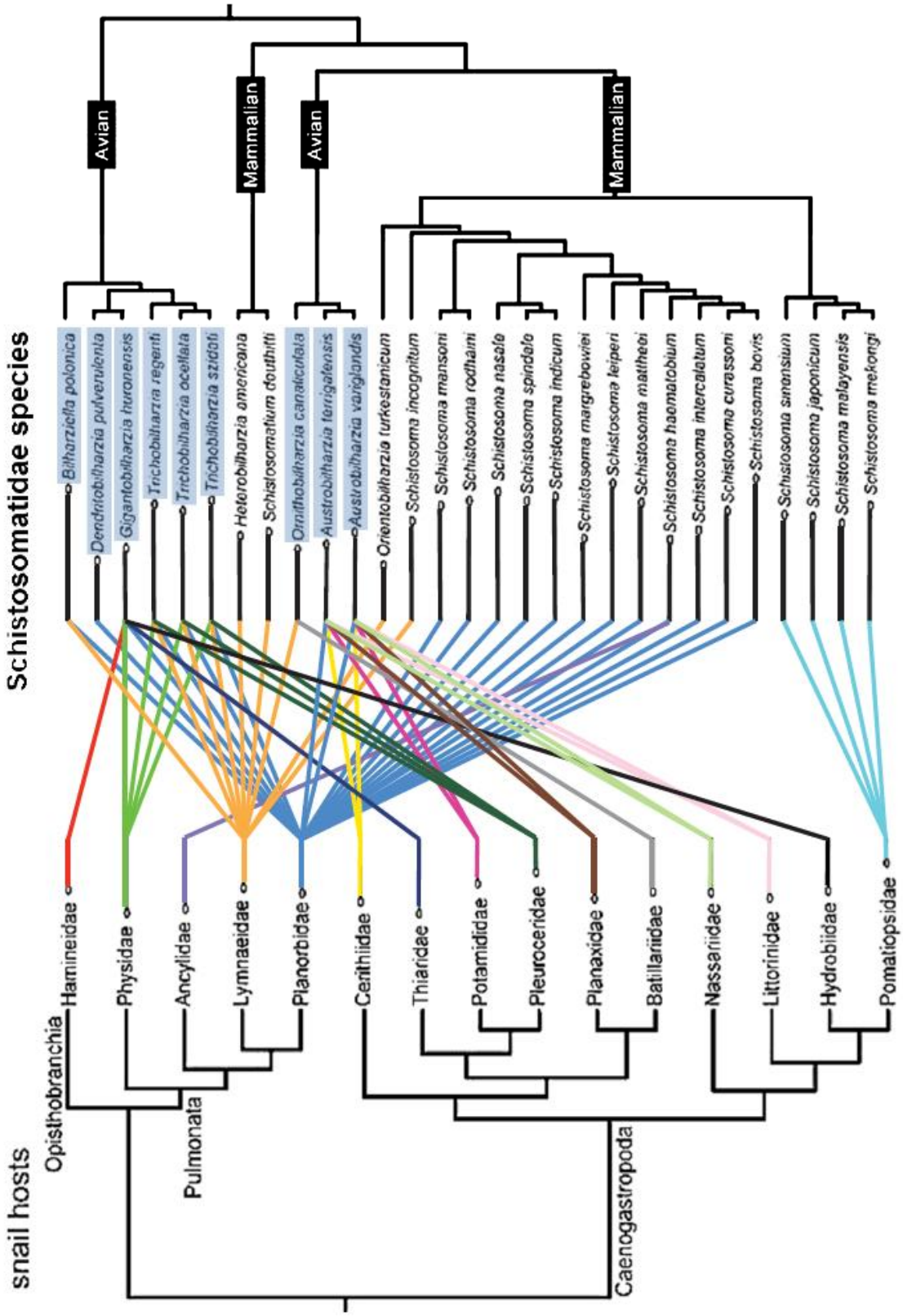

Figure 5. Phylogeny of snail hosts and their associated schistosomatidae parasites. Avian schistosomatidae species are sheded in blue. (Lockyer et al 2003, modified).

egg, it induces a mechanical response that breaks open the shell and releases the miracidium into the environment.

Some species of *Trichobilharzia* have a somewhat different migratory path in the definitive host that involves migration through the bird's nervous system. These species are often referred to as nasal schistosomatids. In this life cycle, schistosomula enter the peripheral nerves after penetrating the skin of birds and migrate through the central nervous system. They move up the spinal cord, into the brain, and then to the nasal blood vessels where they finally mature into adults and release their eggs (Horak et al. 2002). The eggs pass through the nasal blood vessels and into the nasal tissue. Eggs are released back into the environment when the bird places its head in the water (Platt and Brooks 1997).

Snail Hosts

In the United States, both marine and freshwater snails have been implicated in outbreaks of cercarial dermatitis. Freshwater snails are the most common intermediate hosts and include the families Lymnaeidae, Planorbidae, Physidae, Pleuroceridae, Valvatidae and Chilinidae (Lockyer et al. 2003, Brant and Loker 2013). Overall, snails belonging to 15 families have been reported to be infected with avian schistosomatids (Brant and Loker 2013; Fig. 5). According to Brant et al. (2006), Lymnaeidae and Physidae are the hosts most frequently used by North American AS species. In snails, the infection rate is usually between 1-5% (Loy and Haas 2001) but in some locations around the world, it has been estimated to be up to 50% during certain times of the year (Larsen et al 2004, Valdovinos and Balboa 2008).

Avian Hosts

While waterfowl are the most common definitive hosts for AS's, some species can also parasitize shorebirds, gulls, terns, pelicans, cormorants and songbirds. Several waterfowl species

are known to be infected with AS, some of these species include members of the genera *Anas* (dabbling ducks including teals, mallards, wigeons, shovelers), *Anser* (grey geese), *Cygnus* (swans), *Branta* (black geese), *Aythya* (diving ducks including scaup, redhead, canvasback, ring-necked) *Bucephala* (bufflehead and goldeneye), *Lophodytes* (hooded merganser) and *Mergus* (mergansers) (Brant and Loker 2013, Vande Vusse 1980).

The prevalence of AS in birds is believed to be much higher compared to the snail intermediate host (Horak and Kolarova 2011). For instance, in one of the studies in France, 60.5% of aquatic birds were infected with AS (Jouet et al 2009) and in Iceland, 35.5% of birds in the orders Gaviiformes, Podicipediformes and Anseriformes were infected (Skirnisson and Kolarova 2008). Studies in the United States have also found high prevalence within waterfowl including 83.9% prevalence of AS in common mergansers in Flathead Lake, Montana (Loken et al 1995), 24.5% AS prevalence in 46 species of birds (n=378) collected throughout North America (Brant and Loker 2009b) and 92.8% of three gull species in Connecticut (Barber and Caira 1995). A study conducted by Brant in 2007 also found that 92% of screened tundra swans from New Mexico and Nevada were infected with AS. Songbirds also may have high prevalence of AS infection; e.g., 60% of red-winged blackbirds in Michigan (Strohm, Blankespoor and Meier 1981) and 83% of yellow-headed blackbirds in Wisconsin and Michigan (Brackett 1942) were infected with AS.

Avian Health Concerns

The pathological effects of AS on their definitive bird hosts are known to cause inflammation and provoke immune reactions during their migration through tissue. Antigens released from eggs, adults and dead worms can initiate immune responses from the parasitized birds (Horak and Kolarova 2005). The severity of AS infection depends on parasite migratory

behavior, parasite load and host immune status (Rau, Bourns and Ellis 1975, Horak et al 2002). Initial infection occurs when cercariae penetrate the skin and cause minor damage to the epidermal layers. Subsequent phases of infection occur during schistosomula, adult worm and egg migration throughout the visceral organs of the body (Horak and Kolarova 2011). In lung tissue, inflammation, hemorrhage and build-up of scar tissue can result in a compromised respiratory system (Mcmullen and Beaver 1945). Avian schistosomatid adults within the hepatic portal veins can cause liver damage when eggs are deposited into the tissue resulting in a buildup of granulomatous tissue (scar tissue) and inflammation (Mcleod and Little 1942). Infection with blood flukes can also result in blood clots, hemorrhages, and damage to the mesenteric blood vessels (Wojcinski et al 1987) which can increase the susceptibility of infected hosts to other pathogens. Birds with a high parasite load, compromised immune system, or suffering from increased AS pathogenicity as a result of AS migration to other organs are more susceptible to muscle atrophy, reduced kidney and liver function, loss of fat and emaciation (Van Bolhuis et al 2004). The reduction in fitness may affect the overall reproductive potential of their avian host and chronically infected individuals are able to carry and introduce the parasite into new aquatic environments where other individuals can be infected.

In the case of nasal schistosomatids, increased pathogenicity is seen in both specific and non-specific, non-normal avian hosts due to the migration of AS through the CNS. In contrast to visceral schistosomatids in which infection with a low number of AS can be asymptomatic, even a small number of nasal schistosomatids in a host may cause severe neurological symptoms (Kolarova 2007). Some symptoms of nasal schistosomatid infection can include nasal hemorrhage, balance disorders, paralysis and damage to the spinal cord, brain, ocular nerves and nasal tissue (Horak et al 1999).

Effects of Avian Schistosomes in Humans and Other Mammals

Avian blood flukes can also cause cercarial dermatitis in humans (Brant and Loker 2009a). This occurs when avian schistosomatid cercariae penetrate human epidermal tissue during their search for an avian host and cause an inflammatory reaction commonly referred to as 'swimmer's itch'. The cercariae die in the skin and cause an allergic reaction resulting in a self-limited, non-communicable skin rash. Initially there is a Type I immediate hypersensitivity reaction followed by a late phase inflammatory reaction as a result of the death of cercariae within the skin (Kourilova et al. 2004). No medical treatment is necessary and the rash usually resolves within 1-2 weeks (Kourilova et al. 2004).

Cercarial dermatitis is more commonly reported in the upper Midwest with high numbers seen near the Great Lakes region of the United States (Jarcho and Burkalow 1952.) There have been reports of cercarial dermatitis throughout most of the United States including Pennsylvania, California and Colorado (Brant et al 2010, Brant and Loker 2009a, Wills et al 1977). Worldwide, cercarial dermatitis has also been reported in Europe, Canada, India, Australia and Japan (Kolarova 2007).

At this time, very little is known about the survival and migration of AS to other organs of the body in infected mammals. Research has shown migration of some schistosomatid species into the lungs, liver and nervous tissue of mice, rabbits and monkeys (Olivier 1953). It was discovered that three *Trichobilharzia* species, normally found in waterfowl but known to penetrate the skin of mammals, could migrate to the pulmonary tissue and cause hemorrhage in monkeys (Bacha et al. 1982). One study in Europe found another *Trichobilharzia* species, *T. regenti*, in the nervous system of experimentally infected mice. In immunocompromised mice, higher numbers of cercariae were shown to penetrate the skin, migrate to the brain tissue and in

some cases, cause paralysis in the infected mouse (Hradkova and Horak 2002). The pathogenicity of AS infection in mammals is still a subject of current research and more studies are needed before we can determine the health risks of AS exposure to mammals.

Factors Influencing Avian Schistosomatid Diversity and Distribution

The distribution of avian schistosomatids is dependent on several abiotic and biotic factors including climate, availability of intermediate and definitive hosts, ecosystem stability, interspecific competition and host immunity. In order for AS to persist in a water body, there must be susceptible intermediate and definitive hosts available to complete the life cycle. An unstable habitat in which rapid changes occur in water level, water quality, vegetation, or species composition can affect AS presence within a water body.

There are also factors that can influence avian schistosomatid diversity including the diversity of intermediate and definitive hosts within a region, their spatial and temporal patterns and the introduction of new potential hosts.

Current Status of Avian Schistosomatid Research in the United States

Currently, the following genera of avian schistosomatids are known to occur in the United States: *Dendritobilharzia, Trichobilharzia, Gigantobilharzia, Allobilharzia, Ornithobilharzia*, *Anserobilharzia* and *Austrobilharzia* (Barber and Caira 1995, Lockyer et al 2003, Brant et al 2010). The largest and most broadly distributed genus is *Trichobilharzia* with 14 reported species. *Trichobilharzia physellae* is the most common AS species found in snails followed by *T. stagnicolae* with most cercarial dermatitis outbreaks attributed to one of the two species (Brant and Loker 2009b).

Species misidentification based on morphology alone has become a topic of concern for avian schistosomatids due to insufficiently described specimens from previous research. Before

the development of molecular techniques, accurate identification of cercariae and other larval stages was nearly impossible and required experimental infections of intermediate and definitive hosts which was time consuming and costly. This has resulted in incomplete data on the distribution and host associations of avian schistosomatids. Current research utilizes both morphological characteristics and DNA analysis for species identification. This has resulted in the discovery of new species and genera as well as changes to the phylogenetic tree of the Schistosomatidae (Lockyer et al 2003, Brant and Loker 2009b).

North Dakota as an Ideal Region to Study Avian Schistosomatids

Avian blood flukes have not been previously studied in North Dakota. At the same time, the natural conditions as well as diversity and abundance of aquatic birds in the state makes North Dakota an ideal region to study avian blood fluke diversity, distribution and host associations. The diversity of bird species that use the migratory stopover sites in North Dakota may also have a large effect on the diversity of schistosomatid species by introducing new species from other regions. Shallow water bodies also facilitate contact between cercariae and their avian hosts and may increase the prevalence of infection.

To the best of our knowledge, there are no records of identified blood flukes or their distribution in North Dakota, although there have been reports of cercarial dermatitis (Jarcho and Burkalow 1952; Chu 1958). The situation regarding avian schistosomatid knowledge in the neighboring states (South Dakota, Minnesota, Montana) and Manitoba is almost the same, although some sampling was done in Minnesota as part of a molecular phylogenetic study of the genus *Trichobilharzia* (Brant and Loker 2009b). Considering the abundance of waterfowl and other potential avian hosts of schistosomatids in North Dakota, a relatively diverse fauna and broad distribution of these parasites can be predicted.

Research Objectives

The main objectives of this study are:

- To obtain knowledge of avian schistosomatids, their diversity, distribution and host associations in North Dakota.
- Create a DNA sequence dataset to enable identification of avian blood flukes found in North Dakota at any stage of their life cycle.
- Conduct a phylogenetic analysis using newly obtained sequences from North Dakota and previously published sequences from the GenBank database.
- Perform a spatial analysis of snail distribution in different types of habitats to determine any distinct patterns and possible correlations with AS infection.

In order to achieve these objectives, snails and birds were collected and examined for the presence of AS cercariae (in snails) or adult blood flukes (in birds) to determine diversity, distribution and host associations in North Dakota. Molecular and morphological techniques were utilized for avian schistosomatid species identification and diversity study. Phylogenetic analysis including North Dakota AS specimens was done in order to determine their relationships with other schistosomatid taxa. Spatial analysis of snail collection sites involved detection of spatial autocorrelation and analysis of snail occupancy.

CHAPTER II

METHODS AND MATERIALS

Snail Collection

The sampling protocol for snail collection was designed so that samples would be a good representation of the different regions in the state, while also maintaining adequate spatial distribution between sites. Locations were selected by organizing the state into a 56 cell grid (approximately 45x30 miles) and selecting at least one area per cell (when possible) which contained several bodies of water in close proximity (Fig. 6). These locations were then designated as areas to visit during a collection trip. Most sites on the eastern half of the state were sampled throughout several day trips between May and September. Sites in the northern, western and southern part of the state were sampled during four longer (3-5 days) collection trips that occurred in June, July and August of 2013. Water bodies from each of the selected areas were then sampled based on (1) accessibility, (2) water body size, (3) the presence of perennial vegetation (old cattails), and (4) vegetation cover suitable for snail and waterfowl habitation. At each site, GPS coordinates were recorded and pictures of the sampled water bodies were also taken.

Most sites were sampled only once throughout the season. Due to its proximity, the central-eastern part of the state received increased sampling, particularly the region between Devils Lake and Grand Forks. Fewer samples were collected from the south-western (Badlands) and south-central (Missouri plateau) parts of the state due to the low number of aquatic habitats available in the region. At each site, the sampling protocol

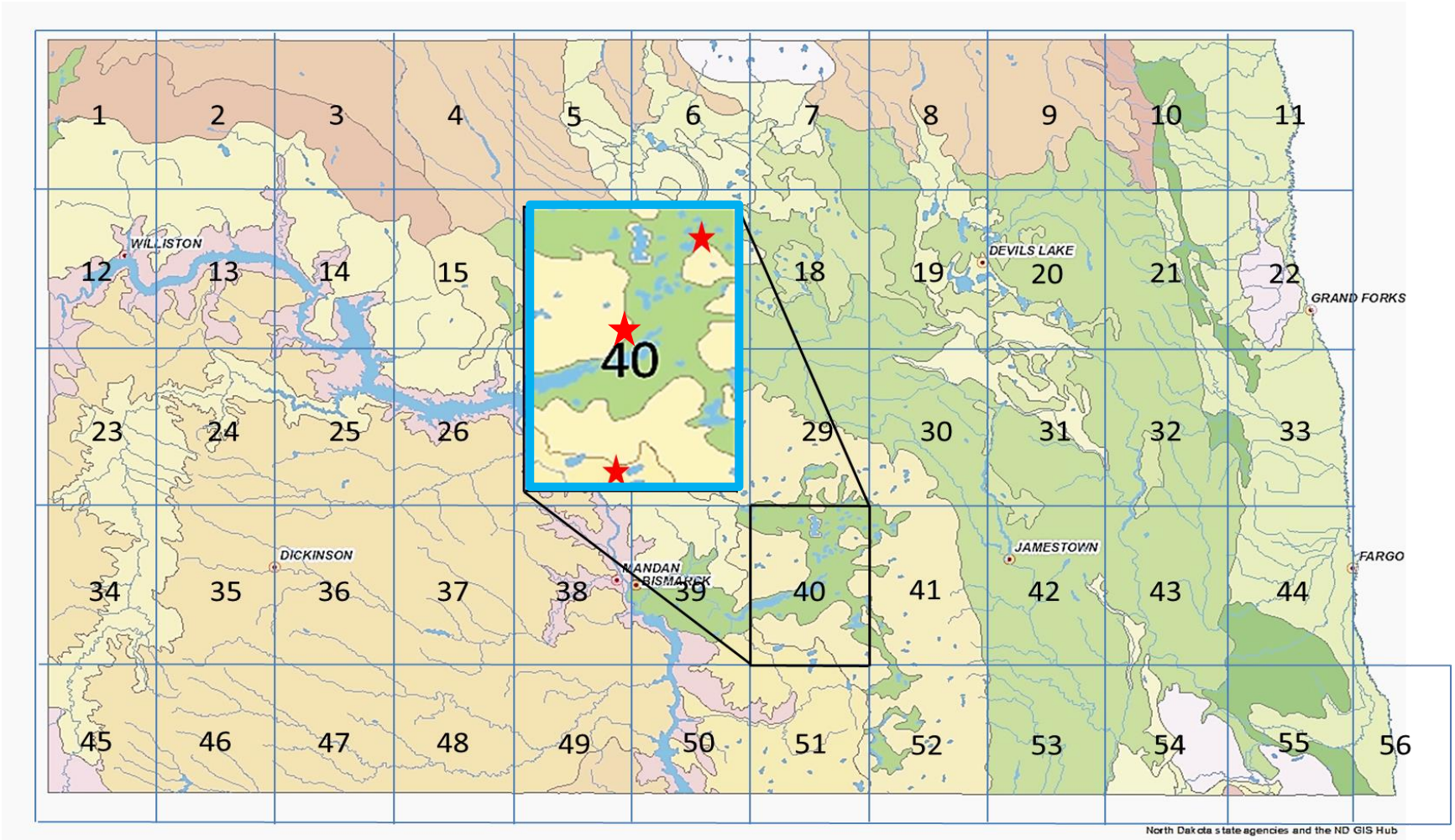

Figure 6. Representation of the sampling design used for snail collection. The state was divided into a grid with 56 cells (~45mi x 30mi). For each cell, at least 1 area with multiple water bodies was selected as a possible sampling site. During collection trips, these designated areas were visited and snails were collected if sites met specified criteria. Cell 40 shows a region with high densities of water bodies present, in these regions multiple locations were selected for sampling. Base map is of the North Dakota ecoregions (level IV) from the ND GIS Hub.

required the collection of at least 100 snails of each species per site (if possible) in order for an accurate depiction of AS presence/absence. This sampling size was based on species accumulation curves of cercarial types and AS infection rates of two snail genera obtained from previous snail collections in the central-eastern part of the state.

Snails were collected by gloved hand or dip net. When possible, snails were collected from several locations along the wetland's shoreline. Water bodies in close proximity (~200ft of each other) were considered one site and snails collected from each water body were pooled and designated as one sample. All collected snails were kept in labeled containers and placed in a cooler to keep snails alive until they could be transported back to the laboratory and examined for AS infection.

Snail Husbandry

Upon collection, snails were housed and screened in the Parasitology Core Facility at the Department of Biology, University of North Dakota. Snails were rinsed, counted and separated by genus into individual glass jars. Each jar contained 1-4 snails depending on snail size. Jars were filled with specially conditioned tap water to remove chloramine and all jars were cleaned daily or every two days to prevent snail mortality. Snails were fed green leaf lettuce ad lib and maintained at room temperature.

Cercarial Screening in Snails

Two protocols were used to screen snails for cercariae. In the first protocol, snails were kept for 2-5 days and monitored for cercarial shedding once a day for at least two days. Screening occurred after the snails were exposed to artificial light for $\geq$ 1 hour. Each jar was screened using a dissecting microscope by examining the bottom, middle and surface water layers for the presence of cercariae. All snails believed to be shedding AS cercariae were

dissected using a dissecting microscope to verify AS infection in the snail and to collect larval stages. In the second protocol, snails were not kept in jars or monitored for cercarial shedding. Instead, all collected snails from a site were immediately dissected and screened for AS infection using the dissecting microscope. The choice of the protocol depended on 1) the duration of the collection trip; longer trips required immediate snail processing because of the difficulty in keeping live snails under field conditions, 2) time constraints; snails were processed as efficiently as possible so that new snails could be collected and screened, and 3) the number of laboratory personnel available for snail processing.

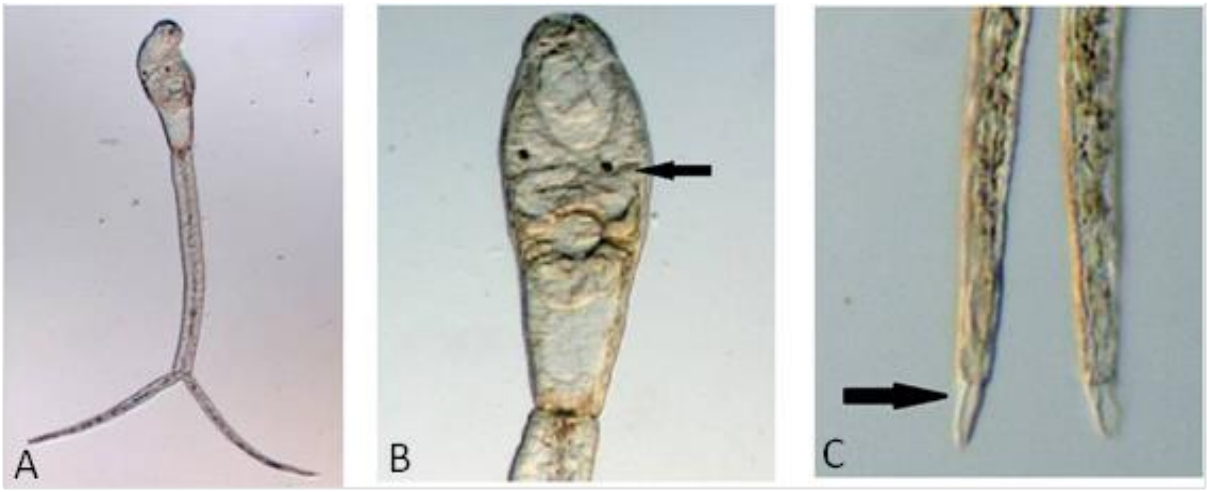

Figure 7. Larval cercarial stage of an avian schistosomatid with identifying morphological features. Features include a forked tail (A), eyespots (B) and knoblike projections on the tips of the tail (C).

Schistosomatid cercariae were identified by using morphological characteristics unique to the group. These morphological characteristics include a forked tail, the presence of eyespots and the presence of knob-like projections on the tips of their tail (Fig. 7). A compound microscope was used in some instances to visualize some of these characteristics. Once identified as AS cercariae, cercariae were preserved in 70-90% ethanol for DNA extraction. Some cercariae were

also fixed in hot formalin for scanning electron microscope (SEM) study or photographed using an Olympus BX51 research compound microscope equipped with DIC optics and a digital imaging system utilizing Rincon software.

Bird Collection

Unlike snails, birds were not collected following a specific sampling protocol and were collected opportunistically from 2003-2014. Bird species that are most likely to host AS were targeted when possible. All bird collecting was done according to the obtained federal and state collecting permits as well as approved IACUC protocol. Most birds were collected in the central-eastern part of the state, between Devil's Lake and Grand Forks, North Dakota. Birds were collected between April and November and included both resident and migratory birds. Most individuals were collected using firearms but some smaller passerines were also captured using mist nets. A number of bird carcasses were provided for examination by local hunters.

Bird Dissection Protocol

All birds were dissected on the day of collection in order to increase the chances of obtaining quality specimens of adult schistosomatids. Birds were identified to species using field guides. During dissection, the liver, kidneys and intestines were removed and kept in a solution of citrated saline to prevent blood clot formation. Sedimentation technique was used to recover blood flukes from kidneys, liver and body wash. The liver and kidneys were each broken up into smaller pieces by gloved hand, and the solution was shaken and allowed to set for 5-10 minutes to allow helminths and tissue time to sink to the bottom of the cylinder. The majority of the supernatant was then discarded and more citrated saline was poured into the cylinder, shaken and allowed to set. This process was repeated until the solution was clear enough to see through under the dissecting microscope. Larger tissue pieces were removed from the solution and small

amounts of the solution were poured into a shallow dish and examined under a dissecting microscope. The body cavity of each bird was also rinsed with citrated saline and the resulting body wash also underwent the sedimentation protocol described above. In some cases, it was necessary to pool samples in order to process them in a timely manner. This occurred during the waterfowl hunting season when several waterfowl specimens were donated in a single day. Pooled samples consisted of birds of the same species that were collected on the same day and from the same site. They were not separated by sex or age.

The mesenteric veins and the blood vessels in the wall of the intestinal tract were carefully examined for the presence of schistosomatid adults. The intestines were removed intact and placed in a large dish containing citrated saline. The mesenteric veins were examined under the dissecting microscope and any worms present were extracted from the blood vessel by excising the blood vessel and pushing the adult flukes out from one end of the vein. Due to the time constraints associated with dissecting multiple birds on the day of collection, it was not always possible to examine the intestines of every bird on instances where several individuals needed to be processed. In such cases, intestines from at least 2-3 birds of each species were examined under the dissecting microscope.

The adult schistosomatids were killed with hot water and preserved in 80% ethanol which permitted both morphological and molecular study. When the number of specimens allowed, some worms/fragments were fixed in 95% ethanol for DNA extraction. Some worms that were intact and in good condition were heat killed and preserved in formalin for SEM study.

DNA Extraction

Schistosomatid cercariae were extracted using two methods. In both cases, 25-30 cercariae were put in an Eppendorf tube. Most of the ethanol was aspirated with a pipette and the

specimens were dried for 30 minutes at 60° to remove any remaining ethanol from the tissues of the specimens. Next, 60µl of pure H_2O was added to the tube to rehydrate the cercariae. The cercariae were then broken apart by sonication using a UP100H compact ultrasonic processor (Hielscher USA, Inc., Ringwood, NJ) at 80-100% for 20 seconds. Following sonication, 250µl of Zymo Cell Lysis Buffer was added to the tubes and the samples were allowed to lyse for at least one hour. In the first method of DNA extraction, the protocol established by Tkach and Pawlowski (1999) was followed by 1) precipitating DNA with isopropanol for at least two hours or overnight 2) centrifugation and removal of the supernatant 3) rinsing the resulting DNA pellet with 70% ethanol (2x) 4) drying the DNA pellet in a 60° heat block to remove any traces of ethanol. Alternatively, a Zymo micro DNA extraction kit was used according to manufacturer's instructions. The DNA extraction kit was used for only a few specimens. At the final step of both extraction methods, the DNA was eluted with ≥ 25µl of pure H2O and stored at -20°C.

