Legacy parasite collections reveal species-specific population genetic patterns among three species of zoonotic schistosomes (Trematoda: Schistosomatidae)

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Abstract

Multi-host helminth systems are difficult to study at a population level due to inherent spatial and temporal sampling challenges. Consequently, our understanding of the factors affecting gene flow, genetic drift and effective population size is limited. Population genetic parameters (Ne, Θ, π) are necessary in understanding fundamental processes in host-parasite evolution such as co-evolutionary dynamics, spread of resistance alleles and local adaptation. This study used museum specimens collected over 20-years of three congeneric trematode (Schistosomatidae) species: Trichobilharzia querquedulae, T. physellae, and Trichobilharzia species A . All contribute to the worldwide zoonotic disease cercarial dermatitis (i.e. swimmers Itch). Populations of each species were sampled for two mitochondrial (cox1 and nad4) and one nuclear loci (ITS1) to estimate population genetic structure, genetic diversity, effective size and population history. Significant differences in these measures were revealed among the three congeners. Trichobilharzia querquedulae maintained a well-connected globally diverse metapopulation, with an effective size approximately three times that of the other two species, which were characterized by lower overall genetic diversity and greater population structure, mediated by the definitive duck host. We hypothesize that the species-specific patterns are due to distinctive ecological preferences and migratory behaviors of their respective definitive hosts. This study demonstrates the value of natural history collections to facilitate population genetic studies that would otherwise be infeasible. Applying population genetic data within this comparative congeneric framework allows us to tease apart particular aspects of host-parasite natural history and its influence on microevolutionary patterns within complex helminth systems, including contributions to zoonotic disease.

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RUNNING TITLE: Population genetics of congeneric trematodes.

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ABSTRACT

Multi-host helminth systems are difficult to study at a population level due to inherent spatial and temporal sampling challenges. Consequently, our understanding of the factors affecting gene flow, genetic drift and effective population size is limited. Population genetic parameters (*Ne,* Θ, π) are necessary in understanding fundamental processes in host-parasite evolution such as co-evolutionary dynamics, spread of resistance alleles and local adaptation. This study used museum specimens collected over 20-years of three congeneric trematode (Schistosomatidae) species: *Trichobilharzia querquedulae, T. physellae,* and *Trichobilharzia* species A . All contribute to the worldwide zoonotic disease cercarial dermatitis (i.e. swimmers Itch). Populations of each species were sampled for two mitochondrial (*cox* 1 and *nad* 4) and one nuclear loci (ITS1) to estimate population genetic structure, genetic diversity, effective size and population history. Significant differences in these measures were revealed among the three congeners. *Trichobilharzia querquedulae* maintained a well-connected globally diverse metapopulation, with an effective size approximately three times that of the other two species, which were characterized by lower overall genetic diversity and greater population structure, mediated by the definitive duck host. We hypothesize that the species-specific patterns are due to distinctive ecological preferences and migratory behaviors of their respective definitive hosts. This study demonstrates the value of natural history collections to facilitate population genetic studies that would otherwise be infeasible. Applying population genetic data within this comparative congeneric framework allows us to tease apart particular aspects of host-parasite natural history and its influence on microevolutionary patterns within complex helminth systems, including contributions to zoonotic disease.

KEYWORDS: *Trichobilharzia,* Schistosomes, Host Ecology, Congeneric Species, Museum Collections, Parasite Population Genetics,

1. INTRODUCTION

Identifying factors that shape microevolutionary forces in natural populations is a fundamental goal of evolutionary biology(Slatkin, 1985). In multi-host helminth systems, determinants of gene flow, genetic drift and effective size (*Ne*) are poorly understood (Nadler, 1995; Criscione & Blouin, 2005a; Rey et al., 2021). These parameters affect evolution of drug resistance (Blouin, 1995; Chevalier et al., 2019), local adaption (Dybdahl & Lively 1996; Nuismer & Gandon, 2009), probability of host-switching (Matthee, Engelbrecht, & Matthee 2018) and ultimately speciation of parasite lineages (Huyse, Poulin, & Théron, 2005; Garcia, Melin, Aureli, & de Leon 2019). Considering the ubiquity of parasitism (Weinstein & Kuris, 2016), this lack of knowledge represents a significant gap in our understanding of microevolutionary processes within natural populations.

In host-parasite systems, microevolutionary forces are mediated by one or more host(s) and relate largely to the specific physiology, immunology, genetics and natural history of the individual host species (Barrett, Thrall, Burdon, & Linde 2008; Criscione & Blouin, 2004; Criscione, Poulin, & Blouin, 2005; Nadler 1995; Nieberding, Morand, Libois & Michaux 2006; Whiteman, Kimball & Parker 2007). Assuming compatibility, host traits (i.e. range, migratory behavior, feeding preferences, vagility) have been shown to shape the population genetic structure of associated parasite populations (Criscione & Blouin 2004; Blasco-Costa, Waters & Poulin 2012; Blasco-Costa & Poulin, 2013; Garcia, Melin, Aureli, & de Leon 2019) ultimately influencing microevolutionary forces. Comparative population genetic studies are ideal for studying parasite population

structure and microevolution. Previous work has primarily considered broad life history differences among distantly related parasite species (as reviewed by Blasco-Costa & Poulin, 2013); autogenic versus allogenic life cycles (Criscione & Blouin, 2004) or migratory versus non-migratory hosts (Blasco-Costa, Waters & Poulin 2012). Ideally, to tease apart the relative influence of host natural history on parasite populations, one should control for disparate evolutionary histories among the parasite populations in question by comparing sister species (Benton 2009; Dawson, Louie, Barlow, Jacobs & Swift 2002). However, the requisite taxon sampling and systematics of few host-helminth systems are sufficiently resolved for this approach due to inherent challenges in working with complex helminth systems (e.g. identifying infected hosts, low prevalence, obtaining sufficient parasite tissue and cryptic variation). In general, population genetic studies of multi-host helminth systems are limited due to inherent challenges in acquiring sufficient parasite material both spatially and temporally. Largely through the utilization of collections housed at the Museum of Southwestern Biology (Parasites Division) and targeted field collections, this study overcame some of these challenges to evaluate the population genetics of the avian schistosome genus *Trichobilharzia* .This study utilized single loci (*cox1, nad4,* ITS1) markers to allow the inclusion of historical collections, and prior genetic surveys of *Trichobilharzia* , where only little or highly degraded material was available for destructive sampling. For most *Trichobilharzia*species it is not uncommon to obtain less than 1ng of DNA following standard extraction protocols (*see methods section*).

Prior studies sampled species of *Trichobilharzia* across its global range, resulting in an updated and robust understanding of the systematics and host associations of these parasites (Brant & Loker, 2009; Ebbs et al., 2016). The present research focuses on North American *Trichobilharzia* species complex known as Clade Q (*sensu*Brant & Loker, 2009) to characterize and compare population genetic patterns of three common Clade Q species. As adults, *Trichobilharzia* develop and reproduce sexually in a duck definitive host (Anatidae) and usually release parasite eggs in their hosts' feces. A free-swimming larval stage (miracidium) hatches from the egg to find the freshwater snail (Pulmonata) intermediate host where asexual amplification occurs. Free-swimming larvae (cercariae) are released from the snail to find a duck host, thus completing the life cycle.

Trichobilharzia is a globally distributed, species-rich genus which infects a broad ecological and taxonomic range of definitive anatid hosts (ducks, geese and swans(Brant & Loker, 2009; Brant & Loker, 2013). This study surveyed 1,358 bird hosts for *Trichobilharzia* infections, in an effort to elucidate host-parasite associations and subsequently characterize population genetic patterns. Here we compare phylogeographic, population genetic and demographic patterns of the three most prevalent *Trichobilharzia* species (Clade Q) recovered from North America (Brant & Loker, 2009, this study): *T. physellae* (TP), *T. querquedulae*(TQ), and *Trichobilharzia* sp. A (TA). Patterns of definitive host-use for the three species will be described in detail below. With respect to their molluscan hosts, TP and TQ are transmitted by physid snails and TA by lymnaeid snails (McLeod, 1937; Brant & Loker, 2009).