Adult blood flukes were also extracted using the same protocols as above. DNA was extracted from adults using only partial fragments of adult specimens. In cases of larger specimens like *Dendritobilharzia,* a posterior or lateral section of the worm was excised and used for DNA analysis. For smaller, filiform specimens like *Trichobilharzia,* posterior sections of longer worms or fragments of individual worms were used for DNA analysis.

Polymerase Chain Reaction

Following DNA extraction, amplification of a ribosomal and mitochondrial gene was done using polymerase chain reaction (PCR). The two DNA regions chosen for species identification and phylogenetic analysis were nuclear large ribosomal subunit gene (28s) and mitochondrial cytochrome oxidase 1 gene (cox1). In both cases, partial gene sequences were used. It should be mentioned that the sequenced fragment of the cox1 gene included the "barcoding" region widely

used for species identification among digeneans and other groups of invertebrates. Fig. 8 (nuclear) and Fig. 9 (mitochondrial) show gene layout and the regions that were amplified and sequenced as well as the positioning of PCR and sequencing primers. The nuclear 28s gene is a component of the large ribosomal subunit and has been used in numerous molecular systematic and phylogenetic studies (Brant and Loker 2009, Snyder 2004). It has both conserved regions as well as variable domains and proved to be useful at different taxonomic levels, from differentiation among congeneric species to phylogenies up to the level of digenean orders. However, in some digenean lineages the interspecific variability in the 28S gene is not sufficient for reliable species differentiation/identification. Therefore the mitochondrial cox1 gene was chosen as the additional marker characterized by a much higher interspecific variability that allows for differentiation between closely related species.

The PCR protocol included a 20µl reaction consisting of: 10µl of New England Biological MasterMix Taq Polymerase, 1µl of a forward primer (10pm/µl concentration), 1µl of a reverse primer (10 pm/µl concentration), 1-3 µl of DNA template and enough water to bring the reaction to 20µl. The same protocol was followed for PCR amplification of the mitochondrial cox1 gene. The PCR conditions for both genes are described in Tables 1 and 2.

In most cases, we have also attempted to amplify the nuclear ribosomal ITS1+5.8S+ITS2 region which has intermediate variability when compared to the 28S and cox1 genes. However, due to difficulty in PCR amplification and DNA sequencing associated with the presence of tandemly arranged repeats and/or multiple copies of the ITS1 region, we were unable to produce an ITS dataset matching that obtained for the 28s and COX1 genes.

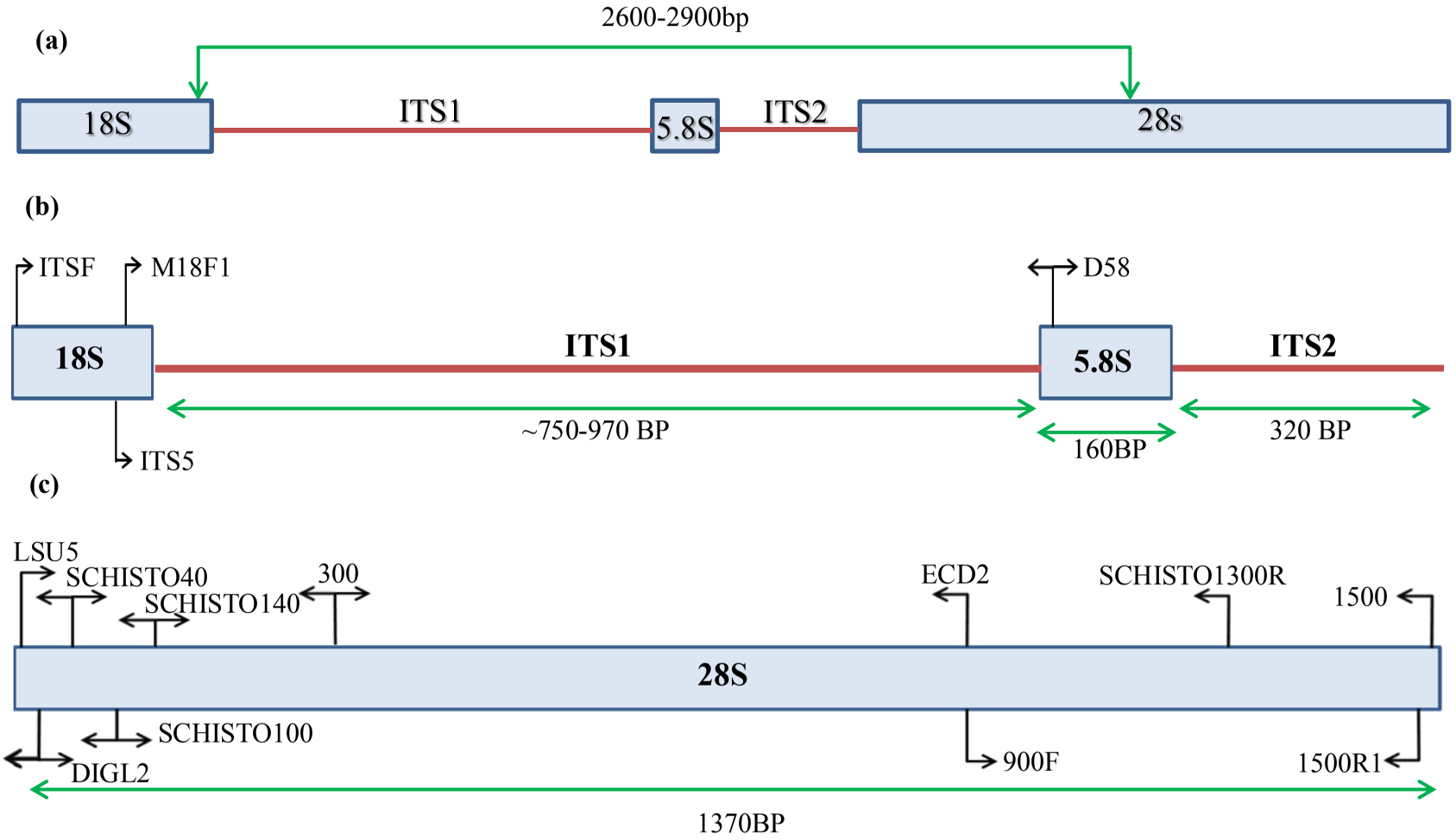

Figure 8. Ribosomal ITS and 28s nuclear regions showing positions of PCR and sequencing primers. (a) Ribosomal complete ITS and 28s nuclear regions showing the 2600-2900bp region that was sequenced. (b) Positions of PCR and sequencing primers at the 5' end of 18S gene and 5.8S gene. (c) Positions of PCR and sequencing primers in the fragment of 28S gene targeted in this study.

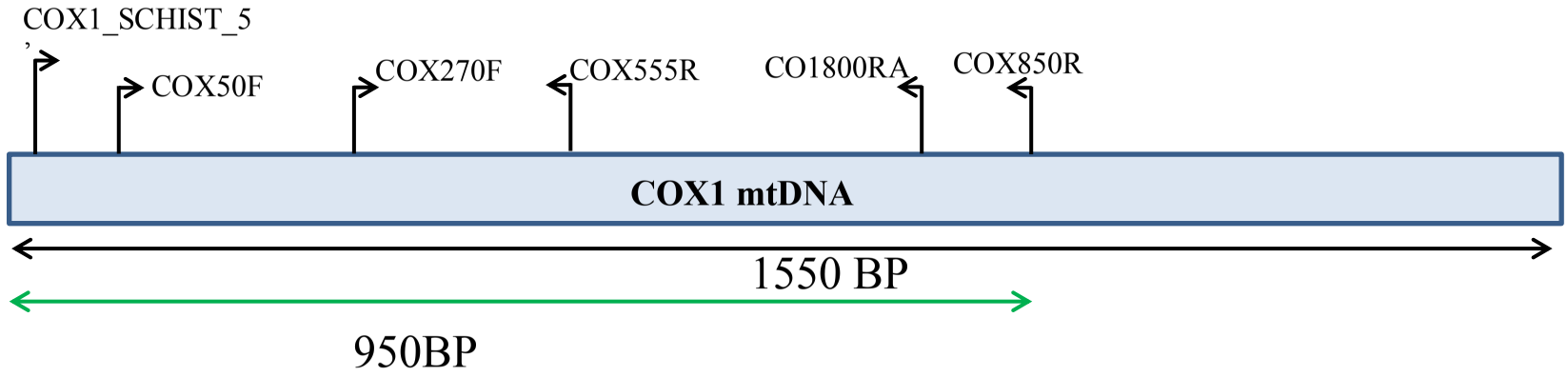

Figure 9. Positions of PCR and sequencing primers in the cox1 mitochondrial gene.

Table 1. Thermocycling conditions for PCR amplification of 28s rDNA of avian schistosomatid cercariae and adult worms.

Step		Conditions
1	**Initial Denaturation**	Temp: 94°C Time: 30 seconds
2	**Denaturation**	Temp: 94°C Time: 30 seconds
3	**Annealing**	Temp: 56°C Time: 40 seconds
4	**Extension**	Temp: 68°C Time: 1 minute per kb
5	Repeat steps 2-4 X 40 cycles	
6	**Final Extension**	Temp: 68°C Time: 5 minutes

Table 2. Thermocycling conditions for PCR amplification of cox1 mDNA of avian schistosomatid cercariae and adult worms.

Step		Conditions
1	**Initial Denaturation**	Temp: 94°C Time: 40 seconds
2	**Denaturation**	Temp: 94°C Time: 30 seconds
3	**Annealing**	Temp: 40°C Time: 45 seconds
4	**Extension**	Temp: 68°C Time: 1 minute per kb
5	Repeat steps 2-4 X 40 cycles	
6	**Final Extension**	Temp: 68°C Time: 5 minutes

After species identification of all collected specimens, only a few individuals from each species were then chosen for PCR amplification and sequencing of the ITS region.

The PCR products were stored at -20°C and cleaned up using Affymetrix ExoSap enzyme based kit or Zymo DNA Clean and Concentrator column based kit. In cases when multiple bands were present, the Zymo DNA Gel Extraction kit was used to isolate the desired band. All methods were followed according to the manufacturer's instructions.

Table 3. PCR and sequencing primers for nuclear 28s and mitochondrial cox1 genes used in this study.

PCR amplification and sequencing	**Primer sequence (5' – 3')**
NUCLEAR	
LSU5	TAGGTCGACCCG CTGAAYTTAAGCA
1500R	CGAAGTTTCCCTCAGGATAGC
1500R1	GCTACTAGATGGTTCGATTAG
ECD2	CCCGTCTTGAAACACGGACCAAG
900F	CCGTCTTGAAACACGGACCAAG
300F	CAAGTACCGTGAGGGAAAGTTG
300R	CAACTTTCCCTCACGGTACTTG
SCHISTO100F	GTAACTGCGAGTGAACAGGG
SCHISTO100R	CCCTGTTCAGTCGCAGTTAC
SCHISTO1300R	GCTCTTGCTCCGCCCCGACG
SCHISTO40F	GCGGAGGAAAAGAAACTAACAAGG
SCHISTO40R	CCTTGTTAGTTTCTTTTCCTCCGC
DIGL2	AAGCATATCACTAAGCGG
DIGL2R	CCGCTTAGTGATATGCTT
ITSF	CGCCCGTCGCTACTACCGATTG
ITS5	GGAAGTAAAAGTCGTAACAAGG
D58F	GCGGTGGATCACTCGGCTCGTG
D58R	CACGAGCCGAGTGATCCACCGC
M18F1	CGTAACAAGGTTTCCGTAG
SCHISTO140R	CTAAACACCACATTGCCTTGC
SCHISTO140F	GCAAGGCAATGTGGTGTTTAG
MITOCHONDRIAL	
COX1_SCHISTO5'	TCTTTRGATCATAAGCG
COX770R	ACCATAAACATATGATG
COX555R	CCAAATTTWCGATCAAA
COX270F	GTTTTATATGGARTTGAG
COX850R	GAAAAAACCTTTATACC
CO1800Ra	CAACCATAAACATATGATG

DNA Sequencing

The cleaned up PCR products were then cycle-sequenced using Life Technologies BigDye chemistry in a 10µl reaction. The sequencing reaction protocol consisted of: 1-2µl of DNA template, 1.5µl of primer (2pm concentration), 5µl of 5x BigDye sequencing buffer, 1µl of BigDye Terminator v3.1 Cycle Sequencing Mix and enough water to bring the reaction to 10µl. Thermocycling conditions are described in Table 4. Sequencing reactions were alcohol-precipitated and sequenced directly on an ABI Prism 3100™ automated capillary sequencer.

Table 4: Thermocycling conditions for sequencing reactions of nuclear 28s and mitochondrial cox1 DNA for all avian schistosomatid cercariae and adult worms.

Step		Conditions
1	Denaturation	Temp: 96°C Time: 15 seconds
2	Annealing	Temp: 50°C Time: 10 seconds
3	Extension	Temp: 60° Time: 4 minutes
4	Repeat steps 1-3 X 25 cycles	

Sequence Editing and Alignment

Sequences were assembled using Sequencher 4.2 software. BioEdit software (Hall 1999) was used for sequence alignment using ClustalW plug-in with default. Subsequent manual adjustment in BioEdit was done when needed. All chromatograms were verified by eye to ensure quality of resulting contigs. Poor quality sequences with background interference were not used. The nuclear 28s sequences were used for genus identification of collected specimens while the mitochondrial cox1 sequences were used for species identification. Both genes were used for phylogenetic analyses. The ITS regions were not used for phylogenetic analysis due to the

presence of tandem repeats. These repeats resulted in difficulty aligning contigs from multiple species and errors in alignment may result in false inferences from any phylogenetic analysis using a misaligned dataset.

Cercarial Morphology

Cercariae preserved in 90% ethanol were rehydrated with water and photographed using a compound microscope utilizing Rincon software. Images were taken of ten cercariae for each haplotype, collected from two different snails (5 from each snail). Measurements were taken from the cercariae images using Rincon software and used for comparison of morphological features between haplotypes. Measurements included body length and body width, oral sucker length and width, ventral sucker length and width, forebody length, tail length, furcae length, and length of the terminal projections of furcae. A few cercariae were also prepared for SEM study. Fixed specimens in formalin and 70% ethanol were dehydrated in a graded series of ethanol and dried with hexamethyldisilazane (Ted Pella Inc., Redding California) as a transition fluid. Cercariae were mounted on an aluminum stub using conductive double-sided tape, coated with gold-palladium, and examined with the use of a Hitachi 4700 scanning electron microscope (Hitachi U.S.A., Mountain View, California) at an accelerating voltage of 5-10kV.

Adult Fluke Morphology

Quality adult worm specimens collected from birds were stained, mounted and photographed. Adult worms preserved in 70% ETOH were first rehydrated in dH_2O then stained with alum carmine. Acid ethanol was used as a de-staining fluid to remove any excess stain and worms were then dehydrated using a series of ETOH solutions of increasing concentration. Afterward, specimens were cleared in clove oil and permanently mounted in xylene-based

Damar gum solution. Some adults were also used for SEM study and were prepared following the same protocol described above for cercariae.

Phylogenetic Analysis

Alignments for analysis were composed of at least two sequences for most of the identified species. When possible at least one sequence was taken from cercariae and one from an adult blood fluke. In addition, sequences from GenBank were also added in the alignment to ensure that as many of the schistosomatid taxa collected from around the world would be represented in the analysis. There were a total of four alignments assembled including 1) nuclear 28s nucleotide sequences representing all species from the derived avian schistosomatid clade (*Trichobilharzia, Gigantobilharzia, Bilharziella, Dendritobilharzia, Allobilharzia, Anserobilharzia*) collected in North Dakota and available from GenBank, 2) nuclear 28s nucleotide sequences from all species collected in North Dakota and available from GenBank, 3) mitochondrial cox1 nucleotide sequences of *Trichobilharzia* species from North Dakota and GenBank, and 4) cox1 nucleotide sequences of *Dendritobilharzia* from North Dakota and GenBank,

All alignments were trimmed to the same length for analyses. jModelTest (Darriba et al 2012, Guindon and Garcuel 2003) and MEGA 6 (Tamura et al 2013) were used to determine the best nucleotide substitution model for each alignment. Phylogenetic analysis using maximum likelihood (ML) were carried out using MEGA 6, and Bayesian inference (BI) using MrBayes 3.2.2 (Huelsenbeck and Ronquist 2001, Ronquist et al 2011). The model GTR+I+G was used for all analyses. Nodal support was estimated by bootstrap (10,000 replicates) and all codon positions were used for ML analyses. For BI (nst=6, rates=invgamma, ngammacat=4) four chains were run simultaneously for 5×10^6 generations and trees were sampled every 1000

cycles with the first 1,250 trees discarded as burnin. Trees were visualized and edited using FigTree v1.4.0 and Adobe Illustrator CS5 software.

Spatial and Occupancy Analysis of 2013 Snail Data

The data gathered from snail collections in North Dakota (2013) was also used to assess spatial patterns of snail and parasite distribution. I tested for the presence of distinct snail distribution patterns through spatial autocorrelation and occupancy in relation to ecological context using logistic regression (Guisan et al 2002). For spatial autocorrelation, SAM (Spatial Analysis in Macroecology) v4.0 (Rangel et al 2010) was used to calculate Moran's I for sites where *Lymnaea*, *Stagnicola* or *Aplexa* snails were collected and for sites where AS infected *Lymnaea*, AS infected *Stagnicola* or AS infected *Aplexa* snails were collected. Distribution data on *Physa*, *Helisoma* and *Promenetus* snails were not included in this analysis due to small sample size and lack of AS infections. *Physa* were infected in only two sites while no AS infections have been detected in the latter two genera. For spatial autocorrelation analysis, the 105 sites were separated into 12 distance classes containing equal numbers of pairs and 500 permutations were run.

Snail occurrence based on ecoregion and land cover was performed using Fisher's exact-test. Land cover and ecoregion for each collection site was determined by plotting sites on Omernik ecoregion (2013) and LandCover (2013) maps using ArcGIS 10.2.1. Logistic regression, using R 3.1.1, was applied to determine the best model for predicting snail presence. The possible predictor variables included land cover, ecoregion, collection date, latitude and longitude. The same analysis was performed for schistosomatid occurrence in which the presence of an infected snail genus was the response variable.

CHAPTER III

RESULTS

Collected Snails and Avian Schistosomatid Prevalence

A total of 17, 653 snails were collected between May – September of 2013 from 105 sites throughout North Dakota (Fig. 10, Appendix A and B). Prolonged cold temperatures during the spring of 2013 delayed the onset of snail sampling for the season and only one site was sampled in late May. The months with the highest number of sampled sites were July (40 sites) and August (29 sites). Eighteen sites were sampled in June and 17 sites were sampled in September. Thirty six out of 105 sites were positive for avian schistosomatid infected snails (Fig. 11). A large portion of the sampled sites (91%) were located in the Northwestern Glaciated Plains and Northern Glaciated Plains ecoregions (Fig. 11).

Collected snails represent 6 genera and 3 families (Table 5). There were no avian schistosomatid infected snails from the genus *Helisoma* and *Promenetus*. The avian schistosomatid prevalence rate within snail genera was highest in *Lymnaea* (1.7%) and *Aplexa* snails (2.9%), while both *Stagnicola* and *Aplexa* had AS prevalence rate < 0.5%. A total of 134 (0.8%) snails were shedding cercariae. The distribution of infected *Stagnicola*, *Lymnaea* and *Aplexa* snails are shown in Figs. 12–14.

In addition, 195 snails were collected from 5 sites in eastern-central Minnesota with 2 sites positive for avian schistosomatid infected snails. They included one *Physa* snail and two *Lymnaea* snails. Snails shedding mammalian schistosomatids (*Schistosomatium douthitti*) were

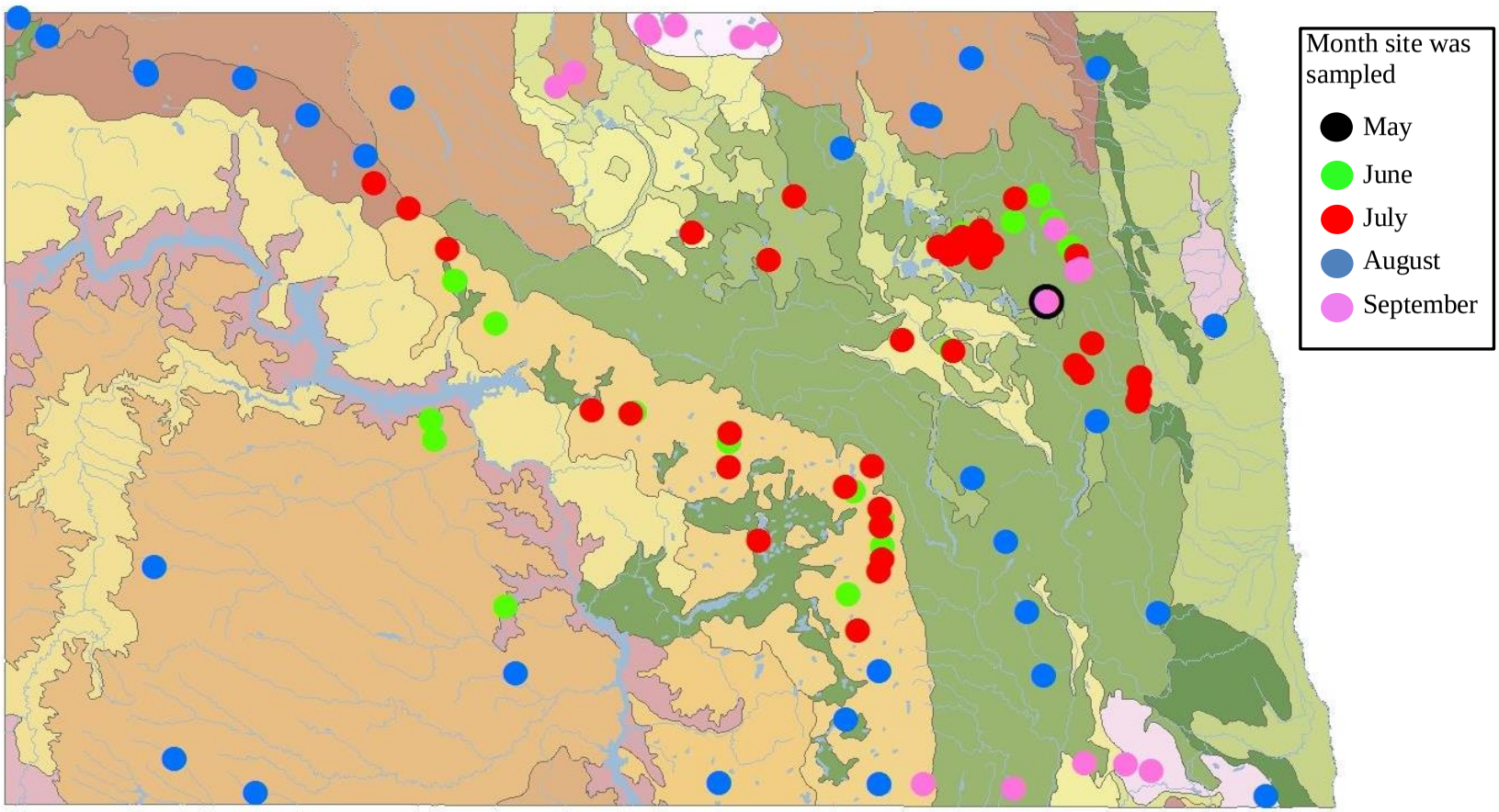

Figure 10. All North Dakota snail collection sites from 2013 and the time period (by month) sites were visited and sampled. Most sites were only visited once during the year. Base map is of the North Dakota Omernik ecoregions (level IV) from the NDGIS Hub.

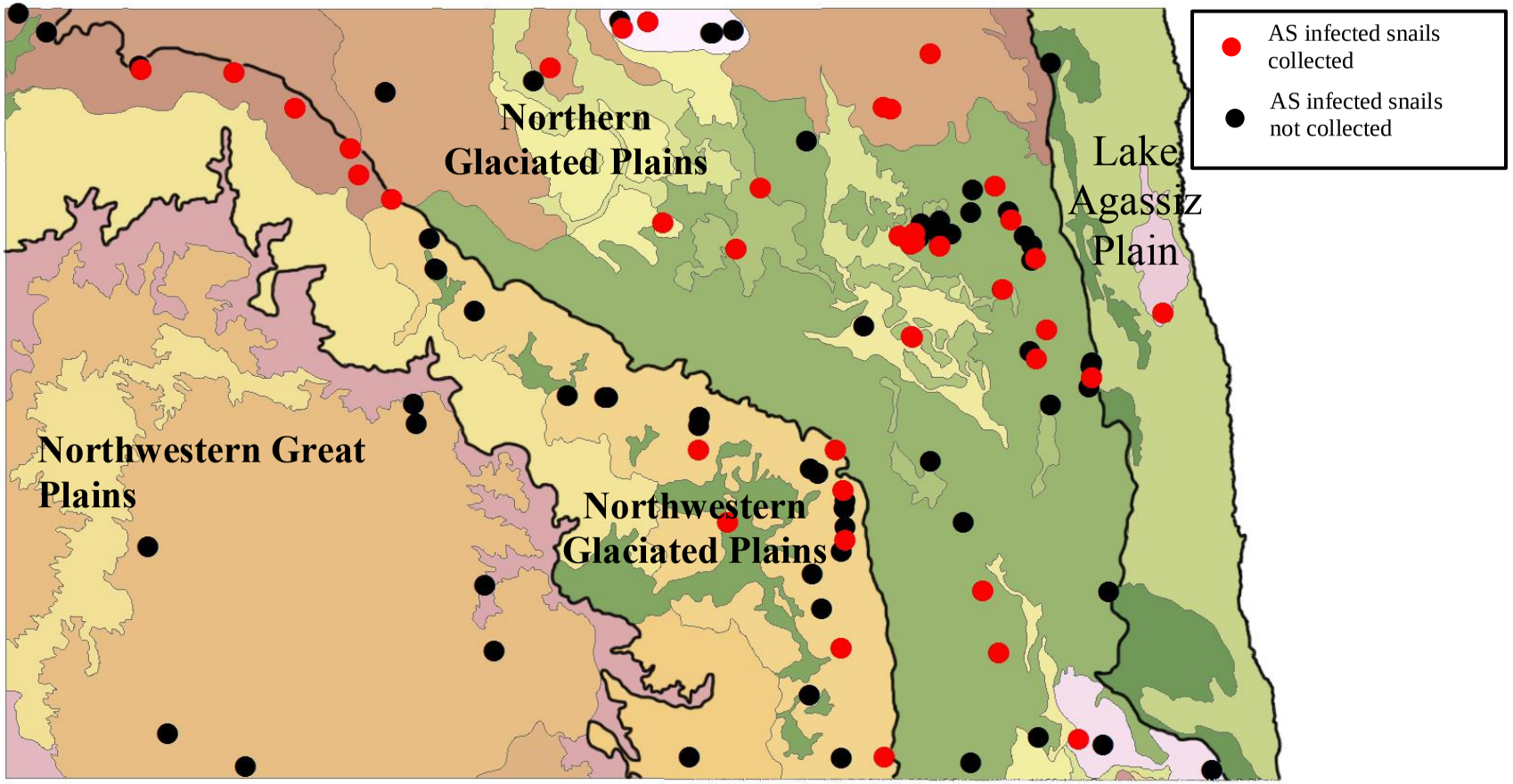

Figure 11. Map of North Dakota showing 2013 collection sites where AS infected snails were found. Red circles represent collection sites where avian schistosomatid infected snails were collected. Black circles represent sites where no avian schistosomatid infected snails were collected. Base map of Omernik ecoregions (level II) taken from ND GIS hub.