Species of *Trichobilharzia* are notable for their public health importance as etiological agents of human cercarial dermatitis (HCD), a global zoonotic disease (Horak et al.l, 2015). The etiology and transmission dynamics of HCD are complex and poorly understood. For example, it is not known if all *Trichobilharzia* species contribute to outbreaks equally or if there are species, or host- or habitat-specific factors that determine outbreak emergence. The data presented herein suggest that *Trichobilharzia* species may be more constrained to specific duck host species than previously appreciated, and accordingly have species-specific population genetic structure, gene flow/drift, effective size, and genetic diversity. It is yet to be studied how these differences might relate to differences in transmission or likelihood of HCD outbreaks, but several hypotheses are discussed here. Broadly, comparative population genetic studies may prove useful in elucidating host traits or conditions that are relevant to parasite transmission and emergence of zoonotic diseases like HCD.

2. Materials and Methods

Host-Parasite Survey

This study surveyed 1,358 avian hosts across 94 species (73 anatids) and 7 countries (Additional File 1), for *Trichobilharzia* infection. Host and parasite records are housed in the Museum of Southwestern Biology (MSB), Parasite Division, at the University of New Mexico, Albuquerque. Hosts were either donated by hunters or obtained from MSB Bird Division. All records are maintained within the Arctos database (https://arctos.database.museum/) and genetic data was obtained through subsampling. All work with vertebrate hosts was conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico, USA (IACUC # 11- 100553-MCC, Animal Welfare Assurance # A4023-01). Most species of *Trichobilharzia*are parasites of the venous system and primarily reside in the mesenteric and hepatic portal veins, with some exceptions, notably *T. regenti* which occupies the nasal mucosa. Mesenteric veins were inspected for adult worms with the aid of a dissecting microscope, and were removed using microscissors (Vanna). Adult worms were also recovered by perfusing the hepatic portal vein with saline and then crushing the liver. Worms were then isolated in a series of decantation steps. *Trichobilharzia* samples were preserved in 95% ethanol for genetic and 80% ethanol for morphological assessment. In addition to the schistosome species recovered from the anatid hosts, co-occurring parasites (liver, kidney, heart and gastrointestinal tract) were also collected. All samples were deposited as vouchers in MSB Division of Parasites (Additional File 1).

For infected hosts we calculated, *prevalence* , the proportion of infected hosts (Bush, Lafferty, Lotz & Shostak 1997), and *observed host range,*calculated as the number of host species infected by a parasite species. A *Trichobilharzia* species was determined to have *ecological specificity* if >90% of infections were recovered from a single ecological group (e.g., dabbling or diving ducks as defined by Sibley 2000). Intensity and consequently abundance are important parameters to consider when estimating host associations (Poulin & Mouillot, 2005). However, due to inherent difficulties of completely sampling *Trichobilharzia* infrapopulations it was not feasible to obtain accurate measures of intensity (infrapopulation size). A parasite*infrapopulation* is defined as all the induvials of a given species within a host at a given time (Bush et al. 1997).

DNA Extraction, PCR Amplification and Sequencing

Trichobilharzia DNA was extracted using the QIAmp DNA Micro Kit (Qiagen, Valenicia, California, USA) or the DNeasy Blood and Tissue Kit (Qiagen, Valenicia, California, USA). We amplified two mitochondrial and one nuclear gene region using primarily the Takara *Ex Taq*kit (Takara Biomedicals, Otsu, Japan). For samples that were difficult to amplify, we used either the Go*Taq* Flexi (Promega) or the Platinum *Taq* kit (Invitrogen) using 0.4 ul of *Taq* and 4ul of 2.5 mM $MgCl_2$ per reaction. We sequenced a 743 bp (5' end) region of the cytochrome oxidase I gene (*cox1*), a 409 bp (5' end) region of the NADH 4 gene (*nad4*) and 733 bp of the internal transcribed spacer region 1 (*ITS* 1, including the 3' end of 18S rRNA and the 5' end of 5.8S). PCR protocols and primers used were identical to those used by Brant and Loker (2009) (*cox1* ,*ITS* 1) and Ebbs et al. (2016) (*nad4*).