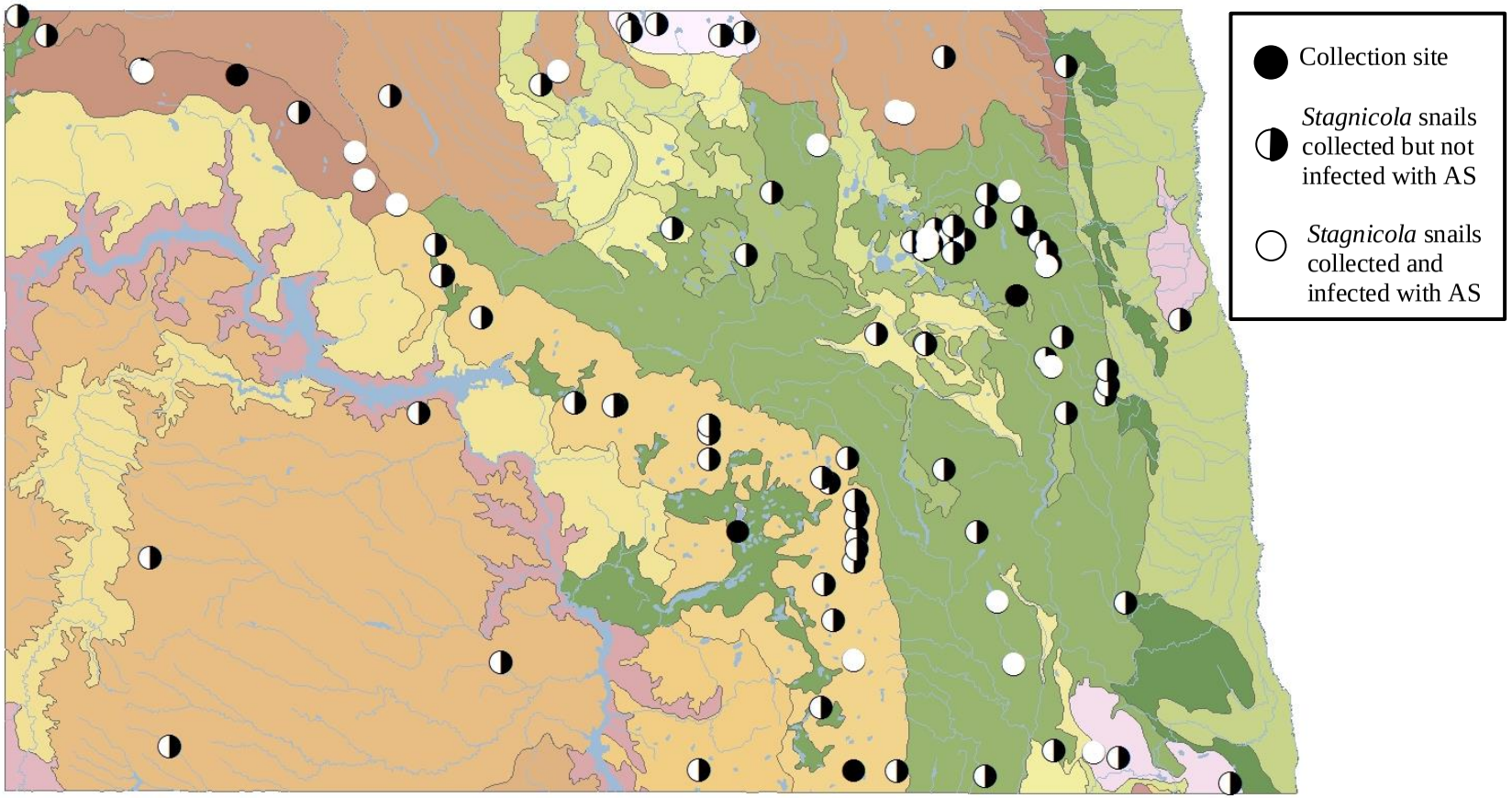

Figure 11. Distribution of avian schistosomatid infected *Stagnicola* snails in North Dakota. Snail collection sites are divided into three categories 1) site that was sampled but no *Stagnicola* snails collected (black circles), 2) collection site where *Stagnicola* snails were collected but none were infected with avian schistosomatids (½ black, ½ blue circles), 3) site where *Stagnicola* snails were collected and at least one was infected with avian schistosomatid cercaria (blue circles).

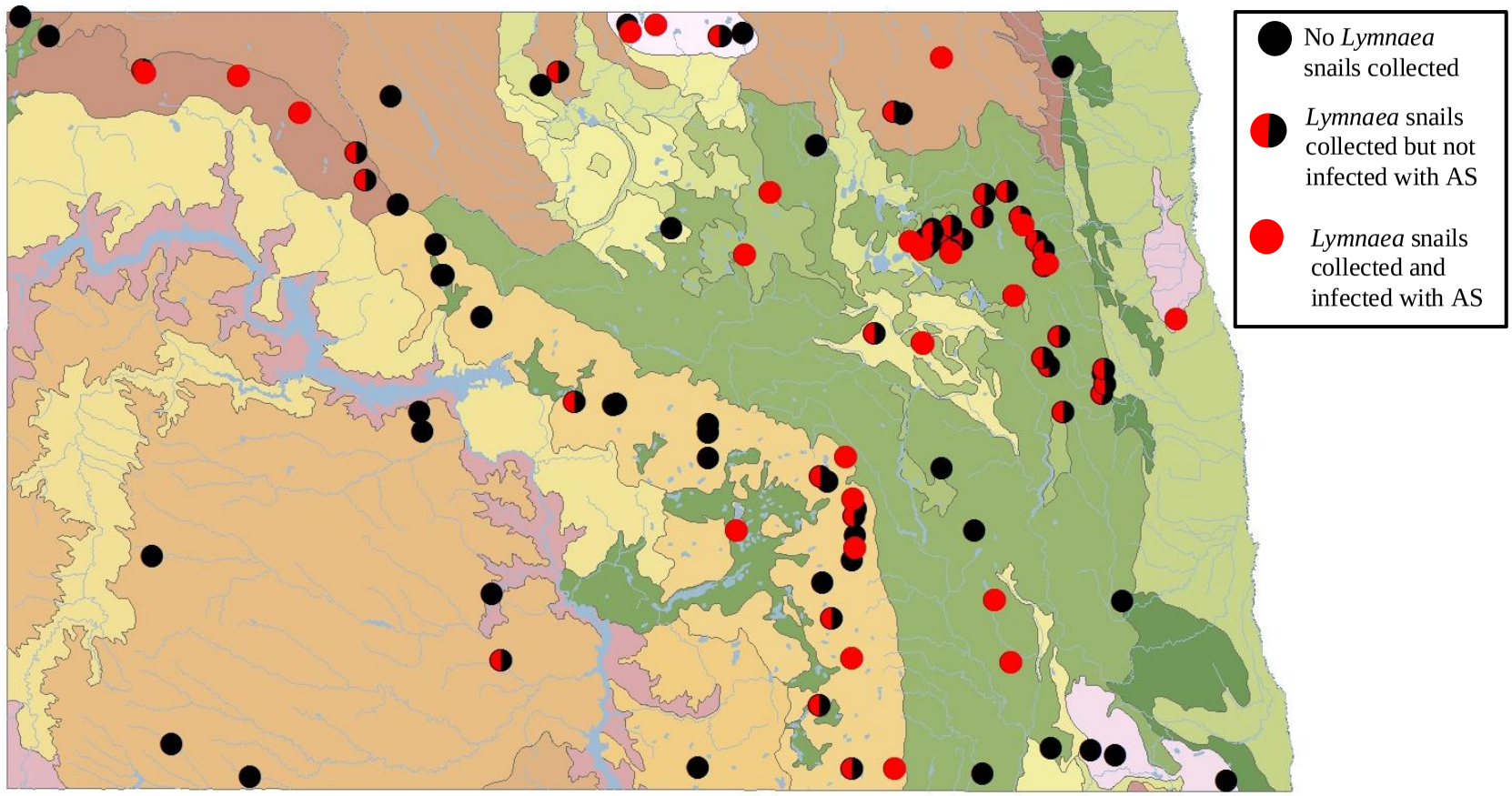

Figure 12. Distribution of avian schistosomatid infected *Lymnaea* snails in North Dakota. Snail collection sites are divided into three categories 1) site that was sampled but no *Lymnaea* snails collected (black circles), 2) collection site where *Lymnaea* snails were collected but none were infected with avian schistosomatids (½ black, ½ red circles n=36), 3) site where *Lymnaea* snails were collected and at least one was infected with avian schistosomatid cercariae (red circles n=25).

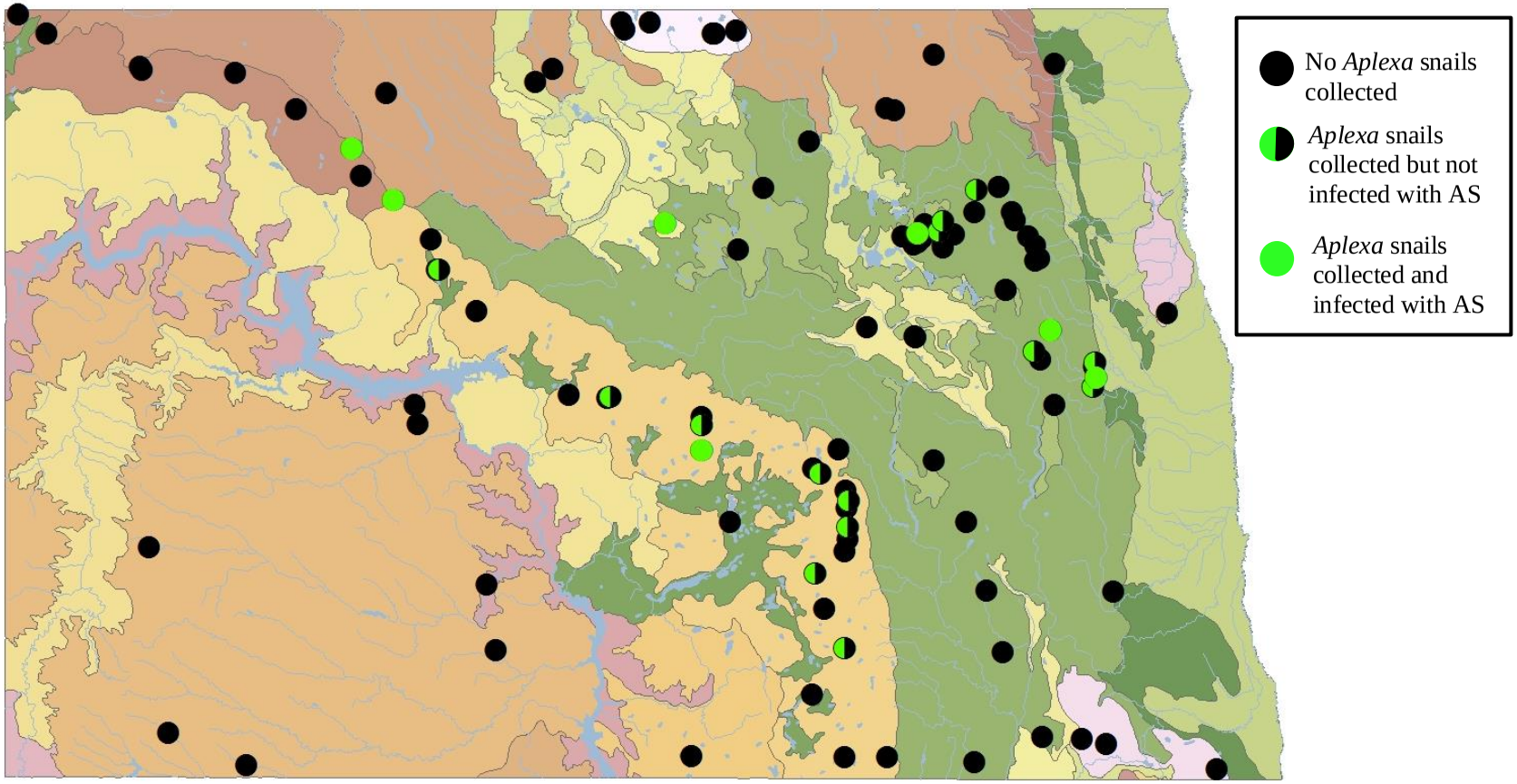

Figure 13. Distribution of avian schistosomatid infected *Aplexa* snails in North Dakota. Snail collection sites are divided into three categories 1) site that was sampled but no *Aplexa* snails collected (black circles), 2) collection site where *Aplexa* snails were collected but none were infected with avian schistosomatids (½ black, ½ green circles, n=14), 3) site where *Aplexa* snails were collected and at least one was infected with avian schistosomatid cercariae (green circles, n=7).

also collected in North Dakota and included four *Stagnicola* snails and 9 *Lymnaea* snails. These snails are not included in Table 5. Differentiation of mammalian and avian cercariae was based on DNA sequencing.

Table 5. Snail genera collected from North Dakota between May-September of 2013 and the number infected with avian schistosomatid cercariae.

Snail Family	Snail Genus	Total Collected	N. infected with AS cercariae
Lymnaeidae	***Stagnicola***	10893	38 (0.4%)
	Lymnaea	4260	73 (1.7%)
Physidae	***Physa***	1157	2 (0.2%)
	Aplexa	732	21 (2.9%)
Planorbidae	***Helisoma***	495	0
	Promenetus	116	0
	Total	17653	134 (0.8%)

Cercariae: Species Identification and Host Associations

A total of 8 schistosomatid species were identified from cercariae collected from snails, seven AS species and one mammalian schistosomatid (Fig. 15, Appendix C). *Stagnicola* served as intermediate hosts for the highest number of schistosomatid species with a total of 6 species detected form this genus. *Lymnaea* snails had four species detected with only one positive snail for *Trichobilharzia* sp. A and *Trichobilharzia* sp. E. AS species were identified to species by matching the newly obtained sequences to sequences already available in the GenBank database. Five out of the 7 sequenced AS species, matched GenBank sequences of identified species. Two AS species did not have close matches to any already identified species using GenBank sequences and are designated as *Trichobilharzia* sp. 2 and *Trichobilharzia* sp. 4. *Trichobilharzia szidati* was the most prevalent avian schistosomatid species collected from 71 *Lymnaea* snails.

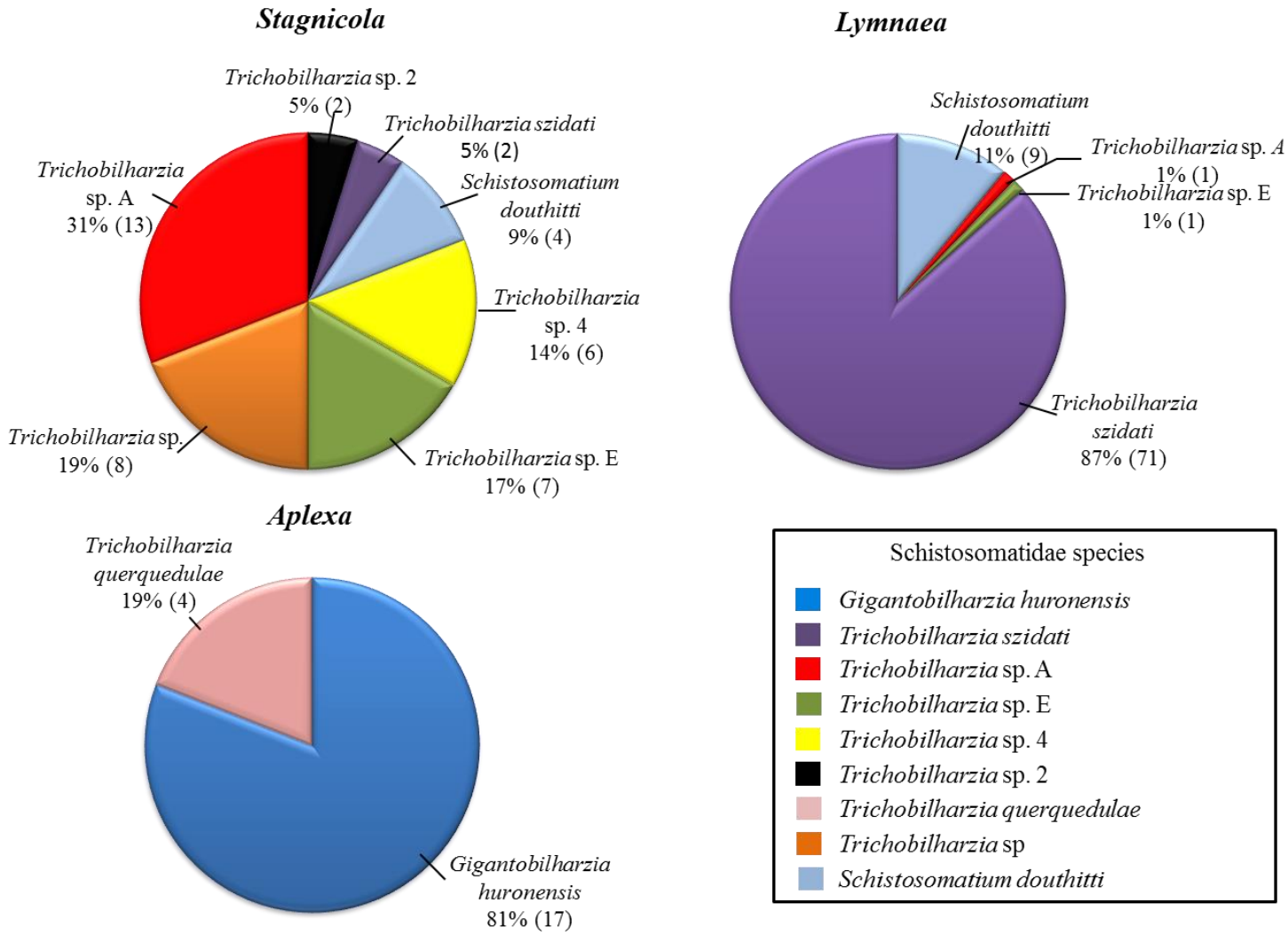

Figure 15. Schistosomatidae species found within each snail genus and their prevalence. Two *Physa* snails were infected with *Gigantobilharzia huronensis* and are not shown in the figure.

The least prevalent was *Trichobilharzia* sp. 2 with only two infected snails collected (both from the same site). The mammalian schistosomatid, *Schistosomatium douthitti,* was found to utilize both *Lymnaea* and *Stagnicola* snails as intermediate hosts and the avian schistosomatid species, *Gigantobilharzia huronensis,* was also found in two snail genera, *Physa* and *Aplexa*. The AS species *Trichobilharzia szidati*, *Trichobilharzia* sp. A and *Trichobilharzia* sp. E were found within two snail genera as well, however, the low prevalence in one of the two snail genera indicate that snail misidentification may be more likely.

Adult Flukes: Species Identification and Host Associations

Avian schistosomatids were found in 72 birds collected in North Dakota between 2003 and 2014. In the fall of 2013, there were nine instances in which samples had to be pooled. For these samples, they are described as coming from a single bird since it is unknown how many total birds within the pooled sample may have been infected. Mallards, Blue-winged teals and Northern shovelers were the bird species with the highest numbers of individuals examined. There were 11 AS species recovered from 22 species of birds collected in North Dakota (Table 6, Appendix D).

Trichobilharzia was the most diverse AS genus in birds with 5 species obtained. One *Trichobilharzia* species, *Trichobilharzia* sp. 4, did not have a close match to any identified species with sequences in the GenBank database. All sequenced *Dendritobilharzia* specimens had identical nuclear 28s rDNA sequences that matched *Dendritobilharzia pulverulenta* sequences available from GenBank. However, they formed two distinct groups when the mitochondrial cox1 gene sequences were analyzed. These two *Dendritobilharzia* groups were named *Dendritobilharzia* sp. 1 and *Dendritobilharzia* sp. 2. Within the genus *Gigantobilharzia*,

Table 6. Avian schistosomatids collected from birds in North Dakota.

Schistosomatidae species	Bird species	Number of infected birds
Allobilharzia visceralis	*Cygnus columbianus* (Tundra swan)	1
	Branta canadensis (Canada goose)	1
***Austrobilharzia* sp.**	*Recurvirostra americana* (Avocet)	1
	Limosa fedoa (Marbled godwit)	1
***Dendritobilharzia* sp. 1**	*Anas clypeata* (Northern shoveler)	1
	Anas platyrhynchos (Mallard)	3
	Anas discors (Blue-winged teal)	2
	Aythya valisineria (Canvasback)	1
***Dendritobilharzia* sp. 2**	*Anas platyrhynchos* (Mallard)	1
	Anas clypeata (Northern shoveler)	1
	Anas strepera (Gadwall)	3
	Bucephala albeola (Bufflehead)	1
	Aythya americana (Redhead)	1
***Dendritobilharzia* sp.**	*Aythya affinis* (Lesser scaup)	1
	Gavial immer (Common loon)	1
	Anas clypeata (Northern shoveler)	1
	Anas platyrhynchos (Mallard)	1
	Aix sponsa (Wood duck)	1
Gigantobilharzia huronensis	*Quiscalus quiscula* (Grackle)	5
	Xanthocephalus xanthocephalus (Yellow-headed blackbird)	1
	Agelaius phoeniceus (Red-winged blackbird)	3
	Molothrus ater (Brown-headed cowbird)	1
***Gigantobilharzia* sp.**	*Gallinago gallinago* (Common snipe)	1
***Trichobilharzia* sp. E**	*Anas crecca* (Green-winged teal)	2
Trichobilharzia querquedulae	*Anas discors* (Blue-winged teal)	6
	Anas strepera (Gadwall)	1
	Anas clypeata (Northern shoveler)	4
***Trichobilharzia* sp.4**	*Anas clypeata* (Northern shoveler)	2
	Anas discors (Blue-winged teal)	1
***Trichobilharzia* sp. A**	*Anas strepera* (Gadwall)	2
Trichobilharzia physellae	*Anas platyrhynchos* (Mallard)	2
	Aythya collaris (Ring-necked duck)	2
	Aythya affinis (Lesser scaup)	2
***Trichobilharzia* sp.**	*Aythya collaris* (Ring-necked duck)	2
	Aythya americana (Redhead)	2
	Anas discors (Blue-winged teal)	1
	Anas clypeata (Northern shoveler)	6
	Bucephala clangula (Common goldeneye)	1
	Aix sponsa (Wood duck)	1
	Anas platyrhynchos (Mallard)	1

all sequences matched the *Gigantobilharzia huronensis* from GenBank except for one adult fluke recovered from a Common snipe. The closest match was to the genus *Gigantobilharzia*, so this specimen was named *Gigantobilharzia* sp. A specimen collected from a Marbled godwit also did not match any available GenBank sequence. The closest match was to the genus *Austrobilharzia*, therefore this specimen is referred to as *Austrobilharzia* sp. The second *Austrobilharzia* specimen was a single complete worm that was stained and mounted instead of undergoing DNA extraction. Since no DNA sequences are available for this specimen, it is also labeled as *Austrobilharzia* sp.

There are 5 avian schistosomatid species for which both the cercarial and larval stages were recovered from the intermediate snail and definitive bird host (Table 7).

Table 7. Avian schistosomatid species identified in both the intermediate snail host and the definitive avian host.

Avian schistosomatid species	**Intermediate snail host**	**Definitive avian host**
***Trichobilharzia* sp. E**	*Stagnicola*	*Anas crecca* (Green-winged teal)
Trichobilharzia querquedulae	*Aplexa*	*Anas discors* (Blue-winged teal) *Anas strepera* (Gadwall) *Anas clypeata* (Northern shoveler)
***Trichobilharzia* sp. 4**	*Stagnicola*	*Anas clypeata* (Northern shoveler) *Anas discors* (Blue-winged teal)
***Trichobilharzia* sp. A**	*Stagnicola*	*Anas strepera* (Gadwall)
Gigantobilharzia huronensis	*Aplexa* *Physa*	*Quiscalus quiscula* (Grackle) *Xanthocephalus xanthocephalus* (Yellow-headed blackbird) *Agelaius phoeniceus* (Red-winged blackbird) *Molothrus ater* (Brown-headed cowbird)

The Identification of Avian Schistosomatid Species by DNA Sequencing

All collected samples of cercariae from snails and the majority of samples of adult blood flukes from birds underwent DNA extraction and PCR amplification. In some instances, quality DNA sequences could only be obtained for either the mitochondrial cox1 or the nuclear 28s large ribosomal subunit. Due to the relatively conserved nature of the nuclear 28s gene, differentiation of some species (e.g., *Trichobilharzia querquedulae*, *Trichobilharzia* sp. A and *Trichobilharzia* sp. 4) was only possible using the cox1 gene. As a result, 8 cercarial and 14 adult blood fluke samples of *Trichobilharzia* could only be identified to the genus level. The same scenario occurred among *Dendritobilharzia* specimens in which differentiation was only possible using the mitochondrial cox1 gene. *Dendritobilharzia* specimens that did not produce quality cox1 sequences (n=5) were only identified to genus.

Intraspecific and Interspecific/Intergeneric Nucleotide Sequence Variation among Collected Avian Schistosomatids

The intraspecific nucleotide variation for all collected AS species is shown in Table 8 for both the nuclear 28s (1034bp) and mitochondrial cox1 (578 bp) genes. No intraspecific sequence variation was detected in the nuclear 28s gene for all collected species except *Gigantobilharzia huronensis* and *Trichobilharzia querquedulae*. The latter two species both had one sequence containing one base pair difference. The intraspecific nucleotide variations, within the mitochondrial cox1 gene, were all higher but averages were still below 1%.

The interspecific nucleotide variation for AS species collected in North Dakota is shown in Table 9 (*Trichobilharzia* species only) and Table 10 (all other collected AS species). When comparing interspecific nucleotide variation for the 28s gene, there was an average variation of 1.21% for all collected species. The average interspecific variation for the mitochondrial cox1

Table 8. Intraspecific variation of avian schistosomatid species collected in North Dakota based on pairwise sequence comparison. The nuclear 28s (1034) and mitochondrial cox1 (578bp) genes were used for comparisons.

Avian schistosomatid species	# Sequences for 28s	Intraspecific variation for 28S, % min-max (avg)	# Sequences for cox1	Intraspecific variation for cox1, % min-max (avg)
Allobilharzia visceralis	2	0	1	-
***Austrobilharzia* sp.**	1	-	1	-
***Dendritobilharzia* sp.1**	7	0	5	0-0.99 (0.47)
***Dendritobilharzia* sp. 2**	4	0	5	0.20-1.38 (0.83)
Gigantobilharzia huronensis	5	0-0.09 (0.03)	6	0-0.60 (0.20)
***Gigantobilharzia* sp. 2**	1	-	1	-
***Trichobilharzia* sp. E**	2	0	5	0.17-2.00 (0.77)
***Trichobilharzia* sp. 2**	2	0	2	0.33
Trichobilharzia querquedulae	3	0-0.08 (0.06)	5	0-1.50 (0.67)
***Trichobilharzia* sp. 4**	4	0	5	0.33-0.83 (0.63)
***Trichobilharzia* sp. A**	2	0	6	0-1.33 (0.72)
Trichobilharzia szidati	5	0	10	0-1.50 (0.50)
Trichobilharzia physellae	2	0	6	0-0.60 (0.27)

gene was 11.64%. To demonstrate the significant level of divergence between the *Austrobilharzia* sp. collected in North Dakota and *Austrobilharzia variglandis*, a sequence of *A. variglandis* from GenBank was used for comparison (Table 10).

Table 11 shows intergeneric variation in studied genera. The 28s intergeneric nucleotide variation was lowest between *Dendritobilharzia* and *Gigantobilharzia* and highest between *Allobilharzia* and *Austrobilharzia*. The cox1 intergeneric nucleotide variation was lowest between the genera *Allobilharzia* and *Trichobilharzia* and highest between the genera *Dendritobilharzia* and *Gigantobilharzia*. The cox1 sequence obtained from the *Austrobilharzia* sp. collected in North Dakota was 200 bp shorter than sequences from all other genera so it was

Table 9. Interspecific variation of 7 *Trichobilharzia* species collected in North Dakota based on pairwise sequence comparison. The variation in the nuclear 28s partial gene (1034bp) is shown above the diagonal and the variation in the mitochondrial cox1 gene (601bp) is shown below the diagonal. The number of 28s/cox1 sequences used for each species included: *Trichobilharzia* sp. E (2/5), *Trichobilharzia* sp. 2 (2/2), *T. querquedulae* (3/5), *Trichobilharzia* sp. 4 (4/5), *Trichobilharzia* sp. A (2/6), *T. szidati* (5/10), *T. physellae* (2/6). Variability is shown as % of the sequence length. The min-max values and (avg) are followed by average in parentheses. Only one number is shown where no variability was detected.