Sequencing reactions were performed using the BigDye ver. 3.1 sequencing kit (Applied Biosystems, Foster City, California, USA). Sequences were edited using Sequencher 5.3 (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned using ClustalW(Larkin, Blackshields, Brown, & Chenna, 2007) and adjusted manually as needed. Sequences generated from this study are accessioned in the NCBI GenBank database under the accession numbers XXXX – XXXX.

Phylogenetic Analyses

To evaluate intraspecific relationships phylogenetic analyses of Clade Q (*sensu* Brant and Loker, 2009) were carried out. Phylogenetic datasets were analyzed by individual gene sequences (*cox* 1*, nad* 4*, ITS1*) or concatenated sequences (*cox* 1*+ nad* 4*+ITS1*), using *Trichobilharzia regenti* as an outgroup (Brant and Loker, 2009, Ebbs et al. 2016). Mitochondrial genes (*cox* 1 and *nad* 4) are well suited for intraspecific studies as there is no recombination and they consequently have a higher sorting rate(Avise, 1987; Vilas, Criscione, & Blouin, 2005). Part of the nuclear rRNA complex,*ITS* 1 was chosen to evaluate potential mitonuclear discordance. Appropriate models of nucleotide substitution were selected using JModelTest2(Darriba,

Taboada, Doallo, & Posada, 2012) based on the Akaike information criterion (AIC(Akaike, 1974). Maximum Likelihood (ML) and Bayesian Inference (BI) were performed on each phylogenetic dataset. ML analyses were performed in RaxML v.8 (Stamatakis, 2014), with 1,000 bootstrap replicates to assess topologies. Bayesian Inference (BI) was performed using the program MrBayes v. 3.2.6(Ronquist & Huelsenbeck, 2003), consisting of two replicated runs for each locus with four Markov chain Monte Carlo (MCMC) chains, one cold and three heated chains. Each analysis ran for 10,000,000 generations and was sampled every 1000 generations. The analysis was terminated when the standard deviation of the split frequencies was or fell below 0.01, indicating search chain convergence. Likelihood parameters and convergence between runs were assessed using the program Tracer v.1.6 (Rambaut, 2018), based on EES values greater than 200. The first 2500 trees from each analysis were discarded as burnin. Resulting phylogenetic trees were visualized and manipulated using Fig Tree v. 1.4.4(Rambaut & Drummond, 2009) and MEGA X (Kumar, Stecher, Li, Knyaz & Tamura, 2018)

Pairwise uncorrected *p*- distances were calculated in MEGA X (Kumar et al., 2018) within and between the three species of*Trichobilharzia* to determine the in-group for all subsequent intra-specific analyses. A cut off of [?]5% divergence for *cox* 1 was chosen to delineate each in-group, based on Brant and Loker (2009). In-group datasets included TQ n_{cox1}= 63, n_{nad4}=41, TP n_{cox1}= 27, n_{nad4}=14, TA n_{cox1}= 12, n_{nad4}=9.

We assessed haplotype relationships of mitochondrial genes for the in-group of TP, TQ and TA using minimum spanning network analysis in the program POPart (Leigh & Bryant, 2015). Haplotypes were coded by migratory flyway and host species. Migratory flyways are a common way of partitioning bird populations (Belkhiria, Alkhamis, & Martinez-Lopez, 2016; Swanson, Hawkins, Hanson, Nelson, & Reeves, 1985) and their symbionts(Lam et al., 2012; Pearce et al., 2009). Four North America flyways (Pacific, Central, Mississippi and Atlantic) have been defined; each was sampled for adult *Trichobilharzia* for this study. All samples collected from the Southern Hemisphere were pooled because of the small sample size.