***Trichobilharzia* species**	***Tricho.* sp. E**	***Tricho.* sp. 2**	***T. querquedulae***	***Tricho.* sp. 4**	***Tricho.* sp. A**	***T. szidati***	***T. physellae***
***Trichobilharzia* sp. E**	****	2.22	2.71-2.80 (2.74)	2.71	2.71	1.84	2.71
***Trichobilharzia* sp. 2**	11.98-12.15 (12.01)	****	2.03-2.13 (2.06)	2.03	2.03	1.35	2.03
T. querquedulae	12.15-12.82 (12.51)	12.48-12.98 (12.70)	****	0-0.1 (0.03)	0-0.01 (0.05)	0.87-0.97 (0.90)	0.19-0.29 (0.23)
***Trichobilharzia* sp. 4**	12.65-13.98 (13.50)	12.48-13.31 (12.93)	4.99-5.99 (5.36)	****	0	0.87	0.19
***Trichobilharzia sp.* A**	13.48-14.98 (14.34)	12.81-14.14 (13.53)	8.82-10.48 (9.53)	9.48-11.31 (10.41)	****	0.87	0.19
T. szidati	10.48-11.81 (11.38)	12.81-13.48 (13.28)	10.82-11.98 (11.38)	10.48-11.65 (11.05)	11.31-12.65 (12.06)	****	0.87
T. physellae	14.31-14.64 (14.50)	14.14-14.31 (14.23)	9.32-9.82 (9.48)	9.48-9.82 (9.68)	12.98-13.31 (13.13)	9.82-11.15 (10.51)	****

Table 10. Interspecific variation among members of *Austrobilharzia*, *Dendritobilharzia* and *Gigantobilharzia* in the nuclear 28s (1034 bp) and mitochondrial cox1 (601 bp) genes. The number of sequences used for 28s/cox1 comparison included: *Austrobilharzia* sp. (1/1), *Dendritobilharzia* sp. 1 (7/5), *Dendritobilharzia* sp. 2 (4/5), *Gigantobilharzia huronensis* (5/6) and *Gigantobilharzia* sp. 2 (1/1). Values are shown as percentages.

Avian schistosomatid species compared	**Interspecific variation for 28S, min-max (avg)**	**Interspecific variation for cox1, min-max (avg)**
***Austrobilharzia* sp. -*Austrobilharzia variglandis* ***	0.30	13.50
***Dendritobilharzia* sp. 1 -*Dendritobilharzia* sp. 2**	0	6.71-7.30 (7.02)
***Gigantobilharzia huronensis* -*Gigantobilharzia* sp. 2**	0-0.37 (0.14)	10.88-11.33 (11.28)

*An *Austrobilharzia variglandis* sequence from GenBank was used for comparison.

Table 11. Intergeneric variation of avian schistosomatids collected in North Dakota. The intergeneric variation in the nuclear 28s partial gene (1098bp) is shown above the diagonal and intergeneric variation in the mitochondrial cox1 gene (578bp) is shown below the diagonal. Variability is shown as % of the sequence length. The min-max values are followed by average in parentheses.

Genera	***Allobilharzia***	***Austrobilharzia***	***Dendritobilharzia***	***Gigantobilharzia***	***Trichobilharzia***
Allobilharzia	*****	11.34	5.28	5.28-5.65 (5.52)	4.10-4.92 (4.61)
Austrobilharzia	-	*****	9.53	9.62-9.98 (9.86)	9.35-10.16 (9.82)
Dendritobilharzia	19.72-20.42 (20.18)	-	*****	2.55-2.73 (2.67)	2.91-4.46 (3.56)
Gigantobilharzia	17.88-20.31 (18.18)	-	20.59-22.67 (22.07)	*****	2.72-4.54 (3.68)
Trichobilharzia	13.74-17.04 (15.42)	-	16.96-21.45 (18.69)	15.80-20.83 (18.34)	*****

excluded from intergeneric comparisons to preserve sequence length of other genera (Table 11). Intergeneric variation for comparisons with *Austrobilharzia* sp. were calculated using only 325 bp and the averages are as follows: *Allobilharzia* (21.54%), *Dendritobilharzia* (23.32%), *Gigantobilharzia* (25.35%) and *Trichobilharzia* (21.18%).

Cercarial Morphology

The measurements for AS cercaria and their variance are shown in Table 12. Not all measurements were taken for all collected species because only a few infected snails were collected for some AS species. There were also lower numbers of cercariae collected from smaller sized snails like *Aplexa*, and *Physa*. *Trichobilharzia querquedulae* was the only AS species for which no measurements were taken due to low numbers of cercaria available after DNA extraction (*Aplexa* is the intermediate snail host) and *Trichobilharzia* sp. 2 only had one snail, with 5 cercariae measured. Difficulty visualizing the oral and ventral suckers for all measured cercariae also produced low, unreliable measurements in some instances, therefore the measurements for oral sucker length/width, and ventral sucker width are not reported.

One way ANOVA tests were performed for all cercarial features measured and values are reported in Table 13. Forebody length was the only feature measured that did not show any significant difference between avian schistosomatid species, $F(3.25) = 0.82$, $p = 0.54$. Post hoc T-tests were then conducted for all other measured cercarial features and results are shown in Table 14. To correct for the problem of performing multiple T-tests simultaneously, the Bonferroni correction method was utilized to adjust the P-value significance cutoff from 0.05 to 0.0006. Most comparisons between *Gigantobilharzia huronensis* and *Trichobilharzia* species resulted in significant differences between cercarial measurements. There were few significant differences in morphological measurements when *Trichobilharzia* species were compared to each other. Comparisons of tail length and body width between *Trichobilharzia* sp. 2 and other *Trichobilharzia* species had the highest number of significant p-values (7). There were also a few (3) significant p-values when *Trichobilharzia* sp. 4 was compared to other *Trichobilharzia* species.

Table 12. Cercarial measurement results for 6 avian schistosomatid species. Measurements were taken for ten cercariae of each species (5 cercariae x 2 snails). Only 5 cercariae were measured from *Trichobilharzia* sp. 2.

Body length	**Avg**	**Median**	**Min-Max**	**StDev**	**CV**	**Tail length**	**Avg**	**Median**	**Min-Max**	**StDev**	**CV**
Gigantobilharzia huronensis	212.8	212.4	195.8-226.5	11.8	5.5	*Gigantobilharzia huronensis*	189.3	192.8	151.0-243.3	30.7	16.2
Trichobilharzia sp. E	291.8	289.1	263.0-326.7	22.7	7.8	*Trichobilharzia* sp. E	337.9	337.3	301.3-365.3	20.6	6.1
Trichobilharzia sp. 2	324.5	316.0	305.0-352.5	19.0	5.8	*Trichobilharzia* sp. 2	393.0	390.0	370.0-416.2	17.3	4.4
Trichobilharzia sp. 4	278.3	271.1	253.1-305.6	16.4	5.9	*Trichobilharzia* sp. 4	294.8	294.5	249.9-322.0	19.6	6.6
Trichobilharzia sp. A	302.1	306.4	269.6-360.0	27.2	9.0	*Trichobilharzia* sp. A	338.3	344.3	308.9-358.0	17.0	5.0
Trichobilharzia szidati	322.7	323.3	284.2-350.7	24.7	7.6	*Trichobilharzia szidati*	314.6	316.8	253.7-373.4	42.7	13.6
Body width						**Furcae length**					
Gigantobilharzia huronensis	74.0	73.8	71.-76.94	1.8	2.5	*Gigantobilharzia huronensis*	102.0	101.1	77.9-122.9	11.9	11.6
Trichobilharzia sp. E	68.3	67.7	52.4-85.1	8.7	12.7	*Trichobilharzia* sp. E	210.1	209.7	174.3-227.8	16.0	7.6
Trichobilharzia sp. 2	90.4	91.4	85.6-92.6	2.8	3.1	*Trichobilharzia* sp. 2	216.6	220.7	202.5-225.1	9.4	4.3
Trichobilharzia sp. 4	67.9	67.6	62.1-77.5	4.5	6.6	*Trichobilharzia* sp. 4	191.3	195.9	170.5-216.8	16.7	8.7
Trichobilharzia sp. A	64.9	62.4	55.7-75.6	7.1	10.9	*Trichobilharzia* sp. A	240.9	235.9	208.4-271.7	21.2	8.8
Trichobilharzia szidati	63.6	65.3	56.068.6	4.6	7.1	*Trichobilharzia szidati*	227.1	230.0	144.9-265.4	34.5	15.2
Forebody length						**Length of furcae projections**					
Gigantobilharzia huronensis	-	-	-	-		*Gigantobilharzia huronensis*	10.8	11.0	7.5-15.6	2.4	22.5
Trichobilharzia sp. E	208.7	209.8	192.8-222.3	12.8	6.1	*Trichobilharzia* sp. E	14.7	15.0	10.5-19.5	2.6	17.6
Trichobilharzia sp. 2	191.2	191.2	145.3-237.1	64.9	33.9	*Trichobilharzia* sp. 2	16.4	16.2	14.5-18.4	1.9	11.4
Trichobilharzia sp. 4	207.1	210.3	194.7-216.4	11.2	5.4	*Trichobilharzia* sp. 4	15.6	15.4	12.6-17.5	1.7	11.0
Trichobilharzia sp. A	222.1	210.2	199.6-255.1	23.9	10.8	*Trichobilharzia* sp. A	16.5	16.8	13.3-20.7	2.5	15.0
Trichobilharzia szidati	226.2	233.3	211.6-233.8	12.7	5.6	*Trichobilharzia szidati*	16.3	16.6	12.4-18.5	2.0	12.2
Ventral sucker length											
Gigantobilharzia huronensis	-	-	-	-							
Trichobilharzia sp. E	20.0	18.9	18.4-22.8	2.4	12.2						
Trichobilharzia sp. 2	25.9	25.9	25.2-26.6	1.0	3.9						
Trichobilharzia sp. 4	31.1	30.3	26.2-36.7	5.3	17.1						
Trichobilharzia sp. A	29.7	29.0	26.2-36.0	3.9	13.0						
Trichobilharzia szidati	23.5	21.8	21.1-27.7	3.0	12.6						

Table 13. Results of ANOVA tests for each cercarial character measured in collected avian schistosomatid cercariae from North Dakota in 2013. P-values below 0.05 are in bold.

Cercariae feature	df between species	df within species	F	F crit	P-value
Tail length	5	48	59.6	2.41	**$2.0x10^{-19}$**
Furcae length	5	48	58.2	2.41	**$3.2x10^{-19}$**
Furcae projection length	5	46	8.47	2.42	**$9.6x10^{-6}$**
Body length	5	48	34.58	2.41	**$8.2x10^{-15}$**
Body width	5	48	18.43	2.41	**$3.6x10^{-10}$**
Ventral sucker length	4	13	5.59	3.18	**0.007**
Forebody length	4	12	0.82	3.26	.54

Table 14. P-value results for cercariae measurements of avian schistosomatids collected in North Dakota. T-tests were used to compare 6 cercariae features measured for 6 avian schistosomatid species. P-values below 0.0006 ($6.0x10^{-4}$) are considered significant and are in bold.

Species being compared	Body width	Body length	Ventral sucker length	Tail length	Furcae length	Furcae projection length
Gigantobilharzia huronensis x Trichobilharzia sp. E	0.068	**$1.7x10^{-7}$**	-	**$1.1x10^{-9}$**	**$5.4x10^{-12}$**	0.003
Gigantobilharzia huronensis x Trichobilharzia sp. 2	**$3.0x10^{-5}$**	**$3.2x10^{-5}$**	-	**$7.0x10^{-10}$**	**$1.6x10^{-9}$**	$6.2x10^{-4}$
Gigantobilharzia huronensis x *Trichobilharzia* sp. 4	0.002	**$1.5x10^{-8}$**	-	**$1.4x10^{-7}$**	**$2.2x10^{-10}$**	**$2.1x10^{-4}$**
Gigantobilharzia huronensis x *Trichobilharzia* sp. A	0.003	**$5.1x10^{-7}$**	-	**$2.2x10^{-9}$**	**$3.8x10^{-11}$**	**$9.3x10^{-5}$**
Gigantobilharzia huronensis x *Trichobilharzia szidati*	**$7.1x10^{-5}$**	**$8.4x10^{-8}$**	-	**$6.8x10^{-7}$**	**$1.5x10^{-6}$**	**$8.0x10^{-5}$**
Trichobilharzia sp. E x *Trichobilharzia* sp. 2	**$8.7x10^{-6}$**	0.015	0.039	**$3.4x10^{-4}$**	0.338	0.189
Trichobilharzia sp. E x *Trichobilharzia* sp. 4	0.922	0.146	0.052	**$1.5x10^{-5}$**	0.020	0.380
Trichobilharzia sp. E x *Trichobilharzia* sp. A	0.357	0.369	0.005	0.970	0.002	0.141
Trichobilharzia sp. E x *Trichobilharzia szidati*	0.163	0.012	0.135	0.279	0.202	0.155
Trichobilharzia sp. 2 x *Trichobilharzia* sp. 4	**$5.5x10^{-8}$**	0.002	0.231	**$3.6x10^{-6}$**	0.003	0.488
Trichobilharzia sp. 2 x *Trichobilharzia* sp. A	**$4.5x10^{-7}$**	0.182	0.200	$8.2x10^{-4}$	0.018	1.869
Trichobilharzia sp. 2 x *Trichobilharzia szidati*	**$1.6x10^{-8}$**	0.884	0.168	**$5.4x10^{-4}$**	0.411	0.946
Trichobilharzia sp. 4 x *Trichobilharzia* sp. A	0.269	0.032	0.718	**$5.2x10^{-5}$**	$2.1x10^{-5}$	0.401
Trichobilharzia sp. 4 x *Trichobilharzia szidati*	0.054	**$4.6x10^{-4}$**	0.116	0.093	0.016	0.459
Trichobilahrzia sp. A x *Trichobilharzia szidati*	0.643	0.102	0.023	0.258	0.322	0.869

Adult Fluke Morphology

There were four species for which complete adult worms were collected, stained and mounted for morphological study. The *Austrobilharzia* sp. collected from an Avocet is shown in Fig 16. Only a single worm was collected so no molecular data is available for this *Austrobilharzia* sp.

Multiple *Dendritobilharzia* sp. 1 and *Dendritobilharzia* sp. 2 specimens were stained and mounted and consisted of both genetically marked and unmarked adult worms. In order to identify morphologic features that could be used to differentiate between the two proposed *Dendritobilharzia* species, genetically marked individuals were examined and are shown in Fig. 17 (female) and 18 (male). Two morphological features that were found to vary between the two species included the position of the genital pore in relation to the cecal bifurcation in male *Dendritobilharzia* specimens. In genetically marked *Dendritobilharzia* sp. 1 specimens, the ceca loop rejoined anterior to the testes and the location of the genital pore was adjacent to the ceca bifurcation point (Fig. 18a). In genetically marked *Dendritobilharzia* sp. 2 specimens, the ceca loop continued into the testes where it rejoined posterior to the first pair of testes (Fig. 18b). The location of the genital pore occurred at the midpoint region of the cecal loop. Another morphological characteristic that appeared to be a possible identifying feature of each species was their overall body shape with *Dendritobilharzia* sp. 1 specimens having a wider body shape and *Dendritobilharzia* sp. 2 specimens having a more slender body shape (Fig 17a,b and Fig 18a,b).

There were few complete adult worms of *Trichobilharzia* due to the difficulty in extracting complete worms from the mesenteric blood vessels of birds and low intensity of infection in most birds. *Trichobilharzia querquedulae* was the only *Trichobilharzia* species in which

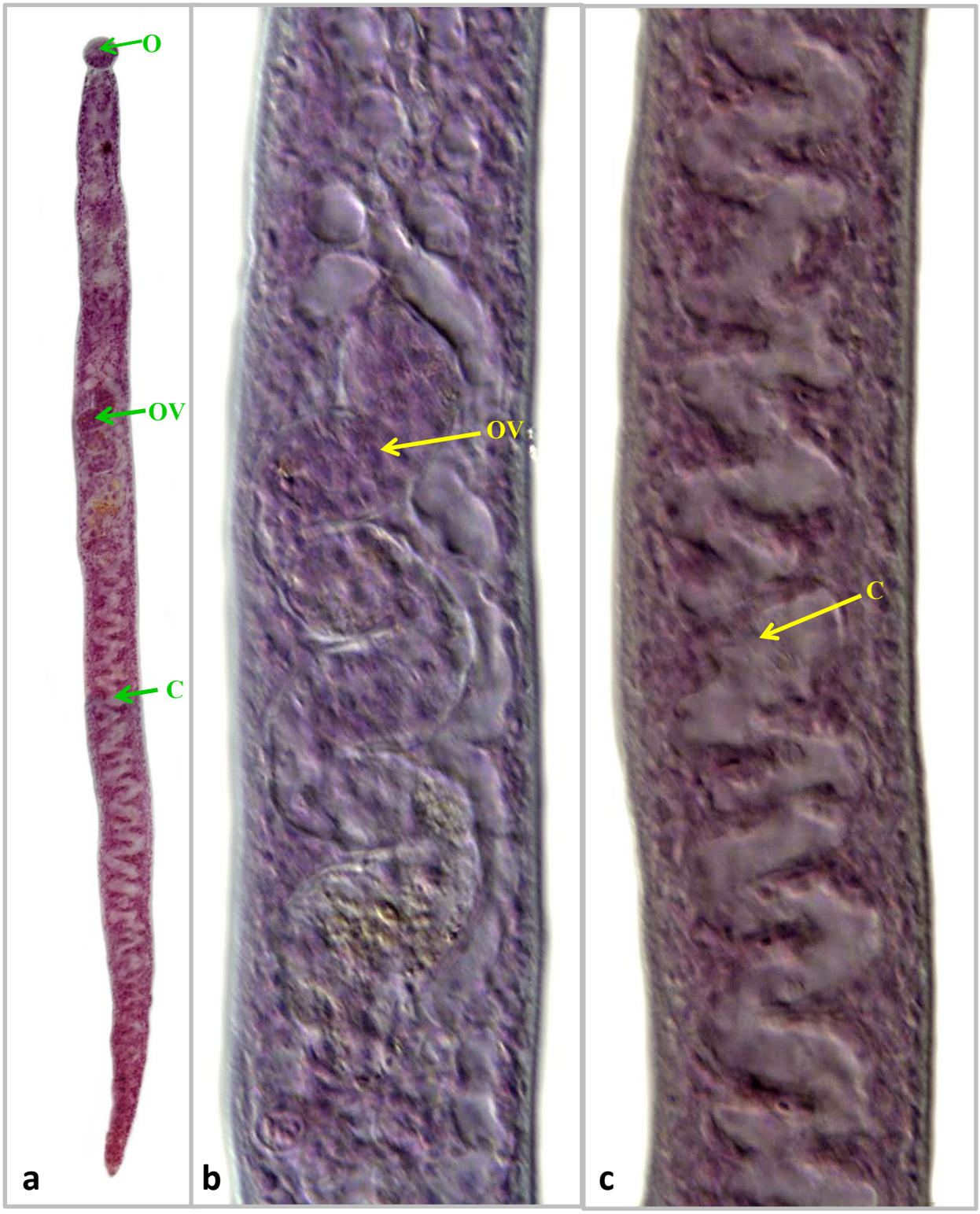

Figure 16. Morphology of a female *Austrobilharzia* sp. collected from an Avocet in North Dakota. (a) Complete specimen, (b) ovary and (c) ceca. OS, oral sucker; OV, ovary; C, ceca.

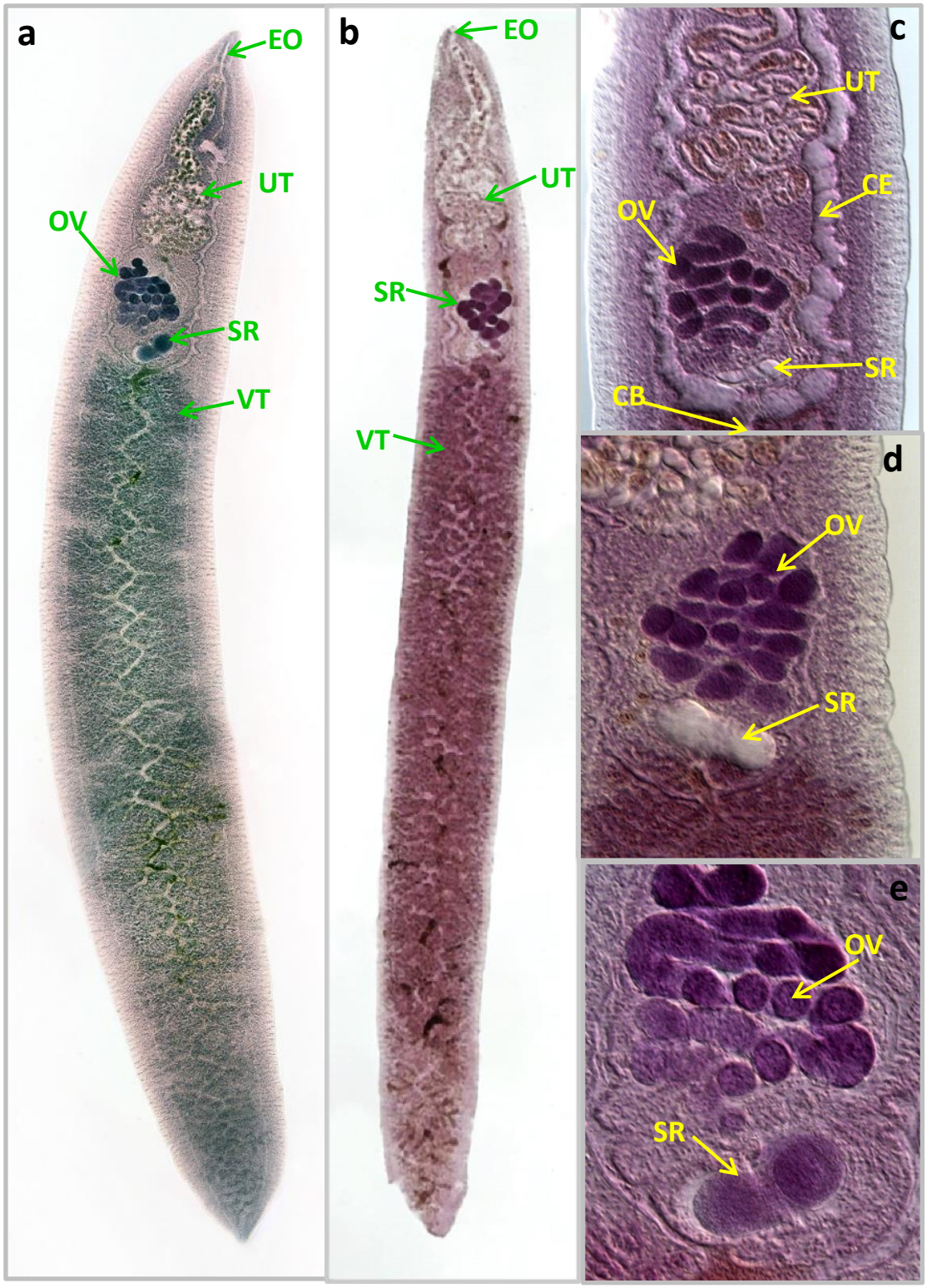

Figure 17. Morphological comparison between *Dendritobilharzia* sp. 1 (a) and *Dendritobilharzia* sp. 2 (b) adult female worms. Reproductive structures are shown in (c). (d) and (e) show an empty and full seminal receptacle respectively. CB, cecal bifurcation; CE, ceca; EO, eosophatus; OV, ovary; SR seminal receptacle; UT, uterus; VT, vitellaria.

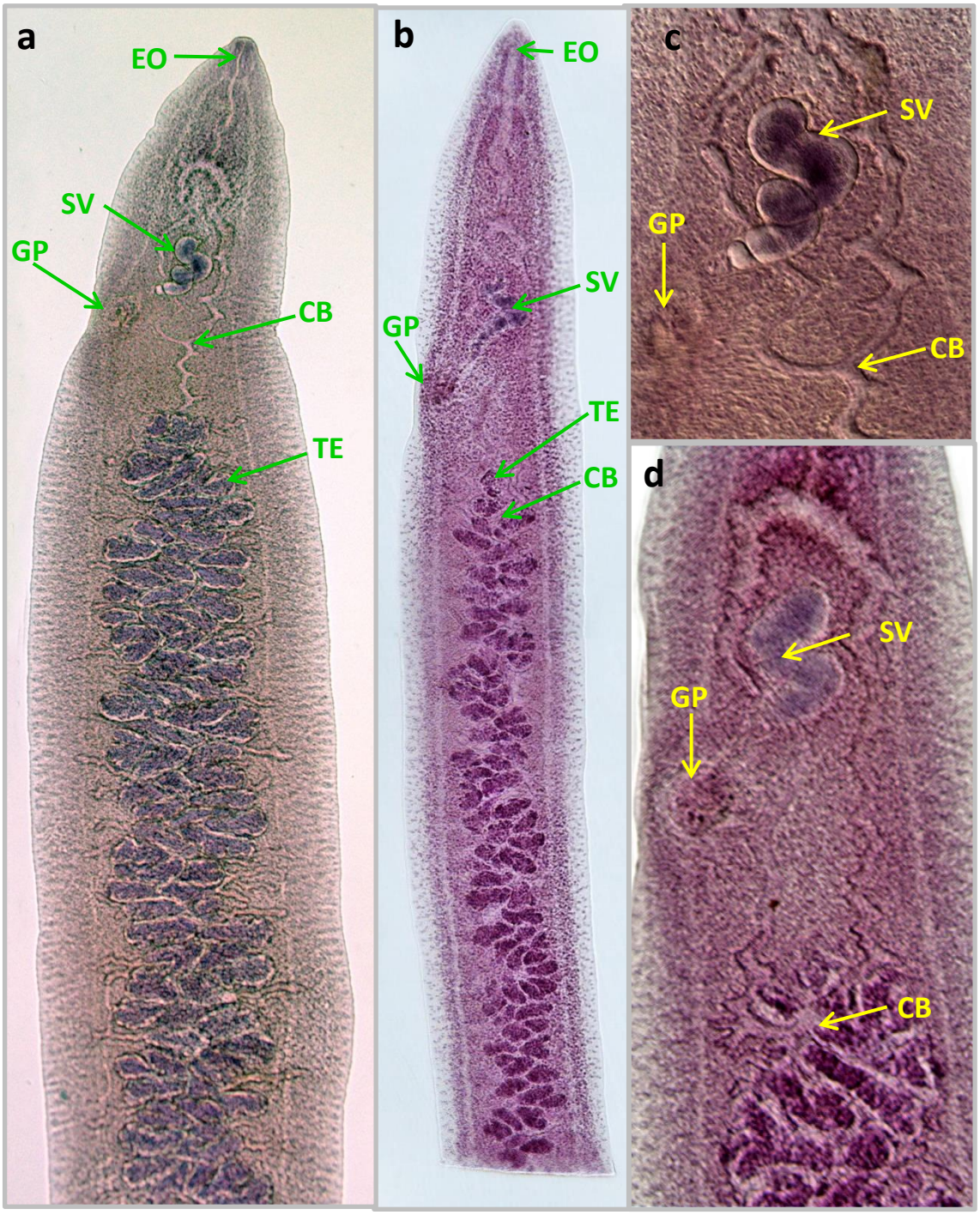

Figure 18. Morphological comparison between *Dendritobilharzia* sp. 1 (a) and *Dendritoblharzia* sp. 2 (b) adult male worms. A closer view of the positioning of the genital pore and cecal bifurcation are shown for *Dendritobilharzia* sp. 1 (c) and *Dendritobilharzia* sp. 2 (d). CB, cecal bifurcation; EO, eosophagus; GP, genital pore; SV, seminal vesicle; TE, testes.

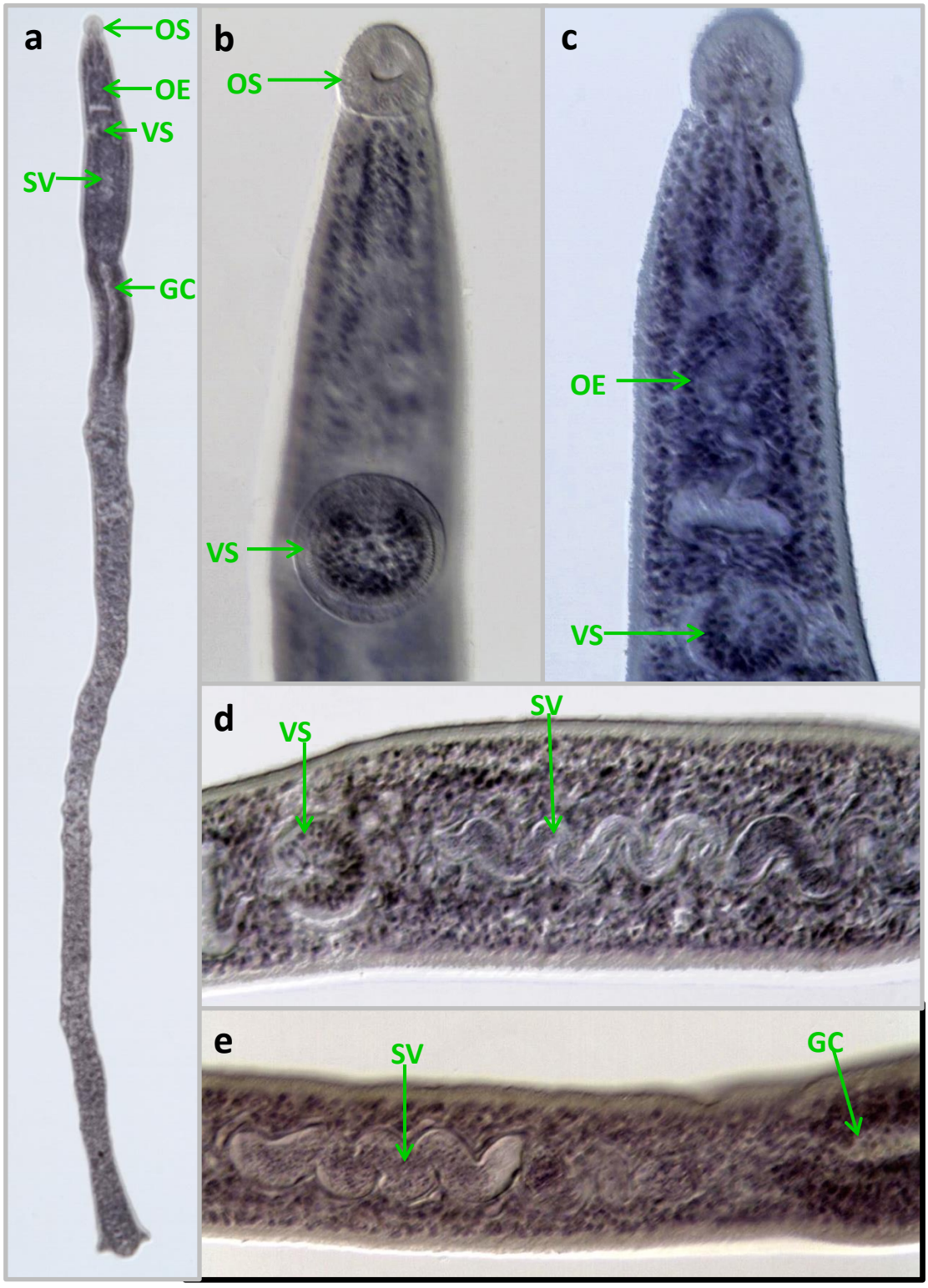

Figure 19. Complete male *Trichobilharzia* specimen (a). Anterior portion of a female *Trichobilharzia* specimen (b)showing the ventral sucker. Anterior portion of a male *Trichobilharzia* specimen (c) Seminal vesicle of a male Trichobilharzia specimen (d and e) and the beginning of the gynaecophoric canal (e). GC,gynaecophoric canal; OE, oesophagus; OS, oral sucker; SV, seminal vesicle; VS, ventral sucker.

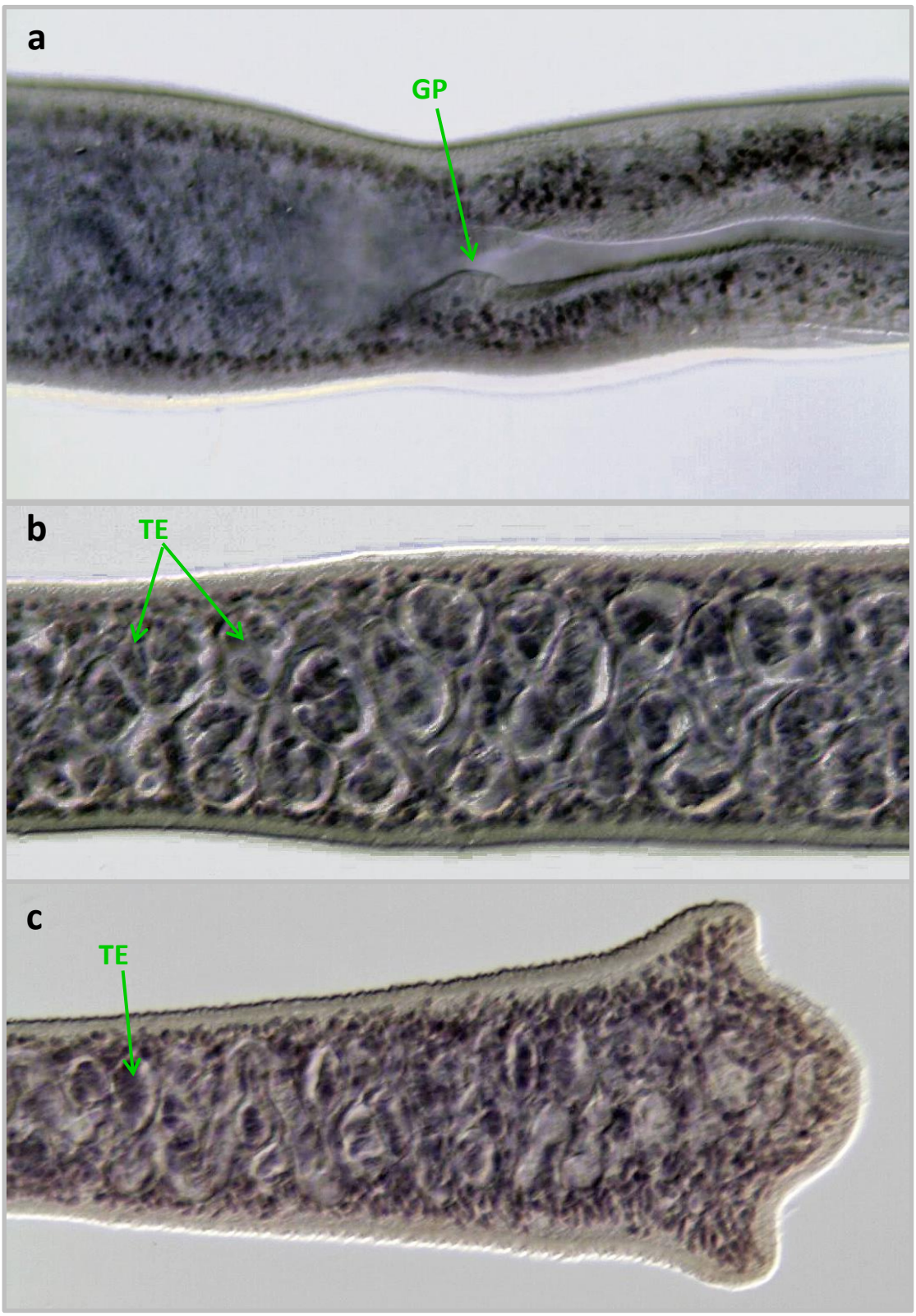

Figure 20. Male *Trichobilharzia* specimen showing the genital opening (a) testes (b) and terminal end of adult (c). GP, genital pore; TE, testes.

complete adult worms were collected in sufficient numbers for morphological study. A few of these worms were used for SEM study. One complete adult worm collected from a shoveler was stained and mounted (Fig. 19 and 20). No molecular data is available for this specimen and it was only identified to genus.

SEM Study of *Trichobilharzia* spp.

We have studied some larval and adult *Trichobilharzia* specimens under scanning electron microscope. SEM study of *Trichobilharzia szidati* cercariae allowed for a detailed observation of the external morphology. Fig. 21a shows the body of the cercaria with a lateral view of the ventral sucker. The fin folds of the bifurcated tail and the projections on the tips of the furcae are shown in Fig. 21b, c. Minute spines were found distributed not only throughout the body of the cercaria (Fig. 21d), but also on the surface of the tail and even furcae (Fig. 21c). Sensory papillae were seen on the body of the cercariae primarily in the zone of suckers (Fig. 21e, f).

SEM study of an adult *Trichobilharzia querquedulae* (Fig. 22) allowed us to examine the external structure of both the oral and ventral suckers, the tegumental spination as well as the external structure of the gynecophoric canal in the male worm (Fig 22). Sensory papillae were also seen distributed throughout the tegumental surface of the adult worm (Fig. 22c).

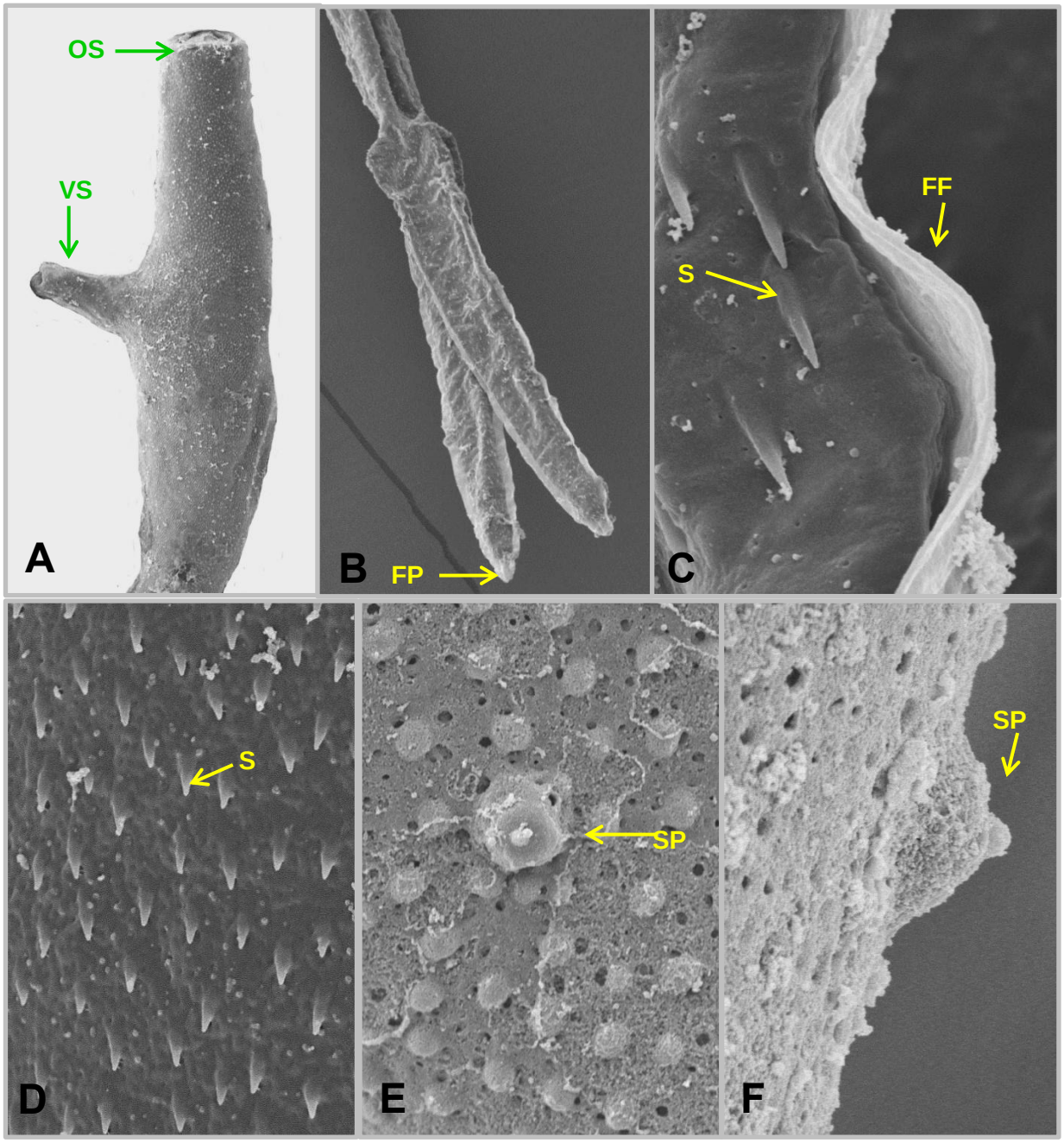

Figure 21. SEM images of a *Trichoiblharzia szidati* cercaria. A) Body of cercaria with ventral (VS) and oral sucker (OS). B) Tail furcae (FC) and furcae projections (FP) on the tail tips. C) Closer view of spines (S) and fin fold (FF) present on tail. D) Spines (S) on body of cercariae. E) Ventral and F) lateral view of sensory papillae (SP) on body of cercaria.

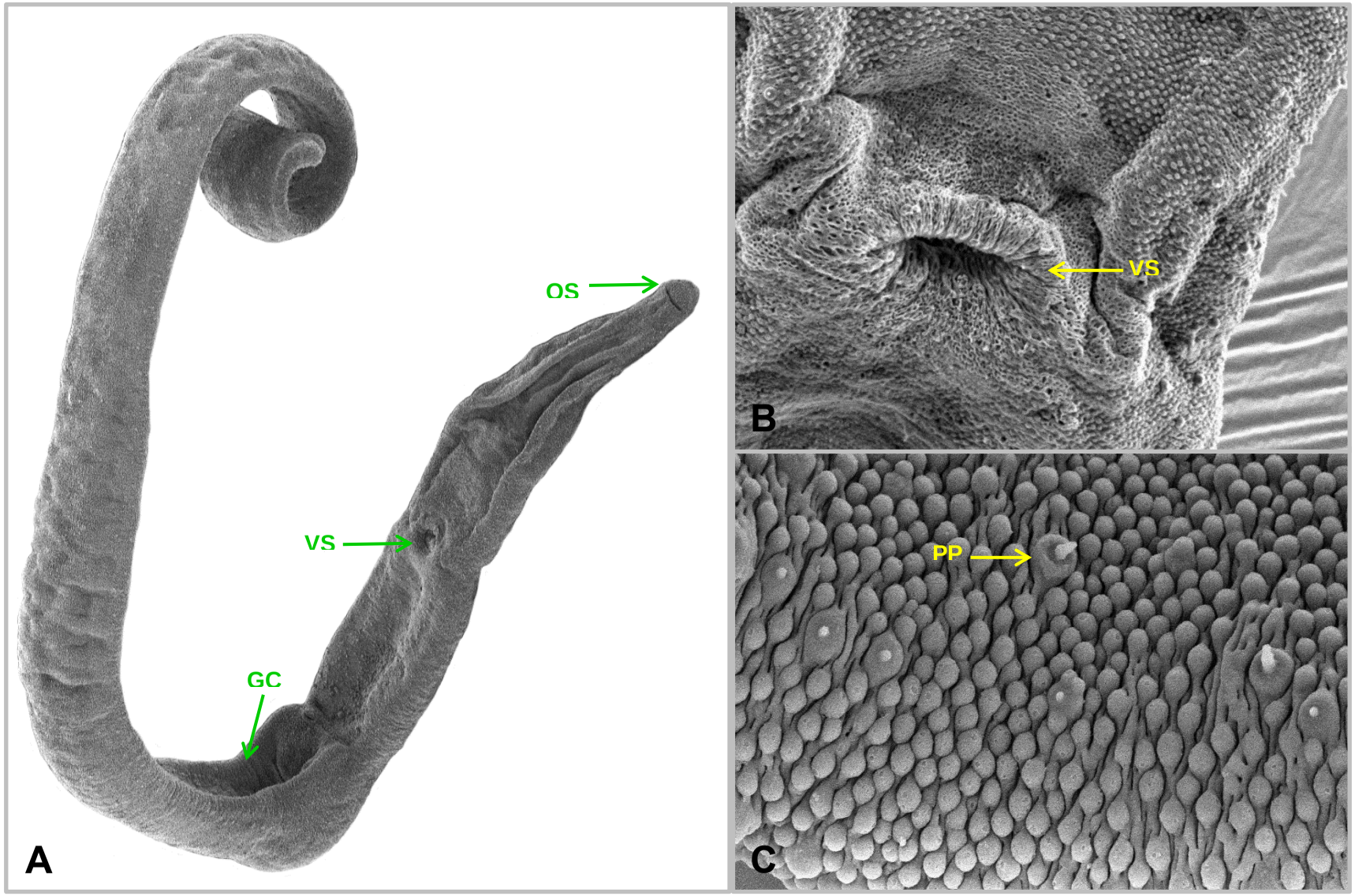

Figure 22. SEM images of a *Trichoiblharzia querquedulae* adult worm. A) Partial anterior portion of an adult male with visible oral (OS) and ventral (VS) sucker and the anterior portion of the gynecophoric canal (GC). B) Closer view of the ventral sucker (VS). C) Papillae (PP) on tegumental surface.

Phylogenetic Analysis

A total of 8 trees were generated using maximum likelihood and Bayesian analysis for the 4 alignments described in Chapter II. The nuclear 28s rDNA alignment of Schistosomatidae genera included 91 sequences (1100 bp) with 26 sequences obtained from avian schistosomatid specimens collected in North Dakota (13 species, 5 genera). Sequences also included 1) one sequence of a mammalian schistosomatid species collected from a lymnaeid snail in North Dakota, *Schistosomatium douthitti*, 2) one sequence of a *Gigantobilharzia* species collected from a Sandwich tern (*Thalasseus sandvicensis*) in Mississippi, 3) one sequence from a *Bilharziella polonica* obtained from a Pochard (*Aythia ferina*) collected in the Ukraine and 4) two *Macrobilharzia macrobilharzia* sequences obtained from Anhingas (*Anhinga anhinga*) collected in Mississippi. The remaining sequences were taken from the GenBank database. A total of 14 Schistosomatidae genera and >30 recognized species are represented as well as 16 avian schistosomatid sequences with unknown systematic placement. Several regions throughout the world are represented including North America, South America, Asia, Africa, Europe and Australia with the majority of sequences obtained from snails and birds collected in North America.

Bayesian and Maximum Likelihood both produced trees of similar topologies with Bayesian analysis resulting in more strongly supported nodes. The Bayesian and Maximum Likelihood nuclear 28s rDNA trees are each separated into two figures due to better visualization of relationships between taxa (Figs. 23-26). The systematic positions of *Trichobilharzia*, *Allobilharzia* and *Anserobilharzia* are shown in Fig. 24 (Bayesian analysis) and Fig. 26 (Maximum Likelihood analysis). There is a difference in topology between the two trees regarding the placement of *Trichobilharzia physellae* within the *Trichobilharzia* sp. A,

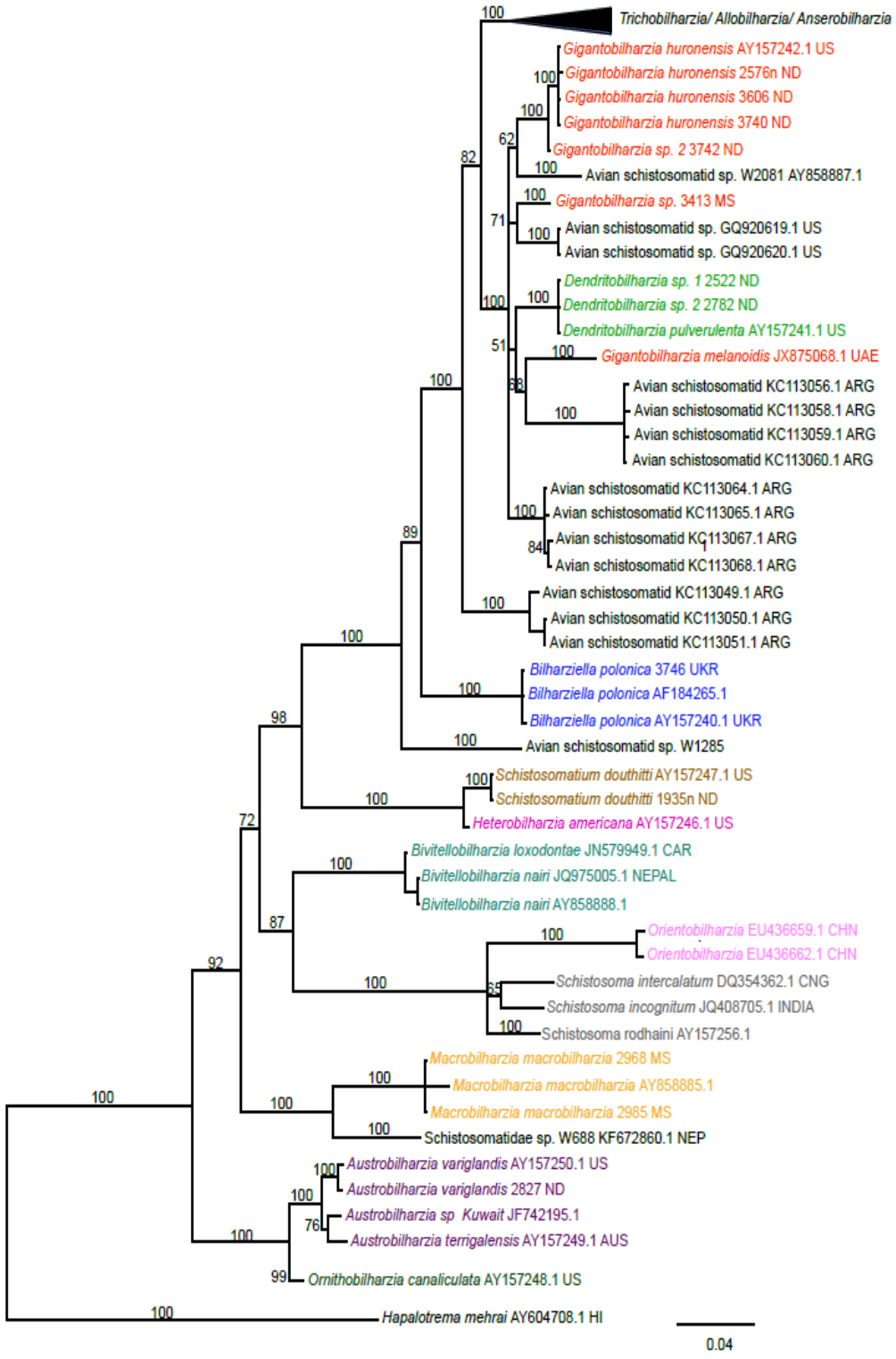

Fig 23. Nuclear 28s rDNA Bayesian tree showing interrelationships among genera of the Schistosomatidae. Sequences obtained in this study are marked with "ND". Posterior probability values are shown above the internodes. Collapsed part of the tree is presented on Figure 23. Genera are color coded to show monophyletic and non-monophyletic lineages. Posterior probability values are shown.

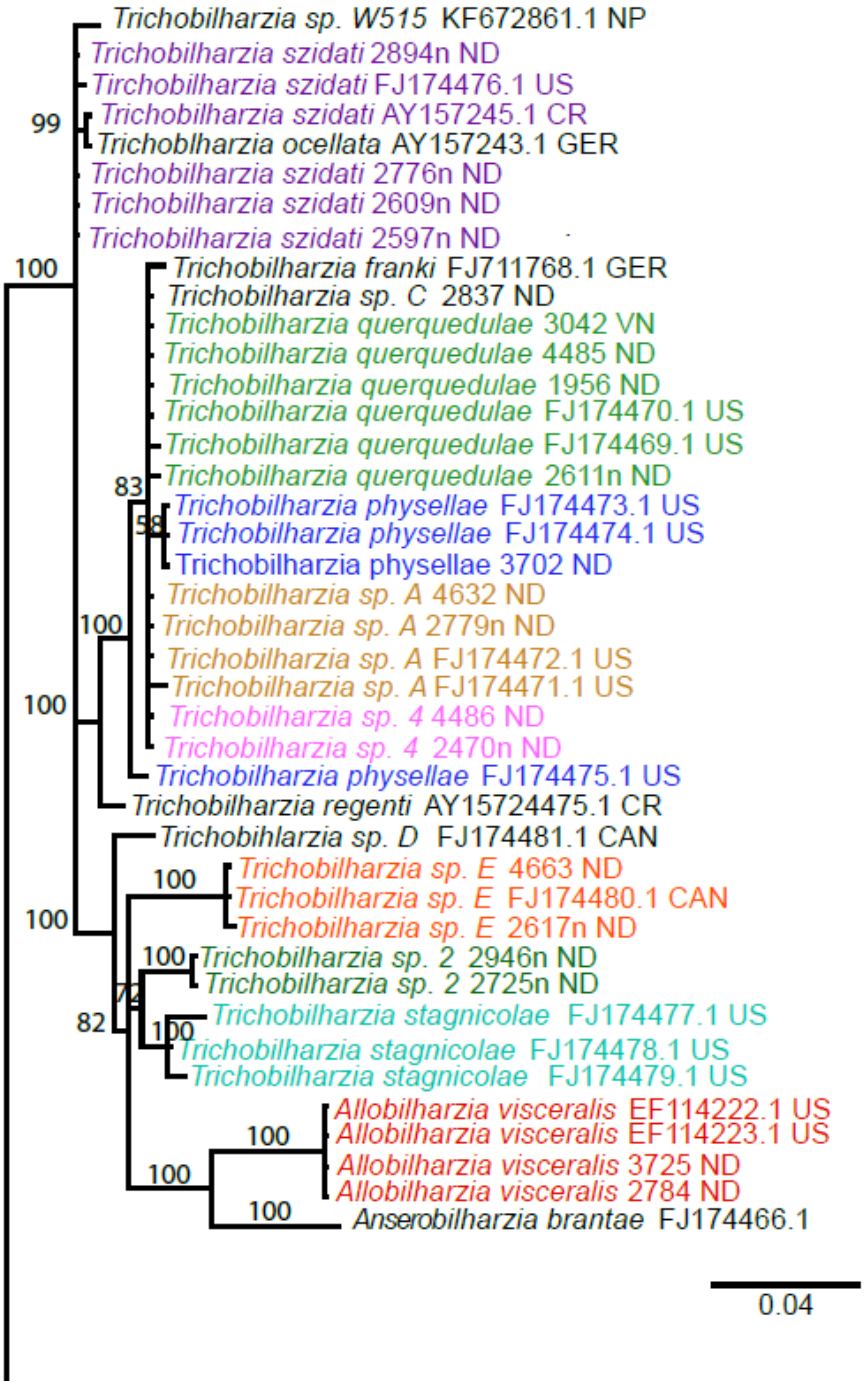

Figure 24. A fragment of the 28S Bayesisn tree from Fig. 22 showing interrelationships among derived genera *Trichobilharzia*, *Allobilharzia* and *Anserobilharzia*. *Trichobilharzia* species are color coded to show monophyletic and non-monophyletic lineages. Posterior probability values are shown at the internodes.