Genetic diversity and population structure

To estimate the genetic diversity and population genetic structure of the TP, TQ and TA infrapopulations, samples were grouped by the state or province the host was collected from and then regionally by the migratory flyway from which the host was collected. Indices of genetic diversity for each species and their respective populations were estimated in DNAsp v. 5 (Librado & Rozas, 2009) for both *cox* 1 and *nad* 4; number of polymorphic sites (S), average number of nucleotide differences (K) and nucleotide diversity (π). Uncorrected *p*- distances were calculated between flyways, regions and host species in Arlequin 3.5 (Excoffier & Lischer, 2010).

One-way AMOVAs were performed in Arelquin v. 3.5 (Excoffier & Lischer, 2010) to estimate overall population genetic structure (*cox1*). A set of population genetic hypotheses was tested for each species:

H_1: Structure by flyway, where infrapopulations were grouped by host locality and regionally by flyway. For the TQ dataset AMOVAs were run both with the inclusion and exclusion of Southern Hemisphere populations.

H_2: Structure by host species, where infrapopulations were grouped by duck species.

H_3: Structure by latitude, where infrapopulations were separated into high vs. low latitude groups rather than the longitudinal groups designated by flyway.

Estimates of Φ_{ST} variation were generated from all samples across all localities sampled, among flyways, host species, latitudinal group and among localities within the flyways, host species, and latitudinal groups. We also estimated pairwise Φ_{ST}(Reynolds, Weir, & Cockerham, 1983) by locality, flyway and host species. Significance ($P<0.05$) was determined by permutation tests of 10,000 random permutations.

To test for isolation by distance patterns we constructed a geographic distance matrix by converting geographic coordinates to Euclidean distances between localities as implemented in Primer 7(Clarke & Gorley,

2015). Correlation between geographic distances and both uncorrected$p$- distances and pairwise Φ_{ST} matrices were calculated using Spearman's rank correlation also in Primer 7.

Demographic analyses

To assess contemporary and historical demographic patterns, Watterson's estimator (Θw) and Tajima's D (D), were calculated (*cox1*) and assessed statistically using coalescent simulations with a 95% confidence interval and 10,000 permutations in DNAsp (Librado & Rozas, 2009).

Changes in *Ne* over time were estimated by fitting a Bayesian Skyline demographic model (BSP, Drummond, 2005) for each of the three species of interest based on *cox1* *BEAST v. 1.7.0 (Drummond et al., 2012). The HKY substitution model was used for two simultaneous MCMC runs for 10,000,000 iterations sampling every 1,000 steps. Mutation rates of 2% and 4% change per million years were used to estimate mitochondrial genome evolution, as has been used for*Schistosoma mansoni* (Morgan et al., 2005; Jones et al., 2020). As substantial divergence within TA collected between the Pacific and Central flyways was estimated, these populations were analyzed separately. Convergence was checked (ESS of 200 or greater) and results visualized using Tracer v1.6 (http://beast.bio.ed.ac.uk/Tracer). BSP data from each analyzed datasets generated in Tracer v. 1.6 were exported and visualized in Microsoft Excel for comparison.

3. Results

3.1 Trichobilharzia survey data

A total of 1,358 birds were examined in this study (Additional File 2). Specimens representing species of *Trichobilharzia* were only recovered from hosts within the Anatidae. Based on our examinations, and those from the literature, there were 464 anatid hosts (n=1271, 36.5%) infected with at least one species of*Trichobilharzia.* Co-infection of multiple *Trichobilharzia*lineages was not uncommon (Additional File 2) and was probably underestimated as not all individual worms were assessed genetically. Of the 73 anatid species examined, 29 were infected with a species of*Trichobilharzia* .

TQ, TP and TA were the three most prevalent *Trichobilharzia*species recovered from our North American surveys. The three species varied substantially in their host species range; however, the infection pattern of each species was found to occur primarily (>90%) in a particular group of ducks (Table 1, Figure 1). These groups of ducks correlate to their general ecological patterns such as dabbling (feeding in shallow, vegetated waters) versus diving ducks (feeding in areas with some depth for diving for plants/invertebrates or fish). This distinction is related to their feeding habitat, behavior, and ecology, rather than exclusively phylogenetic relatedness (Sibley, 2000; Sun et al. 2017; Meehan et al. 2021). TP was found to infect 8 species within 5 genera, with highest prevalence in *Aythya affinis,* 27%, *A. collaris,* 26% and*A. valisineria,* 20%, which can be considered core host species, as collectively they represent 73% of all infections (Bush, 1997). Lower TP prevalence's were found within *Anas platyrhynchos*(12.5%)*, Clangula hyemalis* (10%)*, Mergus merganser*(10%)*, Bucephala albeola* (7.7%)*,* and *Mareca strepera* (2.8%). In concordance with previous studies (Brant and Loker 2009; Ebbs et al. 2016), TQ was found almost exclusively (98.3%) from the dabbling duck genus *Spatula* , (Gonzalez, Düttmann, & Wink, 2009; also described as *Anas* Clade B by Sun et al. 2017), with a single report (2.7%) from an American wigeon (this study,*Mareca americana*). Prevalence of TQ was highest within*Spatula discors* (90%), but all *Spatula* species examined were infected at a rate of [?]74%. *Trichobilharzia* sp. A was recovered exclusively (100%) from the American wigeon (*Mareca americana*), having the narrowest host range of the three species, with a prevalence 27%. Interestingly however, *M. americana* was found to harbor the highest *Trichobilharzia* species diversity of any of the host species examined, with 5 *Trichobilharzia* lineages recovered (Additional File 2), compared to an average of 1.5*Trichobilharzia* species per host species within North America (1.8 per host species globally, Additional File 2).

3.2 Phylogeographic patterns among congeners

3.2.1 TRICHOBILHARZIA QUERQUEDULAE : Phylogenetic analysis of TQ supports its global distribution within *Spatula* (Ebbs et al. 2016, Figure 2). Mitochondrial data suggests that three individuals

(W345.3, W750.2 and W771, ˜4.7% divergent) fall basal to the TQ clade (Figure 2, Additional File 3). While genetic distances were below the 5% threshold for in-group TQ analyses, this subclade may warrant further investigation as a putative cryptic species, hybrids, or divergent subpopulations. Interestingly, unlike most specimens which are from *S. discors* , all 3 of these basal individuals were recovered from *S. clypeata* . Apart from the above three samples, minimal phylogenetic structure was observed within TQ with no pattern that suggests there is structure by geography or host use, based on either ML or BI analyses (Figure 2, Additional File 3).

Minimum spanning networks (Figure 3) of *cox1* revealed high haplotype diversity within TQ, signifying genetically diverse and well-connected populations. Infrapopulations (within individual hosts) haplotype diversity was similar to component population (infections of a given life stage across hosts) haplotype diversity, suggesting infrapopulation recruitment occurs randomly over time and space (Nadler, 1995; Sire, Durand, Pointier, & Theron, 2001). TQ haplotypes did not partition according to flyway, and Southern Hemisphere haplotypes did not group together and were not found to be more or less divergent than Northern Hemisphere haplotypes (also see Ebbs et al., 2016). No evidence of genetic structure in association with host species was found. An AMOVA testing for structure by latitude was found to be significant, indicating possible population genetic differentiation between worms acquired from either the summer (breeding) or wintering ranges, related to host philopatry, or potentially differentiation among resident host populations in low latitudes versus migratory populations.

3.2.2 TRICHOBILHARZIA PHYSELLAE: Two subclades (TP1, TP2) were recovered within TP based on *cox* 1 analysis (Figure 2) that corresponds to eastern and western collection sites. These two subclades were recovered as well in *nad4* and *cox1* + *nad4* + ITS1 analyses (Additional File 3) but were not supported statistically. Despite limited geographic sampling, representation within the two subclades might suggest a distinction between eastern and western populations of TP. Haplotypes within TP1 were recovered from 3 of the 4 flyways, 75% (12 of 16) of TP2 individuals were recovered from the Central flyway. Networks were additionally coded by high versus low latitude and host species, which suggested no further sub-structuring. An AMOVA indicated substantial population genetic differentiation in association with migratory flyway (Table 4).