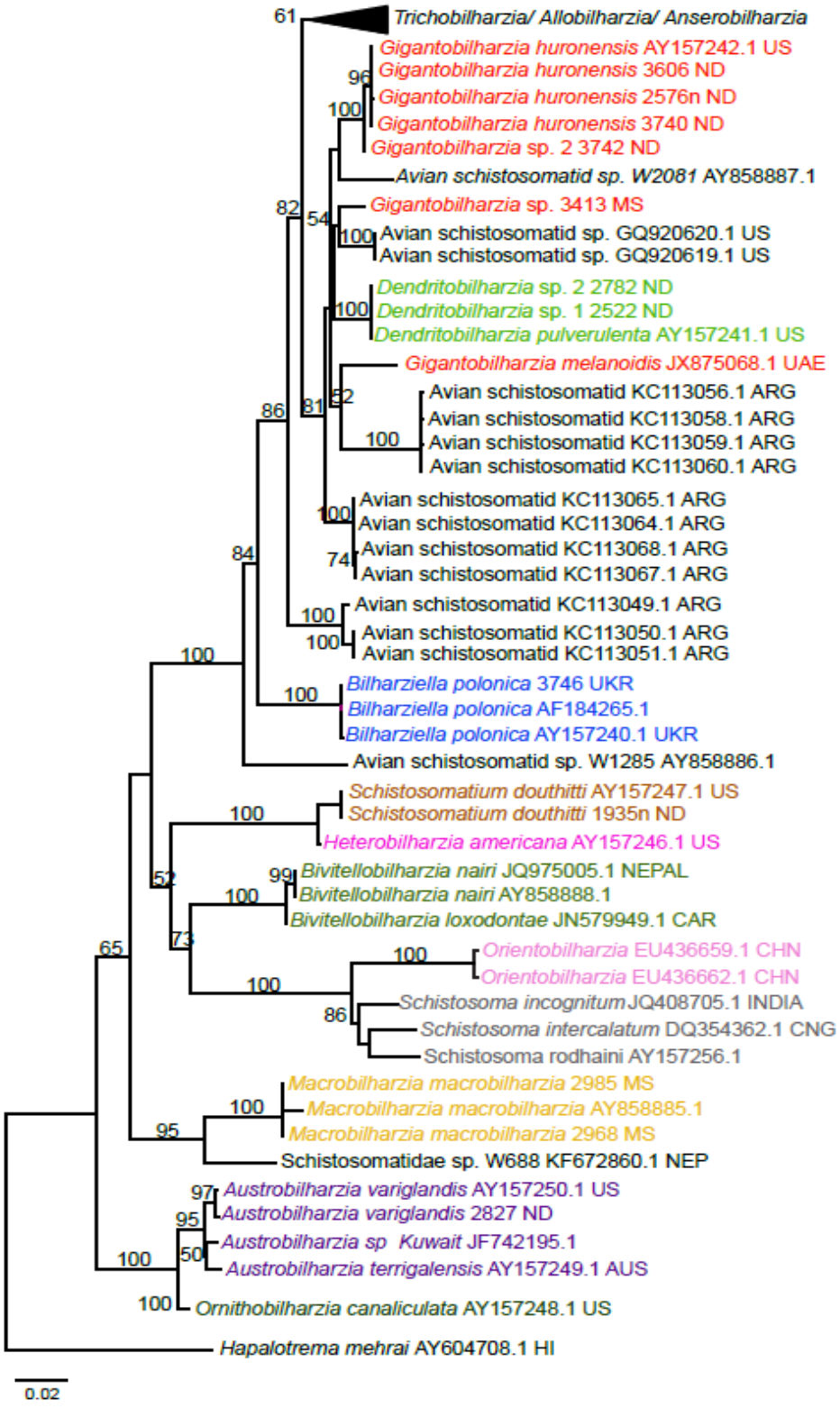

Figure 25. Nuclear 28s rDNA Maximum likelihood tree showing interrelationships among genera of the Schistosomatidae. Sequences obtained in this study are marked with "ND". Bootstrap values are shown above the internodes. Collapsed part of the tree is presented on Figure 25. Genera are color coded to show monophyletic and non-monophyletic lineages. Bootstrap values are shown above the internodes.

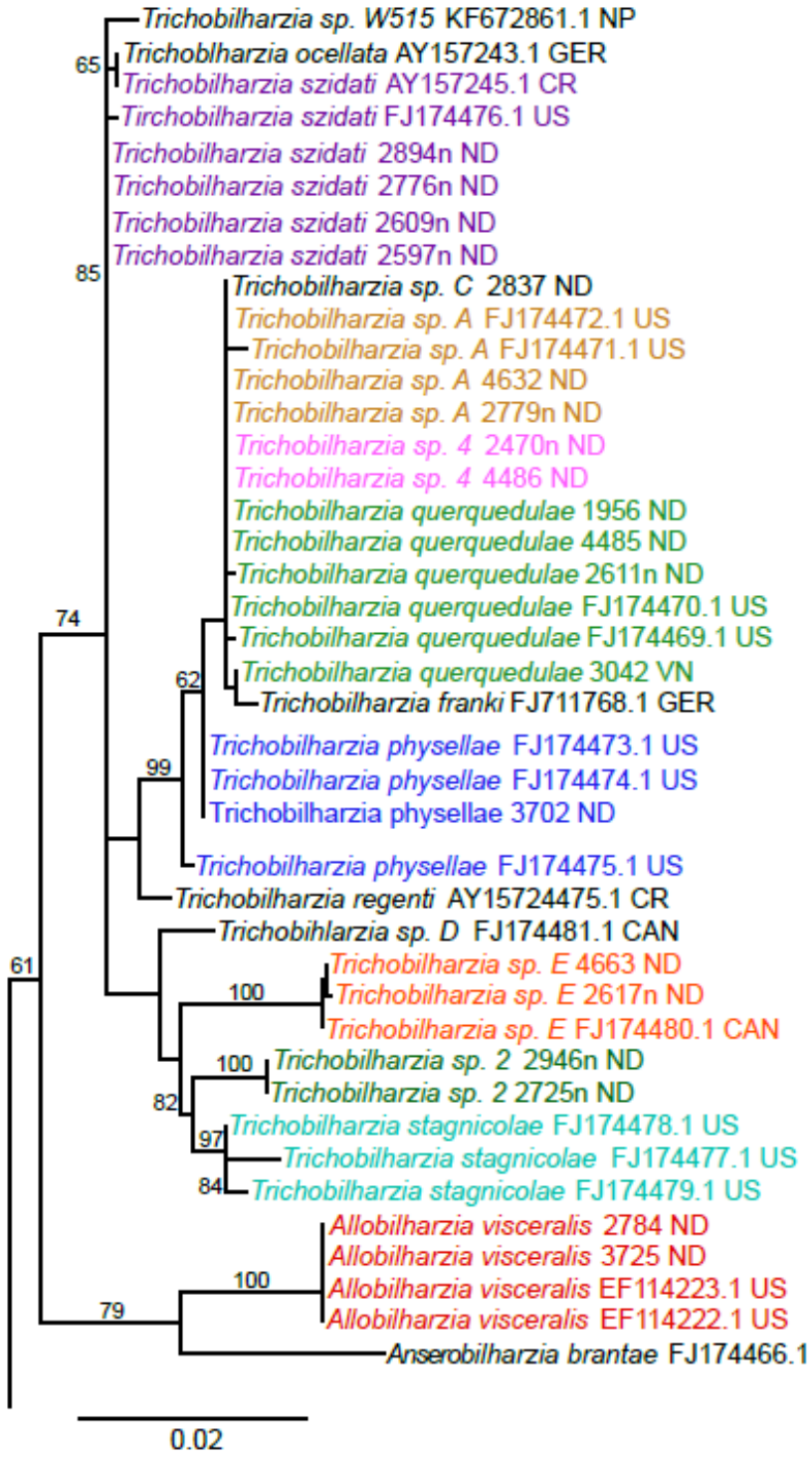

Figure 26. A fragment of the 28s rDNA Maximum Likelihood tree from Fig. 24 showing interrelationships among derived genera *Trichobilharzia*, *Allobilharzia* and *Anserobilharzia*. *Trichobilharzia* species are color coded to show monophyletic and non-monophyletic lineages. Bootstrap values are shown at the internodes.

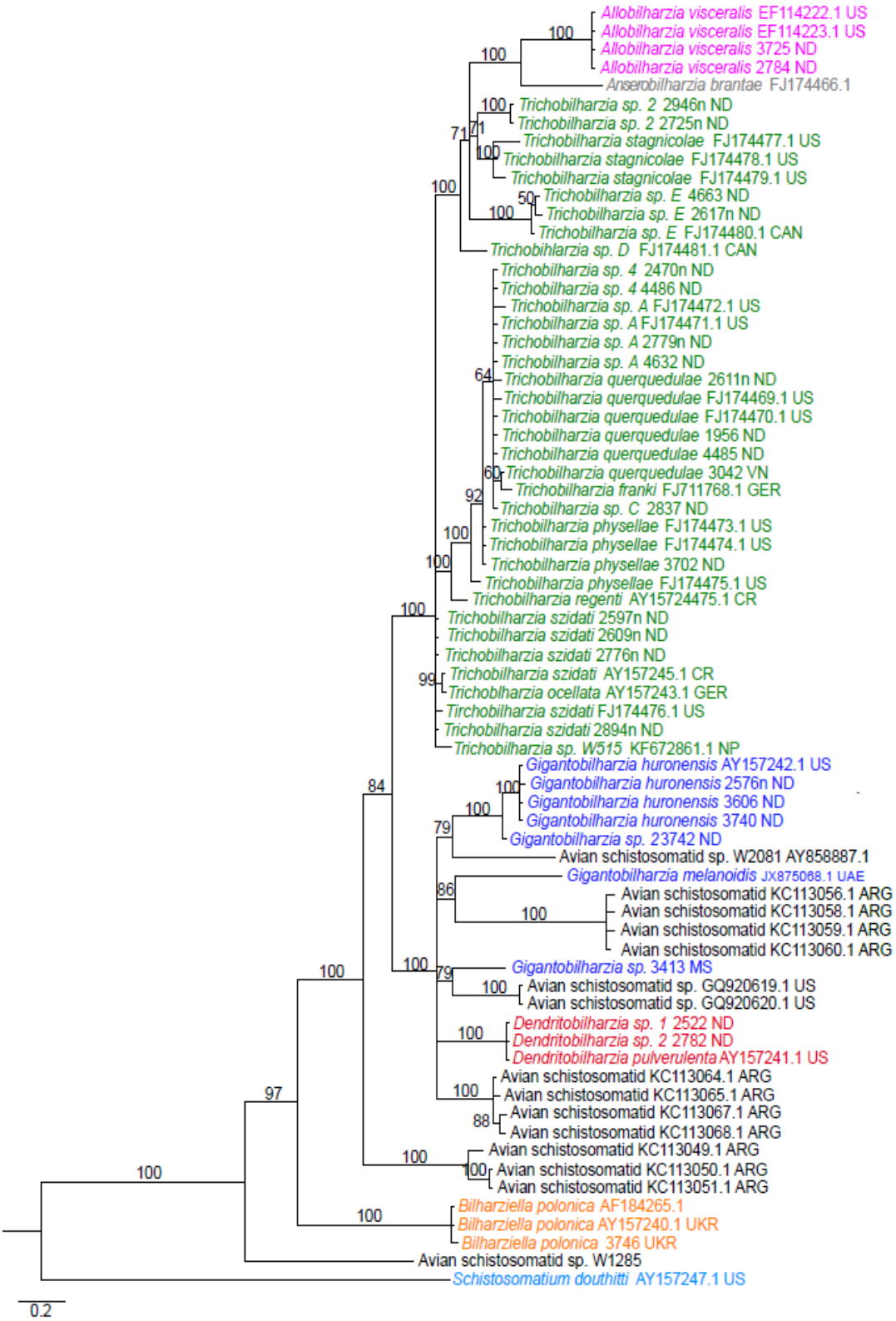

Figure 27. Nuclear 28s rDNA Bayesian tree of the derived avian schistosomatid clade. Genera are color coded to show monophyletic and non-monophyletic lineages. Posterior probabilities are shown.

Trichobilharzia querquedulae, *Trichobilharzia* sp. 4 and *Trichobilharzia franki* clade. There is also a difference in tree topology regarding the positioning of the genera *Allobilharzia* and *Anserobilharzia* in relation to *Trichobilharzia*. These two genera are positioned within the *Trichobilharzia* clade in the Bayesian tree but are positioned as a sister taxon to *Trichobilharzia* in the Maximum Likelihood tree. The remaining Schistosomatidae genera are shown in Fig 23 (Bayesian analysis), and Fig. 25 (Maximum likelihood). The tree topology is almost identical in both trees except for slight changes in relative position of weakly supported branches that are treated as polytomies.

The phylogenetic tree on Fig. 26 is based on Bayesian analysis of 28s sequences of members of the derived avian schistosomatid clade consisting of *Trichobilharzia, Allobilharzia, Gigantobilharzia, Dendritobilharzia, Bilharziella* and *Anserobilharzia* with *Schistosomatium* used as an outgroup. The taxon set in this tree is compatible with that used by Brant et al. (2013) and Brant and Loker (2013). The topology in this tree is almost identical to the 28s Bayesian tree containing all Schistosomatidae genera (Fig. 23) except for slight changes in the polytomies containing *Gigantobilharzia, Dendritobilharzia* and avian schistosomatids from Argentina.

Increased variability within the mitochondrial cox1 gene allowed for finer resolution within genera, however, this high nucleotide variability also produced phylogenetic trees with low node support for both Maximum likelihood and Bayesian analyses when multiple genera were included in the alignments. The mitochondrial cox1 phylogenetic trees of the derived avian schistosomatid clade (*Trichobilharzia, Gigantobilharzia, Bilharziella, Dendritobilharzia, Allobilharzia* and *Anserobilharzia*) produced trees with multiple polytomies and low branch support (trees not shown). Only the Bayesian analyses of alignments containing one genera (570 bp) produced tree topologies with moderate support and they are depicted in Figs. 28 and 29.

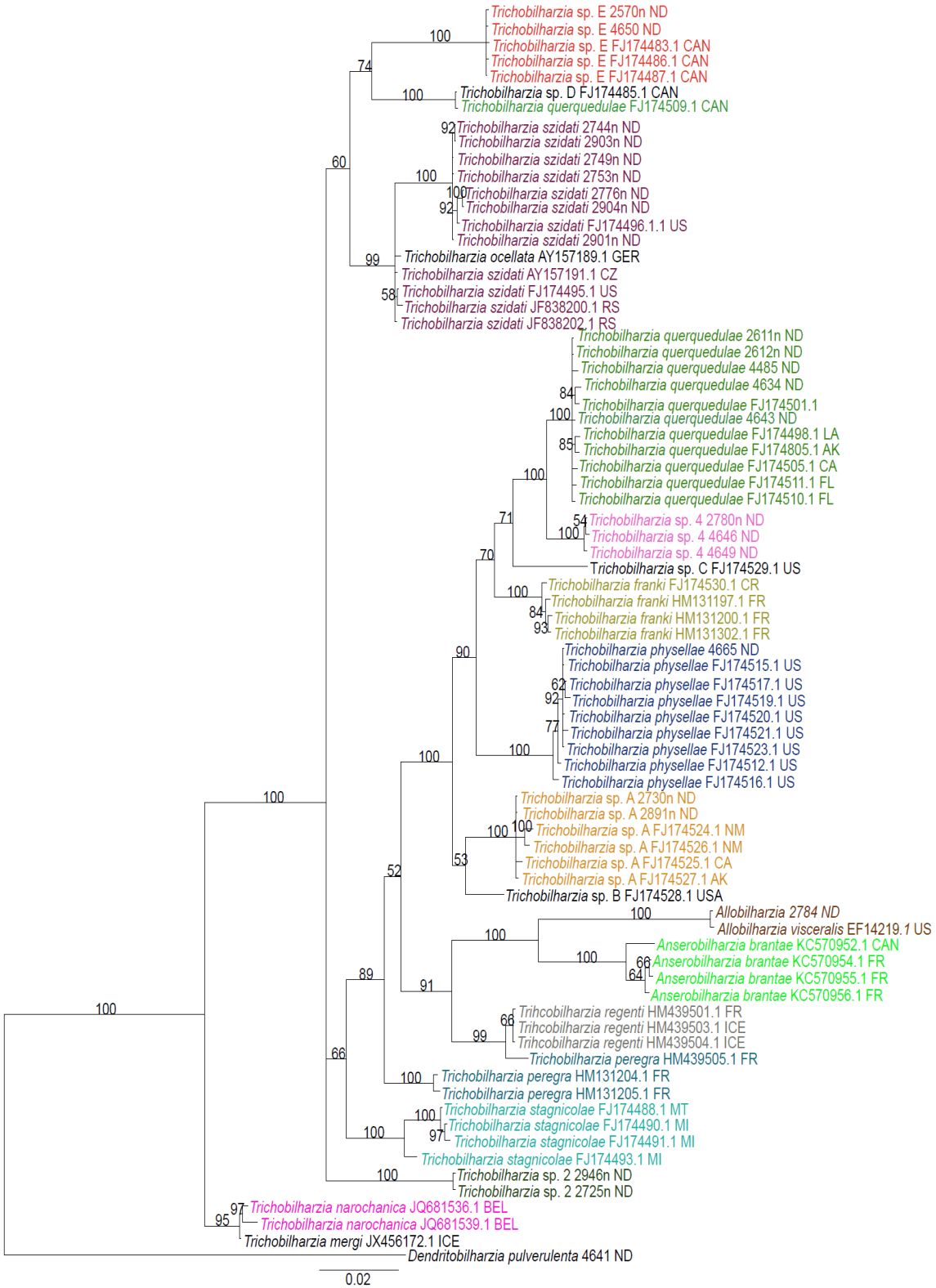

Figure 28. Mitochondrial cox1 Bayesian tree of the genus *Trichobilharzia*. Species are color coded to demonstrate monophyletic and paraphyletic grouping within species. Species with only one representative are in black. Posterior probabilities are shown.

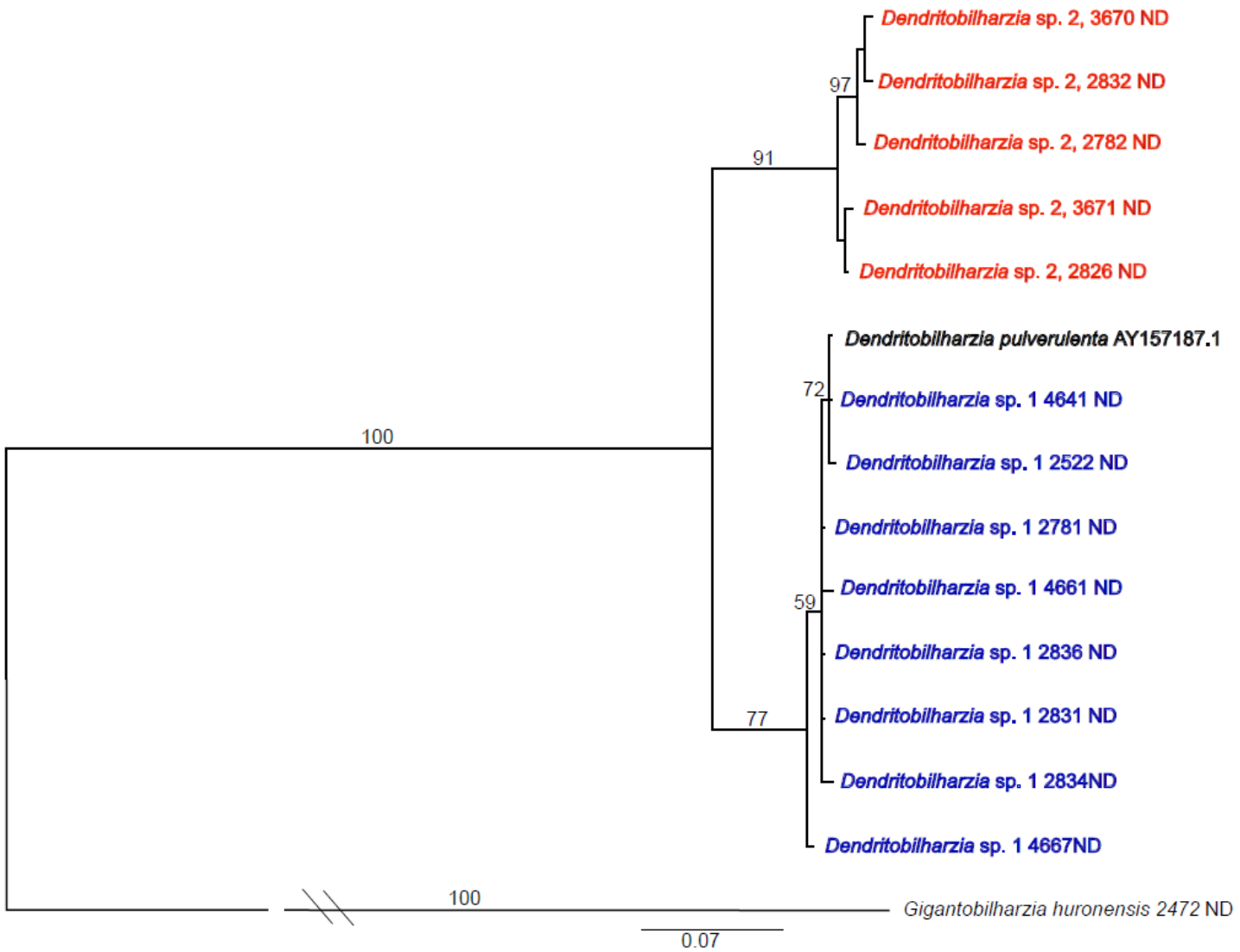

Figure 29. Mitochondrial cox1 Bayesian tree of *Dendritobilharzia* specimens collected in North Dakota and one sequence from GenBank. *Dendritobilharzia* sp. 1 sequences are highlighted in blue and *Dendritobilharzia* sp. 2 sequences are highlighted in red.

Fig. 28 shows the systematic positioning between *Trichobilharzia* species. There is separation between the species *Trichobilharzia querquedulae*, *Trichobilharzia* sp. 4, *Trichobilharzia franki*, *Trichobilharzia physellae* and *Trichobilharzia* sp. A into separate taxa. These species were grouped into the same clade during Bayesian and Maximum Likelihood analyses of the nuclear 28s rDNA gene. *Trichobilharzia szidati*/*Trichobilharzia ocellata* formed a strongly supported (99%) clade. However, the interrelationships among taxa belonging to this clade were not well resolved. Sequences from specimens collected in Europe and Russia (with the exception of a single US specimen) and published as *T. ocellata* and *T. szidati* formed a polytomy. In contrary, all specimens of *T. szidati* collected by us in North Dakota formed a 100% supported clade. This suggests a possibility that the present *T. szidati* may in fact contain more than one species. *Allobilharzia* and *Anserobilharzia* are also positioned within the *Trichobilharzia* clade in this Maximum Likelihood tree of the mitochondrial cox1 gene.

The final tree (Fig. 29) was generated using Bayesian methods and includes only *Dendritobilharzia* mitochondrial cox1 gene sequences from specimens collected in the United States. The proposed species, *Dendritobilharzia* sp. 1 and *Dendritobilharzia* sp. 2, separate into two distinct clades supporting the results of the pairwise sequence comparisons presented above (Table 10).

Spatial and Occupancy Analysis of 2013 Snail Data

Spatial autocorrelation was only seen in sites where *Lymnaea* snails were present, indicating non-independence for these sites (Table 15). Sites within 25 km of each other showed the highest level of spatial autocorrelation (Moran's I=0.348, p-value=0.002). The distance class which included sites within 65 km of each other also showed a significant level of spatial autocorrelation (Moran's I= 0.146, p-value=0.006). No spatial autocorrelation was detected in

sites where *Stagnicola* and *Aplexa* snails were collected or sites where AS infected *Lymnaea*, AS infected *Stagnicola* or AS infected *Aplexa* snails were collected.

Sites were divided into 7 land cover (Table 16) and 4 ecoregions (Table 17). Because of small sample sizes, three land cover (deciduous forest, n=3; developed, n=1; fallow crop, n=2) and one ecoregion category (Lake Agassiz Plain, n=2) were removed from the analysis. The Bonferroni correction method was used to adjust the significance cutoff (exact-p) from 0.05 to 0.0013 (ecoregion) and 0.017 (land cover). The Fisher's exact-test showed a significant association between land cover and the presence of *Lymnaea* snails, exact p=0.0005 (2-tailed test) (Table 18). Herbaceous wetlands had the highest occupancy rate for *Lymnaea* snails (Table 16). There was no significant difference in the occupancy rate of *Stagnicola* and *Aplexa* snails based on land cover or ecoregion.

Logistic regression (Table 19) revealed land cover (herbaceous wetlands, grass pastures and open water) and latitude/longitude to be the best predictors for the presence of *Lymnaea* snails at a site. Date of sampling was also shown to be a good predictor for the presence of *Aplexa* snails. No models were found to be good predictors for *Stagnicola* presence and for the presence of AS infected *Lymnaea* snails, AS infected *Stagnicola* snails and AS infected *Aplexa* snails.

Table 15. Spatial autocorrelation results for Moran's I. This analysis was performed on snail collection sites where *Lymnaea*, *Stagnicola*, or *Aplexa* snails were collected as well as sites where AS infected *Lymnaea*, AS infected *Stagnicola* and AS infected *Aplexa* snails were collected. The 105 sites were divided into 12 distance classes with equal number of pairs (count=908). Significance was tested using 500 permutations. P-values < 0.05 are in bold.

	LymPres		**StagPres**		**AplPres**	
DistCntr (km)	**Moran's I**	**P-value**	**Moran's I**	**P-value**	**Moran's I**	**P-value**
24.381	0.348	**0.002**	0.029	0.482	0.038	0.368
64.739	0.146	**0.006**	0.017	0.726	-0.014	0.732
94.745	0.064	0.134	-0.049	0.278	-0.031	0.494
120.624	-0.058	0.188	-0.044	0.308	0.016	0.722
143.569	-0.12	**0.012**	-0.032	0.454	-0.023	0.608
164.827	-0.103	**0.024**	-0.004	0.936	-0.009	0.858
188.173	-0.018	0.668	-0.034	0.426	-0.056	0.206
216.157	-0.108	**0.028**	-0.057	0.24	0.013	0.758
243.721	-0.121	**0.006**	0.04	0.365	-0.058	0.156
275.975	-0.204	**0.002**	0.018	0.668	-0.017	0.682
329.247	0.19	**0.002**	-0.008	0.826	0.052	0.252
494.422	-0.126	**0.006**	0.007	0.826	-0.014	0.638
	Lym_AS_Pres		**Stag_As_Pres**		**Apl_AS_Pres**	
DistCntr (km)	**Moran's I**	**P-value**	**Moran's I**	**P-value**	**Moran's I**	**P-value**
24.381	-0.026	0.564	0.028	0.49	0.029	0.528
64.739	-0.012	0.764	-0.025	0.556	-0.022	0.588
94.745	-0.035	0.412	-0.005	0.908	-0.048	0.262
120.624	0.004	0.938	0.018	0.662	-0.089	**0.048**
143.569	0.016	0.674	-0.076	0.088	0.038	0.358
164.827	-0.071	0.1	-0.077	0.084	0.048	0.29
188.173	0.037	0.408	0.002	0.968	-0.01	0.836
216.157	-0.042	0.362	0.06	0.162	0.033	0.46
243.721	-0.048	0.254	0.035	0.428	-0.053	0.162
275.975	0.079	0.062	-0.112	**0.02**	-0.077	0.062
329.247	-0.016	0.706	0.038	0.36	0.033	0.416
494.422	0.002	0.926	0.002	0.936	0.004	0.87

Table 16. The number of sites for each land cover category in which *Lymnaea*, *Stagnicola* and *Aplexa* snails were present or absent. n=the number of sites for each category.

Land Cover	Crop (n=24)	Deciduous forest (n=3)	Developed (n=1)	Fallow Crop (n=2)	Grass Pasture (n=36)	Herbaceous wetland (n=29)	Open Water (n=10)
***Lymnaea* Present**	12	2	0	1	15	26	5
***Lymnaea* Absent**	12	1	1	1	21	3	5
***Stagnicola* Present**	20	3	1	2	33	25	8
***Stagnicola* Absent**	4	0	0	0	3	4	2
***Aplexa* Present**	4	0	0	0	10	6	1
***Aplexa* Absent**	20	3	1	2	26	23	9

Table 17. The number of sites for each ecoregion category in which *Lymnaea*, *Stagnicola* and *Aplexa* snails were present and absent. n= the number of sites for each ecoregion category.

Ecoregion	Northwestern Glaciated Plains (n=34)	Northwestern Great Plains (n=7)	Northern Glaciated Plains (n=62)	Lake Agassiz Plain (n=2)
***Lymnaea* Present**	16	4	40	1
***Lymnaea* Absent**	18	3	22	1
***Stagnicola* Present**	30	7	53	2
***Stagnicola* Absent**	4	0	9	0
***Aplexa* Present**	7	0	14	0
***Aplexa* Absent**	27	7	48	2

Table 18. Fisher's exact test results showing any significant associations between presence/absence of snail genera (*Lymnaea*, *Stagnicola* and *Aplexa*) and ecoregion or land cover. Exact p-values < 0.013 (ecoregion) and <0.017 (landcover) are considered significant (2-tailed) and are in bold.

	LandCover		Ecoregion	
	Df	Exact P	df	Exact P
LymPres	3	**0.0005**	2	0.298
StagPres	3	0.761	2	0.641
AplPres	3	0.569	2	0.463

Table 19. Best generalized linear models for the prediction of snail genera presence and infected snail genera presence. LCgp=grass pasture, LChw, herbaceous wetland, LCow, open water, Long=longitude, Jdate=date

	***Lymnaea* presence**	**AS infected *Lymnaea* presence**	***Stagnicola* presence**	**AS infected *Stagnicola* presence**	***Aplexa* presence**	**AS infected *Aplexa* presence**
Best model	LCgp, p=0.003 LChw, p<.001 LCow, p=0.13 Long, p<.001	-	-	-	Jdate, p<.001	-
Null deviance	130.725	-	-	-	93.068	-
Residual deviance	90.315	-	-	-	77.337	-
AIC	102.32	-	-	-	81.837	-

CHAPTER IV

DISCUSSION

This study was primarily focused on the examination of intermediate snail hosts because it would better depict the regional diversity and distribution of avian schistosomatids in North Dakota. Since these aquatic snails are confined to a water body, the assumption can be made that transmission of the AS species found in snails occurs locally. Avian schistosomatids found in birds on the other hand, cannot be assumed to complete their life cycle within North Dakota because the examined birds included adult migratory birds. This has resulted in finding AS species for which adult flukes do not have a corresponding cercarial match from snails. The opposite case, in which AS cercariae are collected but their corresponding adult fluke stage is not found within birds also occurred. In the latter case, two different scenarios are possible. We either did not collect an appropriate bird host species or we could have collected the hosts, but did not find a parasite due to small sample size.

Intermediate Snail Hosts

The AS prevalence in snails collected in North Dakota is similar to what was found in previous studies which have shown an average AS prevalence between 1-5% (Loy and Haas 2001). The majority of our sampling effort was focused on the collection of *Stagnicola* and *Lymnaea* snails. Fewer numbers were collected for other snail genera; therefore, comparisons of AS prevalence in snail genera may be biased.

Stagnicola and *Lymnaea* were the most common snail genera in North Dakota wetlands. Their large size and high numbers make them an ideal intermediate host in these aquatic habitats.

Other snail genera/species that were not sampled or sampled insufficiently (e. g., *Gyraulus*) may also play an important role in AS transmission. Since the majority of wetlands that were sampled in 2013 were of similar size and vegetative structure, it is likely that other snail taxa are present in other types of aquatic habitats. Planorbidae and Physidae snails, which were not collected in large numbers during this study, are known to serve as intermediate hosts for multiple avian schistosomatid species, including *Dendritobilharzia*, *Trichobilharzia*, and *Anserobilharzia* species (Brant 2009a, 2013). These snail genera are present in North Dakota (Fuliss et. al. 1999) and are likely to serve as intermediate hosts for other schistosomatid species not found in this study.

Spatial analysis of sampled sites from 2013 detected the presence of spatial autocorrelation in sites where *Lymnaea* snails were collected, indicating non-independence for these sites. This could indicate a clumped distribution pattern for water bodies containing *Lymnaea* snails or a clumped distribution of the habitat conditions that are favored by *Lymnaea* snails. However, these results could also be an artifact of snail sampling methods because sampling was more extensive in areas with high water body density. Occupancy analysis of collected snail genera also revealed a correlation between *Lymnaea* snail presence and herbaceous wetlands and geographic location (latitude, longitude). Increased sampling in the northeastern part of North Dakota and the selection of similar types of wetlands most likely influenced these results. *Lymnaea* snails were typically found in wetlands with moderate to high vegetative cover while *Stagnicola* snails were found in the majority of water bodies sampled (89% of sampled sites). The low sample size of *Aplexa* snails collected, relative to the area that was sampled, was not sufficient to produce any reliable results on *Aplexa* distribution in North Dakota.

Only one avian schistosomatid species, namely *Gigantobilharzia huronensis*, was found to infect two different mollusk genera, *Stagnicola* and *Lymnaea* (Fig 14). In contrast, all the remaining AS species (including all *Trichobilharzia*) collected in our study were specific to their intermediate host species. *Stagnicola* had the highest diversity of AS among all snail genera in the region followed by *Aplexa* while *Lymnaea* hosted only one AS species (Fig. 14).
No avian schistosomatid infected snails were found in the southwestern part of the state. This region, known as the Badlands, has a semi-arid climate with low densities of water bodies. The inability to collect AS infected snails in this region is most likely due to the limited sampling conducted in the area. Physid snails were the most commonly collected snail genera in this region. Future sampling may reveal AS species utilizing these snails as intermediate hosts in this part of the state.

In our study, the prevalence of AS infections in snails was highest in mid- to late summer. Multiple factors play a role in determining the time period for which AS prevalence is highest. Long winters can delay and shorten the amount of time wetlands are available for snails and birds, and long periods of drought or periods with increased precipitation will also affect AS prevalence in intermediate snail hosts. Relative importance of these factors may vary from year to year; however, multi-year observations would be necessary to adequately study this question. Another factor that could also affect AS prevalence in snails is competition with other parasites for the invertebrate hosts (Bush et al 2001). One potential competitor is *Schistosomatium douthitti*, a mammalian schistosomatid. This species was collected from both *Stagnicola* and *Lymnaea* snails, two snail genera that were also utilized by avian schistosomatids. In some localities that were repeatedly sampled, eastern –central North Dakota, they account for almost half of the Schistosomatidae infected *Lymnaeid* snails collected at these sites. Other cercarial

types that were also seen to utilize these snail genera included xiphidiocercariae, echinostome and monostome cercariae. For instance, redial stages of echinostomatids are well known to directly feed on larval stages of other digeneans including schistosomatids (Lie 1973).

In this study, cercariae were taken directly from the snail's hepatopancreas. This method would have included immature cercariae and would therefore not produce reliable results for avian schistosomatid cercarial measurements. Even though an attempt was made to only include fully formed cercariae, a preferable method would have been the use of free-swimming cercariae from the water column. This would have ensured that cercariae used for measurements were fully formed. Previous studies however, have concluded that using cercarial morphological characteristics is not a reliable method for species differentiation (Brant and Loker 2009a). Size of cercariae can vary depending on snail size and snail species infected. There may also be environmental factors that affect cercarial size causing variation between geographic locations.

Definitive Bird Hosts

A total of 8 avian schistosomatid species were recovered from waterfowl species, 2 from shorebirds and 1 avian schistosomatid species from passerines. Despite a reasonably good coverage of aquatic bird diversity in the study, increased sampling may reveal additional avian schistosomatid species from birds in North Dakota. One of the most intriguing results was the fact that *Trichobilharzia szidati* was the AS species most prevalent in snails, however, none were recovered from birds. Additional bird sampling should occur at locations where *Trichobilharzia szidati* were found in snails in order to determine the avian definitive bird host of this parasite in North Dakota. One previous study in Europe identified teals and mallards as definitive hosts for this species (Rudolfova et al 2005). We expect this to also be the case in North Dakota.

Gigantobilharzia huronensis was the only AS species found in passerine birds. The behavior of *Aplexa* snails (the intermediate host of *G. huronensis*) most likely plays an important role in transmission of this parasite to passerines. *Aplexa* snails were usually found in very shallow areas of water bodies. This promotes prolonged and easy contact between passerine birds and cercariae while birds are searching for food or nesting material. Additional sampling of wetland passerines should reveal more definitive hosts for *Gigantobilharzia huronensis*.

On at least two occasions, we have found AS with marine life cycles in shorebirds (marbled godwit and American avocet) in North Dakota. Avian schistosomatids that utilize molluscs from marine environments cannot complete their life cycle in the freshwater bodies of North Dakota. Therefore, transmission of these AS species could not occur in the state.

The AS diversity seen in North Dakota is due to the high diversity of birds that nest in the region and migrate through it. High avian diversity allows for the introduction and continued maintenance of AS species that may utilize the same intermediate hosts but have the potential to infect multiple bird species. A number of bird species examined during our study were also not found to be infected with avian schistosomatids. These included, for instance, coots, rails, Northern pintails, mergansers, Ruddy ducks, cormorants and various species of herons. The inability to find AS infected individuals for these species may be due to the low number of individuals examined for each species and the difficulty in finding adult worms in birds when the intensity of infection is low. There were five avian schistosomatid species for which both the intermediate and definitive hosts were identified (Table 7). These bird hosts are all common species in the region with sufficient population densities to allow for completion of the AS life cycle in North Dakota.

Sequence Comparison and Phylogenetic Study of Avian Schistosomatids Found in North Dakota

The nuclear 28s gene is a good target for studies of phylogenetic relationships among the Schistosomatidae. In addition, the use of this gene made our results compatible and comparable to previous phylogenetic studies of avian schistosomatids (Snyder 2004, Lockyer et al 2003). However, the relatively low interspecific variability of the 28s gene makes it unsuitable for reliable species differentiation among congeneric species of avian schistosomatids. In order to differentiate between closely related species and produce finer scale intrageneric phylogenies, the high variability in the mitochondrial cox1 gene was also utilized.

Intraspecific variability was practically absent in the 28S gene except for 1-2 nucleotides in one sequence of *Trichobilharzia querquedulae* and *Gigantobilharzia huronensis*. In the cox1 gene the intraspecific variability was more pronounced, but was still under 1% while the lowest interspecific variability varied from 5.36% to 14.5%. This allowed for a reliable resolution for differentiation among closely related species, mostly notably we were able to demonstrate the presence of two species of *Dendritobilharzia* in North America (see below).

Zarowiecki et al (2007) analyzed the mitochondrial genomes of 5 schistosoma species and concluded that both the mitochondrial cox3 and nad5 fragments were better suited for phylogenetic studies of schistosomes as opposed to the commonly used cox1 fragment. Their reasoning was that because these two fragments contain approximately 25% of the variation of the entire mitochondrial genomes, this should then be the most efficient method in capturing genetic variation. In our study, phylogenetic analysis and comparisons required already available sequences from Schistosomatidae taxa. Previous studies on avian schistosomatids have primarily utilized the mitochondrial cox1 barcoding region and so the cox1 partial fragment was sequenced in order to incorporate already available data into the study. Future studies should consider

sequencing the recommended mitochondrial cox3 and nad5 fragments that provide better resolution of interspecific/intraspecific levels. Sequencing these genes may help to resolve the phylogenetic relationships among closely related species within the most species-rich *Trichobilharzia* clade.

In addition, the nuclear ITS region (ITS1, 5.8S, ITS2) may also provide additional resolution to the relationships between species/genera in the derived avian Schistosomatid clade. The ITS region exhibits intermediate variability compared to nuclear 28s rDNA (low variability) and mitochondrial cox1 mDNA (high variability).

Phylogenetic Interrelationships among Avian Schistosomatid Genera

Phylogenetic analyses including 28S sequences of all species found in our study combined with sequences of all schistosomatid genera available in the GenBank and using a spirorchid *Hapalotrema mehrai* as an outgroup have produced overall well-structured phylogenetic trees with high level of support of most topologies (Figs 22-25). In general, they corresponded well to the previously published phylogenies of the Schistosomatidae (Brant and Loker 2009b, Snyder 2004). We have also conducted separate analyses of alignments that included only the derived avian schistosomatid clade to make them comparable to the analysis presented by Brant et al. (2013) and Brant and Loker (2013). In both cases (the larger and the smaller alignments) the only difference between the results of Bayesian and Maximum Likelihood analyses was the position of the *Allobilharzia-Anserobilharzia* clade in relation to the numerous representatives of *Trichobilharzia*. The Bayesian analyses consistently resulted in *Allobilharzia-Anserobilharzia* being nested within one of the sub-clades of the strongly (100%) supported *Trichobilharzia* clade (Figs 22 and 26). However, the topology resulted from the Maximum Likelihood analysis was somewhat different. In this case, both the larger and the smaller alignments produced overall

weaker supported topologies that showed *Allobilharzia-Anserobilharzia* as a sister clade to *Trichobilharzia*. Brant and Loker (2013, Fig. 1) used essentially the same set of taxa and the same 28S gene region. However, in their Bayesian analysis *Allobilharzia-Anserobilharzia* appeared as sister group to the clade of *Trichobilharzia*. We do not have an adequate explanation of this difference. Nevertheless, the unstable position of this clade in different analyses and weak morphological differences between *Trichobilharzia*, *Allobilharzia* and *Anserobilharzia* warrant further detailed consideration of the interrelationships among these digeneans and their taxonomic status.

Trichobilharzia

The pairwise sequence comparison among 7 species of *Trichobilharzia* found in our study from either snails or birds in North Dakota has demonstrated sequence variability ranging from 0% (between *Trichobilharzia* sp. 4 and *Trichobilharzia* sp. A) to 2.74% (between *Trichobilharzia* sp. E and *Trichobilharzia querquedulae*). In some cases only 1-2 nucleotides were different, e.g., between *Trichobilharzia querquedulae* and *Trichobilharzia* sp. 4 and *Trichobilharzia* sp. A (the latter two species had identical 28S sequences). Overall, the *Trichobilharzia* sp. E. was the most divergent species of the genus in our data set (Table 9). Thus, it can be concluded, that the 28S gene can resolve interspecific differences in most cases, but not all.

The cox1 region utilized in our study proved to be a very useful target for differentiation of closely related congeneric species including all species of *Trichobilharzia* found in North Dakota. The levels of interspecific divergence ranged from 5.36% between *Trichobilharzia querquedulae* and *Trichobilharzia* sp. 4 to 14.50% between *Trichobilharzia* sp. E and *Trichobilharzia physellae* (Table 9). Interestingly, the levels of sequence divergence observed in

the cox1 gene did not fully correspond to the picture provided by the 28S gene. For instance, the pair of species (*Trichobilharzia* sp. 4 and *Trichobilharzia* sp. A) that showed no 28S sequence divergence was not the least divergent in the cox1 gene.

Sequences of two *Trichobilharzia* species found in in our study, namely *Trichobilharzia* sp. 2 and *Trichobilharzia* sp. 4, were lacking in the GenBank database and thus represent new genetic lineages available for phylogenetic studies. Their identity requires further clarification. Considering that the genus *Trichobilharzia* has been sequenced quite densely in North America it cannot be excluded that at least one of these species-level genetic forms may represent a yet undescribed species.

Dendritobilharzia

Vande Vusse (1980) revised all four formally described *Dendritobilharzia* species and concluded that all of them should be synonymized with *Dendritobilharzia pulverulenta*. The results from this study, however, demonstrated the presence of at least two species of *Dendritobilharzia* in North America. The variability in the more conserved ribosomal genes did not provide enough resolution for detection/separation of these forms; however, cox1 sequences clearly separated these species (Table 10). One of them (*Dendritobilharzia* sp. 1) was conspecific with a sequence of *Dendritobilharzia* cf. *pulverulenta* obtained from a specimen collected in Europe as well as with the only cox1 sequence of *Dendritobilharzia* available in the GenBank database. The other species was represented by several specimens found so far only in North Dakota. The morphological characteristics that may differentiate between the two species include the location of the cecal bifurcation relative to the anterior testis and the genital pore. While these characteristics are easy to distinguish they may pose a problem when comparing immature adults. No differences have been identified in female anatomy except for the overall

difference in body shape with *Dendritobilharzia* sp.2 having a more slender body shape compared to the larger and wider *Dendritobilharzia* sp. 1. It is most likely that *Dendritobilharzia* sp. 1 represents true *Dendritobilharzia pulverulenta* while *Dendritobilharzia* sp. 2 may represent either *D. anatinarum* described from North America, or a new, yet undescribed, species. Further morphological analysis will clarify this question.

It is also important to identify the snail hosts for *Dendritobilharzia* in North Dakota. Previous studies have identified planorbid snails as intermediate hosts for this genus and these snails are also expected to serve as intermediate hosts in North Dakota.

Austrobilharzia

The sequenced specimen of *Austrobilharzia* showed a significant, species-level divergence in the cox1 gene from sequences of *A. variglandis* and *A. terragalensis* available in the GenBank. Our specimen differed from these species in 13.73% and 17.39% of nucleotide positions, accordingly.

Gigantobilharzia

All nuclear 28s phylogenetic trees produced in this study have shown *Gigantobilharzia* to be polyphyletic (Figs. 22, 24 and 26). This was also seen in the 28s Bayesian tree of the DAS clade published by Brant and Loker (2013). The polyphyletic nature of this taxon indicates the necessity of a thorough taxonomic revision of *Gigantobilharzia* and its separation into several independent genera. Further detailed morphological and molecular study of *Gigantobilharzia* spp. is required to identify the true phylogenetic relationships between current members of this large genus.

Cercarial Morphology

Our morphometric analysis is generally in agreement with the opinion of Horak et al. (2002) who concluded that cercarial morphological features, including metric characters, in most cases are of limited value for differentiation among species of *Trichobilharzia*.

To the best of our knowledge, nobody has studied cercariae of *T. szidati* using SEM. Among members of *Trichobilharzia*, only cercariae of *T. ocellata* were examined using SEM (Kock and Böckeler 1998; Haas and van de Roemer, 1998). These studies did not focus on the surface morphology other than sensory elements. Only one image in the paper by Kock and Böckeler (1998) shows spines on the stem of the cercarial tail without any comments in the text. None of the studies examined furcae or showed the fin fold. Spines on the furcae are documented here for the first time. However, the functional role of the spination of the tail and furcae remains unclear since they cannot assist in attachment to the host or penetration. Spination was also detected on the tail of human schistosomes (Pereira et al. 2013), but they were of very different shape and were arranged in regular rows rather than randomly, as seen in our observation of *T. szidati*.

Cercarial Dermatitis in North Dakota

Based on this study, cercarial dermatitis ("swimmer's itch") should be considered a concern in the majority of the state's water bodies because the most common AS species collected, *Trichobilharzia* spp. and *Gigantobilharzia huronensis*, are known to cause dermatitis in people. These two genera had a widespread distribution throughout the state (Fig. 10). Therefore, any aquatic habitat that provides sufficient resources for snails and birds is a potential source for AS transmission and cercarial dermatitis. The wetlands that were sampled during this study were usually small to moderately sized water bodies. There is a higher risk for AS

transmission in small, shallow water bodies, due to the higher concentration of infected snails and lower physical separation between intermediate and definitive hosts. While these small, shallow water bodies may appeal to snails, birds and avian schistosomatid researchers, they are not very likely to be frequented by swimmers or utilized for any other recreational activities that may result in exposure of skin to cercariae. Lakes, reservoirs and other man-made water bodies that are maintained for human recreational activity pose a greater risk of AS transmission. Monitoring and limiting the number of birds in a location (prohibiting the feeding of ducks) as well as the use of molluscicides are the methods that are being utilized in reducing the risk of swimmer's itch.

APPENDICES

Appendix A

Map of North Dakota Snail Collection Sites

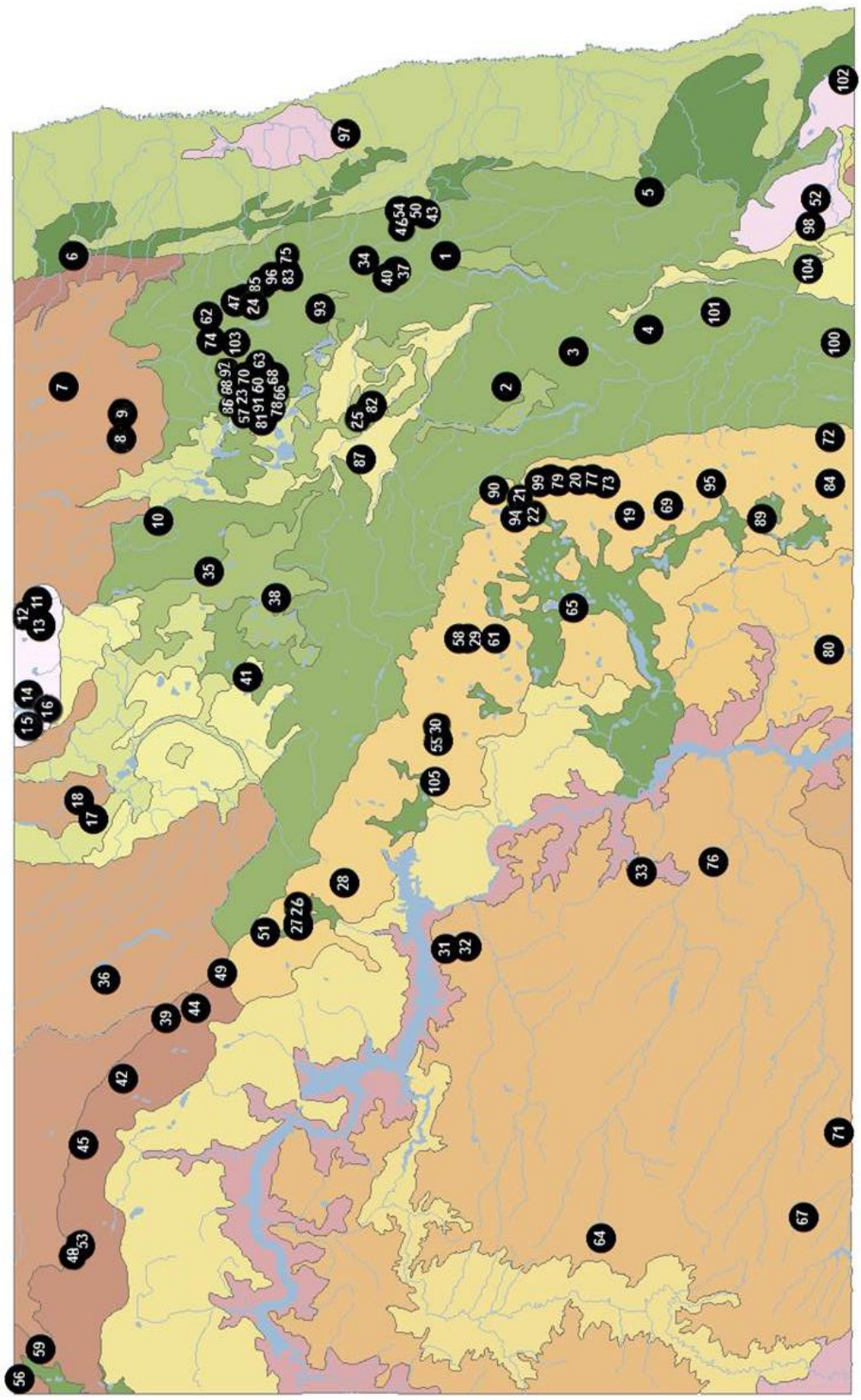

Figure 30. Map of North Dakota showing all sites (numbered) where Schistosomatidae infected snails were collected. The recorded data for each numbered site is available in Appendix B.

Appendix B

Recorded Data for all Snail Collection Sites

Table 20. Recorded data for snail collection sites from North Dakota and Minnesota. Data includes GPS coordinates, date site was sampled, number of snails collected, ecoregion and surrounding land cover.

Site No.	Latitude (dec)	Longitude (dec)	State	Date	Lymnaea	Stagnicola	Physa	Helisoma	Promenetus	Aplexa	Land Cover	Ecoregion
1	47.44764	-97.9003	ND	15-Aug-13	166	58	-	-	-	-	crop	Beach Ridges and Sand Deltas
2	47.22569	-98.6048	ND	15-Aug-13	-	285	-	-	-	-	crop	Collapsed Glacial Outwash
3	46.97956	-98.4141	ND	15-Aug-13	-	152	-	8	-	-	crop	Collapsed Glacial Outwash
4	46.70358	-98.2966	ND	15-Aug-13	136	4	-	1	-	-	crop	Collapsed Glacial Outwash
5	46.70219	-97.5571	ND	15-Aug-13	-	343	-	1	-	-	crop	Collapsed Glacial Outwash
6	48.79003	-97.8983	ND	31-Aug-13	-	66	47	21	-	-	crop	Drift Plains
7	48.82648	-98.6061	ND	31-Aug-13	143	6	-	-	-	-	crop	Drift Plains
8	48.6164	-98.8837	ND	31-Aug-13	63	235	-	-	-	-	crop	Drift Plains
9	48.60907	-98.8402	ND	31-Aug-13	-	145	-	1	-	-	crop	Drift Plains
10	48.48667	-99.3346	ND	31-Aug-13	-	280	-	-	-	-	crop	Drift Plains
11	48.9179	-99.7672	ND	1-Sep-13	-	47	-	-	-	-	crop	Drift Plains
12	48.90672	-99.8884	ND	1-Sep-13	-	71	-	-	-	-	crop	Drift Plains
13	48.90695	-99.8994	ND	1-Sep-13	2	151	80	21	-	-	crop	Drift Plains
14	48.9502	-100.27	ND	1-Sep-13	9	231	-	41	-	-	crop	Drift Plains
15	48.95103	-100.435	ND	1-Sep-13	-	10	1	4	-	-	crop	Drift Plains
16	48.92175	-100.418	ND	1-Sep-13	55	195	-	22	-	-	crop	Drift Plains
17	48.71944	-100.941	ND	1-Sep-13	-	290	-	-	-	-	crop	Drift Plains
18	48.77162	-100.84	ND	1-Sep-13	26	3	2	15	-	-	crop	Drift Plains
19	46.7721	-99.3008	ND	7-Jun-13	-	240	-	-	-	12	crop	Drift Plains
20	46.95957	-99.1095	ND	7-Jun-13	-	48	4	5	-	7	crop	Drift Plains
21	47.06646	-99.1066	ND	7-Jun-13	-	211	-	-	-	94	crop	Drift Plains
22	47.17458	-99.27	ND	7-Jun-13	-	7	12	-	-	13	crop	Drift Plains
23	48.16243	-98.6601	ND	3-Jun-13	-	8	-	-	-	-	crop	Drift Plains
24	48.18011	-98.131	ND	13-Sep-13	106		-	-	-	-	crop	Drift Plains
25	47.72103	-98.7183	ND	3-Jun-13	2	36	-	10	1	-	deciduous forest	Drift Plains
26	47.9846	-101.508	ND	5-Jun-13	-	5	13	-	6	2	deciduous forest	Drift Plains
27	47.98537	-101.518	ND	5-Jun-13	-	10	3	1	7	-	deciduous forest	Drift Plains
28	47.81918	-101.288	ND	5-Jun-13	-	12	-	-	-	-	developed	Drift Plains
29	47.36683	-99.9694	ND	5-Jun-13	-	37	-	-	-	16	fallow crop	Drift Plains
30	47.47977	-100.502	ND	5-Jun-13	-	94	-	-	6	64	fallow crop	Drift Plains

Table 20 cont.

Site No.	Latitude (dec)	Longitude (dec)	State	Date	Lymnaea	Stagnicola	Physa	Helisoma	Promenetus	Aplexa	Land Cover	Ecoregion
31	47.4507	-101.646	ND	6-Jun-13	-	26	28	4	-	-	grass pasture	Drift Plains
32	47.37438	-101.632	ND	6-Jun-13	-	-	13	7	-	-	grass pasture	Drift Plains
33	46.72672	-101.228	ND	6-Jun-13	-	-	44	-	-	-	grass pasture	Drift Plains
34	47.74507	-97.9252	ND	2-Jul-13	22	87	13	8	-	16	grass pasture	Drift Plains
35	48.3049	-99.6048	ND	15-Jul-13	39	128	-	4	-	-	grass pasture	Drift Plains
36	48.6767	-101.812	ND	6-Aug-13	-	210	-	12	-	-	grass pasture	Drift Plains
37	47.62997	-97.9839	ND	2-Jul-13	3	93	3	8	-	-	grass pasture	Drift Plains
38	48.06485	-99.7499	ND	15-Jul-13	56	108	-	14	-	-	grass pasture	Drift Plains
39	48.45842	-102.017	ND	6-Aug-13	155	290	-	5	-	27	grass pasture	Drift Plains
40	47.65988	-98.0205	ND	2-Jul-13	4	240	-	-	-	8	grass pasture	Drift Plains
41	48.1671	-100.178	ND	15-Jul-13	-	157	-	-	-	40	grass pasture	Drift Plains
42	48.61353	-102.343	ND	6-Aug-13	189	5	-	12	-	-	grass pasture	Drift Plains
43	47.52067	-97.673	ND	2-Jul-13	34	92	-	9	5	3	grass pasture	End Moraine Complex
44	48.35387	-101.965	ND	16-Jul-13	4	291	-	3	-	-	grass pasture	End Moraine Complex
45	48.75343	-102.699	ND	7-Aug-13	51	-	-	-	-	-	grass pasture	End Moraine Complex
46	47.60037	-97.6648	ND	2-Jul-13	-	-	-	-	-	-	grass pasture	End Moraine Complex
47	48.21261	-98.1504	ND	22-Jun-13	2	10	1	-	7	-	grass pasture	Glacial Lake Deltas
48	48.77896	-103.257	ND	7-Aug-13	22	4	-	10	-	-	grass pasture	Glacial Lake Deltas
49	48.2585	-101.771	ND	16-Jul-13	-	282	-	-	-	104	grass pasture	Glacial Lake Basins
50	47.5567	-97.6562	ND	2-Jul-13	7	128	-	-	3	48	grass pasture	Glacial Outwash
51	48.10423	-101.556	ND	16-Jul-13	-	71	-	2	-	-	grass pasture	Glacial Outwash
52	46.08053	-97.5933	ND	7-Sep-13	-	27	8	-	-	-	grass pasture	Glacial Outwash
53	48.76512	-103.248	ND	7-Aug-13	231	81	-	-	-	-	grass pasture	Missou coteau
54	47.61543	-97.6582	ND	2-Jul-13	8	8	-	-	-	40	grass pasture	Missou coteau
55	47.47677	-100.522	ND	17-Jul-13	-	110	-	-	-	-	grass pasture	Missou coteau
56	48.97963	-103.97	ND	7-Aug-13	-	9	-	-	-	-	grass pasture	Missou coteau
57	48.09047	-98.703	ND	4-Jul-13	59	155	-	5	-	-	grass pasture	Missou coteau
58	47.39983	-99.9649	ND	17-Jul-13	-	10	-	-	-	-	grass pasture	Missou coteau
59	48.90833	-103.805	ND	7-Aug-13	-	8	-	12	-	-	grass pasture	Missou coteau
60	48.13692	-98.5768	ND	4-Jul-13	19	200	95	11	-	1	grass pasture	Missouri plateau

Table 20 cont.

Site No.	Latitude (dec)	Longitude (dec)	State	Date	Lymnaea	Stagnicola	Physa	Helisoma	Promenetus	Aplexa	Land Cover	Ecoregion
61	47.26833	-99.9693	ND	17-Jul-13	-	217	-	-	-	26	grass pasture	Missouri plateau
62	48.31005	-98.2279	ND	22-Jun-13	12	13	3	-	1	-	grass pasture	Missouri Plateau
63	48.1224	-98.4872	ND	4-Jul-13	39	17	-	-	-	-	grass pasture	Missouri plateau
64	46.881	-103.206	ND	8-Aug-13	-	11	78	-	-	-	grass pasture	Missouri plateau
65	46.98087	-99.8013	ND	17-Jul-13	136	-	-	2	-	-	grass pasture	Missouri plateau
66	48.08425	-98.5519	ND	7-Jul-13	71	-	-	-	-	-	grass pasture	Missouri coteau
67	46.12825	-103.09	ND	8-Aug-13	-	72	-	-	-	-	herbaceous wetlands	Missouri coteau
68	48.07372	-98.5519	ND	7-Jul-13	246	74	-	8	-	-	herbaceous wetlands	Missouri coteau
69	46.63122	-99.2469	ND	23-Jul-13	149	53	-	14	-	-	herbaceous wetlands	Missouri coteau
70	48.1754	-98.552	ND	7-Jul-13	46	32	19	20	7	1	herbaceous wetlands	Missouri coteau
71	45.99395	-102.634	ND	8-Aug-13	-	-	80	-	-	-	herbaceous wetlands	Missouri coteau
72	46.02881	-98.8793	ND	7-Sep-13	40	14	1	2	-	-	herbaceous wetlands	Missouri coteau
73	46.86253	-99.1287	ND	23-Jul-13	-	44	1	3	-	-	herbaceous wetlands	Missouri coteau
74	48.29638	-98.3577	ND	7-Jul-13	12	202	-	1	-	29	herbaceous wetlands	Missouri coteau
75	48.02878	-97.9903	ND	10-Sep-13	100	-	-	-	-	-	herbaceous wetlands	Missouri coteau
76	46.46213	-101.175	ND	9-Aug-13	25	105	-	-	-	-	herbaceous wetlands	Missouri coteau
77	46.90987	-99.1087	ND	23-Jul-13	198	94	-	8	-	-	herbaceous wetlands	Missouri coteau
78	48.0831	-98.7247	ND	11-Jul-13	110	55	-	-	-	-	herbaceous wetlands	Missouri coteau
79	47.03713	-99.1173	ND	23-Jul-13	7	2	245	17	-	-	herbaceous wetlands	Missouri coteau
80	46.02987	-100.025	ND	9-Aug-13	-	4	227	-	-	-	herbaceous wetlands	Missouri Coteau Slope
81	48.11505	-98.7894	ND	11-Jul-13	100	105	-	-	-	-	herbaceous wetlands	Northern Missouri Coteau
82	47.71703	-98.7104	ND	26-Jul-13	56	254	1	3	-	-	herbaceous wetlands	Northern Missouri Coteau
83	48.02106	-98.0131	ND	13-Sep-13	14	47	-	-	-	-	herbaceous wetlands	Northern Missouri Coteau
84	46.02695	-99.1297	ND	9-Aug-13	4	-	75	4	-	-	herbaceous wetlands	Northern Missouri Coteau
85	48.11597	98.05465	ND	22-Jun-13	44	81	2	3	12	-	herbaceous wetlands	Northern Missouri Coteau
86	48.10778	-98.6754	ND	11-Jul-13	173	45	-	-	-	-	herbaceous wetlands	Northern Missouri Coteau
87	47.75922	-98.9971	ND	26-Jul-13	72	3	-	-	-	-	herbaceous wetlands	Northern Missouri Coteau
88	48.15147	-98.6607	ND	11-Jul-13	8	-	-	3	-	-	herbaceous wetlands	Northern Missouri Coteau
89	46.28333	-99.3171	ND	10-Aug-13	3	256	-	47	-	-	herbaceous wetlands	Northern Black Prairie
90	47.2706	-99.1663	ND	26-Jul-13	109	116	3	12	-	-	herbaceous wetlands	Northern Black Prairie

Table 20 cont.

Site No.	Latitude (dec)	Longitude (dec)	State	Date	Lymnaea	Stagnicola	Physa	Helisoma	Promenetus	Aplexa	Land Cover	Ecoregion
91	48.12605	-98.7029	ND	11-Jul-13	-	458	-	-	-	11	herbaceous wetlands	Northern Black Prairie
92	47.90406	-98.1831	ND	29-May-13	97	-	4	8	58	-	herbaceous wetlands	Northern Black Prairie
93	47.90406	-98.1831	ND	6-Sep-13	100	-	-	-	-	-	herbaceous wetlands	Northern Black Prairie
94	47.19318	-99.3128	ND	26-Jul-13	105	165	-	-	-	-	herbaceous wetlands	Northern Black Prairie
95	46.47145	-99.1291	ND	10-Aug-13	81	458	-	8	-	170	herbaceous wetlands	River Breaks
96	48.07893	-98.0117	ND	11-Jul-13	156	95	-	-	-	-	open water	Saline Area
97	47.81408	-97.2399	ND	3-Aug-13	120	112	5	12	-	-	open water	Tewaukon/Big Stone Stagnation Moraine
98	46.10317	-97.7374	ND	7-Sep-13	-	396	2	6	-	-	open water	Tewaukon/Big Stone Stagnation Moraine
99	47.10695	-99.1215	ND	26-Jul-13	23	174	11	12	-	-	open water	Tewaukon/Big Stone Stagnation Moraine
100	46.00847	-98.3705	ND	7-Sep-13	-	91	29	8	-	-	open water	Turtle Mountains
101	46.45537	-98.2019	ND	10-Aug-13	103	138	-	17	-	-	open water	Turtle Mountains
102	45.97768	-96.9499	ND	10-Aug-13	-	555	-	-	-	-	open water	Turtle Mountains
103	48.2095	-98.3682	ND	22-Jun-13	88	53	3	-	3	-	open water	Turtle Mountains
104	46.10975	-97.9723	ND	7-Sep-13	-	171	-	-	-	-	open water	Turtle Mountains
105	47.48748	-100.743	ND	16-Jul-13	1	1	-	-	-	-	open water	Turtle Mountains
106	47.255	-95.2242	MN	21-Jul-13	1	101	-	-	-	-		
107	47.05867	-94.9667	MN	21-Jul-13	-	-	-	-	-	-		
108	47.19	-94.9717	MN	21-Jul-13	2	38	4	-	-	-		
109	47.40017	-94.9376	MN	27-Jul-13	14	-	-	1	-	-		
110	47.10604	-94.4986	MN	27-Jul-13	2	10	-	-	-	-		

Appendix C

Data on Snails Infected with Schistosomatidae Cercariae

Table 21. Data for schistosomatidae infected snails including snail host genus, AS species and sequenced genes.

Site No.	Snail No.	Snail genus	State	Date Collected	Ext No.	AS species	28s	Cox1
4	2410	*Stagnicola*	ND	22-Aug-13	2754neo	*Trichobilharzia*	Y	N
4	2403	*Lymnaea*	ND	15-Aug-13	2747neo	*Trichobilharzia szidati*	Y	N
4	2400	*Lymnaea*	ND	15-Aug-13	2744neo	*Trichobilharzia szidati*	Y	Y
4	2401	*Lymnaea*	ND	15-Aug-13	2745neo	*Trichobilharzia szidati*	Y	Y
4	2405	*Lymnaea*	ND	17-Aug-13	2749neo	*Trichobilharzia szidati*	Y	Y
4	2406	*Lymnaea*	ND	18-Aug-13	2750neo	*Trichobilharzia szidati*	Y	Y
4	2407	*Lymnaea*	ND	19-Aug-13	2751neo	*Trichobilharzia szidati*	Y	N
4	2408	*Lymnaea*	ND	20-Aug-13	2752neo	*Trichobilharzia szidati*	Y	N
4	2409	*Lymnaea*	ND	21-Aug-13	2753neo	*Trichobilharzia szidati*	Y	Y
7	2423	*Lymnaea*	ND	31-Aug-13	2755neo	*Trichobilharzia szidati*	Y	N
8	2425	*Stagnicola*	ND	31-Aug-13	2505neo	*Trichobilharzia* sp E	Y	Y
9	2428	*Stagnicola*	ND	31-Aug-13	2780neo	*Trichobilharzia* sp 4	Y	Y
9	2427	*Stagnicola*	ND	31-Aug-13	2509neo	*Trichobilharzia szidati*	Y	Y
9	2426	*Stagnicola*	ND	31-Aug-13	2889neo	*Trichobilharzia* sp E	Y	Y
10	2429	*Stagnicola*	ND	31-Aug-13	2507neo	*Trichobilharzia*	Y	N
11	2430	*Stagnicola*	ND	1-Sep-13	2495neo	*Schistosomatium douthitti*	Y	N
11	2431	*Stagnicola*	ND	1-Sep-13	2781neo	*Schistosomatium douthitti*	Y	N
14	2433	*Lymnaea*	ND	1-Sep-13	2506neo	*Schistosomatium douthitti*	Y	N
14	2432	*Lymnaea*	ND	1-Sep-13	2498neo	*Trichobilharzia szidati*	Y	Y
16	2435	*Lymnaea*	ND	1-Sep-13	2782neo	*Schistosomatium douthitti*	Y	N
16	2434	*Lymnaea*	ND	1-Sep-13	2502neo	*Trichobilharzia szidati*	Y	Y
18	2498	*Stagnicola*	ND	1-Sep-13	2508neo	*Trichobilharzia* sp E	Y	Y
24	2553	*Lymnaea*	ND	13-Sep-13	2888neo	*Trichobilharzia* sp A	Y	Y
25	1215	*Lymnaea*	ND	3-Jun-13	2777neo	*Trichobilharzia szidati*	Y	N
34	1229	*Aplexa*	ND	2-Jul-13	2469neo	*Gigantobilharzia huronensis*	Y	Y
34	1230	*Aplexa*	ND	2-Jul-13	2473neo	*Gigantobilharzia huronensis*	Y	Y
34	1234	*Aplexa*	ND	2-Jul-13	2474neo	*Gigantobilharzia huronensis*	Y	Y
34	1231	*Physa*	ND	2-Jul-13	2472neo	*Gigantobilharzia huronensis*	Y	Y
35	1597	*Lymnaea*	ND	15-Jul-13	2894neo	*Trichobilharzia szidati*	Y	Y
35	1598	*Lymnaea*	ND	15-Jul-13	2567neo	*Trichobilharzia szidati*	Y	Y
37	1238	*Stagnicola*	ND	2-Jul-13	2512neo	*Trichobilharzia* sp 4	Y	Y
38	1599	*Lymnaea*	ND	15-Jul-13	2568neo	*Schistosomatium douthitti*	Y	N
38	1601	*Lymnaea*	ND	15-Jul-13	2570neo	*Trichobilharzia* sp E	Y	Y
38	1600	*Lymnaea*	ND	15-Jul-13	2569neo	*Trichobilharzia szidati*	Y	Y
39	2129	*Aplexa*	ND	6-Aug-13	2613neo	*Gigantobilharzia huronensis*	Y	Y
39	2131	*Aplexa*	ND	6-Aug-13	2778neo	*Gigantobilharzia huronensis*	Y	Y
39	2143	*Lymnaea*	ND	6-Aug-13	2619neo	*Schistosomatium douthitti*	Y	N
39	2142	*Stagnicola*	ND	6-Aug-13	2618neo	*Schistosomatium douthitti*	Y	N
39	2126	*Aplexa*	ND	6-Aug-13	2611neo	*Trichobilharzia querquedulae*	Y	Y
39	2128	*Aplexa*	ND	6-Aug-13	2612neo	*Trichobilharzia querquedulae*	Y	Y
39	2130	*Aplexa*	ND	6-Aug-13	2614neo	*Trichobilharzia querquedulae*	Y	N
39	2137	*Stagnicola*	ND	6-Aug-13	2617neo	*Trichobilharzia* sp E	Y	Y

Table 21 cont.

Site No.	Snail No.	Snail genus	State	Date Collected	Ext No.	AS species	28s	Cox1
41	1602	*Aplexa*	ND	15-Jul-13	2571neo	*Gigantobilharzia huronensis*	Y	Y
42	2123	*Lymnaea*	ND	6-Aug-13	2609neo	*Trichobilharzia szidati*	Y	Y
42	2124	*Lymnaea*	ND	6-Aug-13	2610neo	*Trichobilharzia szidati*	Y	N
44	1826	*Stagnicola*	ND	16-Jul-13	2595neo	*Trichobilharzia* sp A	Y	Y
44	1619	*Stagnicola*	ND	16-Jul-13	2573neo	*Trichobilharzia* sp E	Y	Y
44	1618	*Stagnicola*	ND	16-Jul-13	2945neo	*Trichobilharzia* sp E	Y	Y
45	2159	*Lymnaea*	ND	7-Aug-13	2620neo	*Trichobilharzia szidati*	Y	Y
45	2160	*Lymnaea*	ND	7-Aug-13	2621neo	*Trichobilharzia szidati*	Y	N
45	2163	*Lymnaea*	ND	7-Aug-13	2623neo	*Trichobilharzia szidati*	Y	Y
45	2165	*Lymnaea*	ND	7-Aug-13	2953neo	*Trichobilharzia szidati*	Y	N
49	1624	*Aplexa*	ND	16-Jul-13	2576neo	*Gigantobilharzia huronensis*	Y	Y
49	1625	*Aplexa*	ND	16-Jul-13	2582neo	*Gigantobilharzia huronensis*	Y	Y
49	1627	*Aplexa*	ND	16-Jul-13	2584neo	*Gigantobilharzia huronensis*	Y	Y
49	1634	*Aplexa*	ND	16-Jul-13	2591neo	*Gigantobilharzia huronensis*	Y	Y
49	1635	*Aplexa*	ND	16-Jul-13	2592neo	*Gigantobilharzia huronensis*	Y	Y
49	1636	*Aplexa*	ND	16-Jul-13	2593neo	*Gigantobilharzia huronensis*	Y	Y
49	1626	*Aplexa*	ND	16-Jul-13	2947neo	*Gigantobilharzia huronensis*	Y	Y
49	1623	*Stagnicola*	ND	16-Jul-13	2575neo	*Trichobilharzia* sp 4	Y	Y
49	1628	*Aplexa*	ND	16-Jul-13	2585neo	*Trichobilharzia querquedulae*	Y	N
49	1622	*Stagnicola*	ND	16-Jul-13	2574neo	*Trichobilharzia* sp A	Y	Y
49	1629	*Stagnicola*	ND	16-Jul-13	2586neo	*Trichobilharzia* sp A	Y	Y
50	1274	*Aplexa*	ND	2-Jul-13	2616neo	*Gigantobilharzia huronensis*	Y	Y
53	2176	*Lymnaea*	ND	7-Aug-13	2627neo	*Trichobilharzia szidati*	N	N
53	2177	*Lymnaea*	ND	7-Aug-13	2628neo	*Trichobilharzia szidati*	Y	Y
53	2178	*Lymnaea*	ND	7-Aug-13	2661neo	*Trichobilharzia szidati*	Y	Y
53	2180	*Lymnaea*	ND	7-Aug-13	2662neo	*Trichobilharzia szidati*	Y	N
53	2181	*Lymnaea*	ND	7-Aug-13	2663neo	*Trichobilharzia szidati*	Y	N
53	2182	*Lymnaea*	ND	7-Aug-13	2664neo	*Trichobilharzia szidati*	Y	Y
53	2183	*Lymnaea*	ND	7-Aug-13	2665neo	*Trichobilharzia szidati*	Y	N
53	2184	*Lymnaea*	ND	7-Aug-13	2666neo	*Trichobilharzia szidati*	Y	Y
53	2185	*Lymnaea*	ND	7-Aug-13	2667neo	*Trichobilharzia szidati*	Y	Y
53	2186	*Lymnaea*	ND	7-Aug-13	2668neo	*Trichobilharzia szidati*	Y	Y
53	2188	*Lymnaea*	ND	7-Aug-13	2687neo	*Trichobilharzia szidati*	Y	Y
53	2174	*Stagnicola*	ND	7-Aug-13	2626neo	*Trichobilharzia szidati*	Y	Y
57	1292	*Stagnicola*	ND	4-Jul-13	2615neo	*Trichobilharzia* sp 4	Y	Y
61	1630	*Aplexa*	ND	17-Jul-13	2587neo	*Gigantobilharzia huronensis*	Y	N
61	1631	*Aplexa*	ND	17-Jul-13	2588neo	*Gigantobilharzia huronensis*	Y	N
62	1216	*Stagnicola*	ND	22-Jun-13	2470neo	*Trichobilharzia* sp 4	Y	Y
65	1632	*Lymnaea*	ND	17-Jul-13	2589neo	*Trichobilharzia szidati*	Y	Y
65	1633	*Lymnaea*	ND	17-Jul-13	2590neo	*Trichobilharzia szidati*	Y	Y
68	1405	*Lymnaea*	ND	7-Jul-13	2531neo	*Trichobilharzia szidati*	Y	N
68	1407	*Lymnaea*	ND	7-Jul-13	2533neo	*Trichobilharzia szidati*	Y	Y
68	1408	*Lymnaea*	ND	7-Jul-13	2534neo	*Trichobilharzia szidati*	Y	Y
68	1436	*Lymnaea*	ND	7-Jul-13	2898neo	*Trichobilharzia szidati*	Y	Y
68	1440	*Lymnaea*	ND	7-Jul-13	2900neo	*Trichobilharzia szidati*	Y	Y
68	1396	*Lymnaea*	ND	7-Jul-13	2901neo	*Trichobilharzia szidati*	Y	Y
68	1406	*Lymnaea*	ND	7-Jul-13	2902neo	*Trichobilharzia szidati*	Y	Y

Table 21 cont.

Site No.	Snail No.	Snail genus	State	Date Collected	Ext No.	AS species	28s	Cox1
68	1438	*Lymnaea*	ND	7-Jul-13	2899neo	*Trichobilharzia szidati*	Y	Y
69	1760	*Lymnaea*	ND	23-Jul-13	2594eno	*Schistosomatium douthitti*	Y	N
72	2504	*Lymnaea*	ND	7-Sep-13	2906neo	*Trichobilharzia szidati*	Y	Y
72	2505	*Lymnaea*	ND	7-Sep-13	2504neo	*Trichobilharzia szidati*	Y	N
75	2509	*Lymnaea*	ND	10-Sep-13	2887neo	*Trichobilharzia szidati*	Y	N
77	1833	*Lymnaea*	ND	23-Jul-13	2597neo	*Trichobilharzia szidati*	Y	Y
77	1834	*Lymnaea*	ND	23-Jul-13	2954neo	*Trichobilharzia szidati*	Y	N
78	1443	*Lymnaea*	ND	11-Jul-13	2552neo	*Trichobilharzia szidati*	Y	N
78	1444	*Lymnaea*	ND	11-Jul-13	2553neo	*Trichobilharzia szidati*	Y	Y
78	1445	*Lymnaea*	ND	11-Jul-13	2554neo	*Trichobilharzia szidati*	Y	Y
78	1447	*Lymnaea*	ND	11-Jul-13	2555neo	*Trichobilharzia szidati*	Y	Y
78	1448	*Lymnaea*	ND	11-Jul-13	2560neo	*Trichobilharzia szidati*	Y	Y
81	1449	*Lymnaea*	ND	11-Jul-13	2561neo	*Trichobilharzia szidati*	Y	N
82	2017	*Physa*	ND	26-Jul-13	2948neo	*Gigantobilharzia huronensis*	Y	N
82	2016	*Lymnaea*	ND	26-Jul-13	2601neo	*Schistosomatium douthitti*	Y	N
82	1957	*Lymnaea*	ND	26-Jul-13	2600neo	*Trichobilharzia szidati*	Y	Y
83	2517	*Stagnicola*	ND	13-Sep-13	2494neo	*Trichobilharzia*	Y	N
90	1956	*Lymnaea*	ND	26-Jul-13	2599neo	*Trichobilharzia szidati*	Y	Y
91	1452	*Aplexa*	ND	11-Jul-13	2893neo	*Gigantobilharzia huronensis*	Y	Y
91	1451	*Stagnicola*	ND	11-Jul-13	2563neo	*Trichobilharzia*	Y	N
91	1450	*Stagnicola*	ND	11-Jul-13	2562neo	*Trichobilharzia* sp A	Y	Y
92	2508	*Lymnaea*	ND	6-Sep-13	2783neo	*Schistosomatium douthitti*	Y	N
93	1183	*Lymnaea*	ND	29-May-13	2471neo	*Trichobilharzia szidati*	Y	Y
93	1184	*Lymnaea*	ND	29-May-13	2776neo	*Trichobilharzia szidati*	Y	Y
94	2020	*Lymnaea*	ND	26-Jul-13	2907neo	*Schistosomatium douthitti*	Y	N
95	2281	*Stagnicola*	ND	10-Aug-13	2693neo	*Trichobilharzia*	Y	N
95	2284	*Stagnicola*	ND	10-Aug-13	2723neo	*Trichobilharzia*	Y	N
95	2279	*Stagnicola*	ND	10-Aug-13	2692neo	*Trichobilharzia*	Y	N
95	2287	*Stagnicola*	ND	10-Aug-13	2725neo	*Trichobilharzia* sp 2	Y	Y
95	2283	*Stagnicola*	ND	10-Aug-13	2946neo	*Trichobilharzia* sp 2	Y	Y
95	2282	*Stagnicola*	ND	10-Aug-13	2694neo	*Trichobilharzia* sp A	Y	Y
95	2286	*Stagnicola*	ND	10-Aug-13	2724neo	*Trichobilharzia* sp A	Y	Y
95	2288	*Stagnicola*	ND	10-Aug-13	2726neo	*Trichobilharzia* sp A	Y	Y
95	2292	*Stagnicola*	ND	10-Aug-13	2728neo	*Trichobilharzia* sp A	Y	Y
95	2293	*Stagnicola*	ND	10-Aug-13	2729neo	*Trichobilharzia* sp A	Y	Y
95	2294	*Stagnicola*	ND	10-Aug-13	2730neo	*Trichobilharzia* sp A	Y	Y
95	2295	*Stagnicola*	ND	10-Aug-13	2731neo	*Trichobilharzia* sp A	Y	Y
95	2275	*Stagnicola*	ND	10-Aug-13	2779neo	*Trichobilharzia* sp A	Y	Y
95	2290	*Stagnicola*	ND	10-Aug-13	2727neo	*Trichobilharzia* sp E	Y	N
95	2272	*Lymnaea*	ND	10-Aug-13	2688neo	*Trichobilharzia szidati*	Y	N
95	2273	*Lymnaea*	ND	10-Aug-13	2689neo	*Trichobilharzia szidati*	Y	Y
95	2274	*Lymnaea*	ND	10-Aug-13	2690neo	*Trichobilharzia szidati*	Y	Y
97	2092	*Lymnaea*	ND	3-Aug-13	2608neo	*Trichobilharzia szidati*	Y	Y
98	2506	*Stagnicola*	ND	7-Sep-13	2500neo	*Trichobilharzia*	Y	N
98	2507	*Stagnicola*	ND	7-Sep-13	2905neo	*Trichobilharzia* sp 4	Y	Y
99	2018	*Lymnaea*	ND	26-Jul-13	2603neo	*Trichobilharzia szidati*	Y	N
99	2019	*Lymnaea*	ND	26-Jul-13	2604neo	*Trichobilharzia szidati*	Y	Y

Table 21 cont.

Site No.	Snail No.	Snail genus	State	Date Collected	Ext No.	AS species	28s	Cox1
101	2303	*Lymnaea*	ND	10-Aug-13	2738neo	*Schistosomatium douthitti*	Y	N
101	2306	*Stagnicola*	ND	10-Aug-13	2741neo	*Trichobilharzia* sp A	Y	Y
101	2297	*Lymnaea*	ND	10-Aug-13	2732neo	*Trichobilharzia szidati*	Y	N
101	2298	*Lymnaea*	ND	10-Aug-13	2733neo	*Trichobilharzia szidati*	Y	N
101	2304	*Lymnaea*	ND	10-Aug-13	2739neo	*Trichobilharzia szidati*	Y	N
101	2305	*Lymnaea*	ND	10-Aug-13	2740neo	*Trichobilharzia szidati*	Y	Y
101	2302	*Lymnaea*	ND	10-Aug-13	2890neo	*Trichobilharzia szidati*	Y	N
101	2301	*Lymnaea*	ND	10-Aug-13	2891neo	*Trichobilharzia szidati*	Y	Y
101	2299	*Lymnaea*	ND	10-Aug-13	2903neo	*Trichobilharzia szidati*	Y	Y
101	2300	*Lymnaea*	ND	10-Aug-13	2904neo	*Trichobilharzia szidati*	Y	Y
102	2307	*Stagnicola*	ND	10-Aug-13	2742neo	*Schistosomatium douthitti*	Y	N
108	1827	*Physa*	MN	21-Jul-13	2596neo	*Gigantobilharzia huronensis*	Y	Y
109	2021	*Lymnaea*	MN	27-Jul-13	2606neo	*Trichobilharzia szidati*	Y	Y
109	2022	*Lymnaea*	MN	27-Jul-13	2607neo	*Trichobilharzia szidati*	Y	Y

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