3.2.3 TRICHOBILHARZIA SP. A: Mitochondrial and concatenated datasets support the monophyly of TA and an affinity to a group with*T. franki* and *Trichobilharzia* sp. B (Additional File 3). However, ITS1 analysis (Additional File 3B) suggests that TA is paraphyletic with TP, *T. franki,* and *Trichobilharzia* sp. B. This result could suggest mito-nuclear discordance within the TA dataset that was not found within either TP or TQ. A single specimen (W249) grouped with support within *Trichobilharzia* sp. C, rather than TP in the *cox* 1 analysis (Figure 2). Geographical sampling was too limited to draw phylogeographic inferences for a minimum spanning network of TA haplotypes.

No supported sister group relationships within Clade Q were found. Bayesian inference of mtDNA supported *Trichobilharzia* sp. B as sister to *Trichobilharzia* sp. A, and both these lineages infected American wigeon (*Mareca americana*). This result however was not found in the analysis of ITS1 or for the concatenated dataset,*cox1+nad4+* ITS1. The *cox1* analysis of Clade Q showed a South American *Trichobilharzia* lineage (W701) transmitted by a physid snail(Pinto, Brant, & Melo, 2014) as a well-supported sister clade to *T. physellae,* however this relationship was not found in the ITS1 gene tree (Additional File 3B). No gene tree supported *T. regenti* or any other *Trichobilharzia* lineage as the root of Clade Q (Figure 2, Additional File 3).

3.3 Comparative population genetic structure

3.3.1 TRICHOBILHARZIA QUERQUEDULAE: Hierarchal AMOVA indicated population structure of *T. querquedulae* is not mediated by migratory flyway (Table 4). To determine if Southern Hemisphere populations were obscuring flyway structure within North American samples, hierarchical AMOVA was repeated excluding non-North American samples, but similar results were obtained. Instead of flyways,

populations were partitioned by latitudinal group (high vs. low) as a proxy for genetic structure associated with breeding vs. wintering range. Analyses including (Φ_{CT}= 0.0441, p=0.00196) and excluding (Φ_{CT}= 0.0072, p= 0.0332, results not shown) Southern Hemisphere populations support latitude in structuring TQ populations. Further significant population structure was found at all levels (Table 4) when latitude was considered. No significant structure by host species was found. Pairwise $_{ST}$ values among flyways are summarized in Table 3. When all populations within a flyway were pooled no significant differentiation between flyway was found within TQ. However, all North American flyways were found to be distinct from the Southern Hemisphere populations. Pairwise $_{ST}$ between localities within flyways and by host species were also measured (results not shown), which identified TQ from northern Manitoba and New Zealand as the most distinct populations. Interestingly, TQ populations collected from different host species (*Spatula clypeata* vs. *S. cyanoptera*) from the same locality (New Mexico) were found to be significantly distinct (p [?]0.01). A Spearman rank test was performed and correlation between geographic distance and both pairwise O_{ST} and uncorrected p- distance was found to be weak (ρ= -0.11 and ρ= 0.002, respectively) suggesting no relationship between geographic distance and genetic diversity/structure.

3.3.2 TRICHOBILHARZIA PHYSELLAE : AMOVA by flyway indicated significant differentiation among flyways (Φ_{CT}= 0.55592,p= 0.02737) (Table 4), supporting the hypothesis that flyways structure TP populations. When populations were grouped according to latitudinal group no significant population genetic structure was observed. When TP individuals were pooled by host species they were collected from and then partitioned by host genera a significant Φ_{ST} was recovered (p= 0.00293), however, neither the Φ_{SC} nor Φ_{CT} were significant suggesting no real influence of host species. The TP dataset is more limited in terms of sample size, yet significant differentiation was found between several flyways (Table 3-4). Pairwise Φ_{ST}between localities within flyways and by host species were also measured (results not shown) which identified that TP recovered from New Mexico were distinct from both Alaskan ($_{ST}$= 0.2952,p <0.001) and Michigan (Φ_{ST}= 0.24159,p <0.001) populations. The Mississippi and Atlantic flyways harbored the highest within flyway divergence values, 1.2% and 0.95% respectively in comparison to the Central (0.01%) and Pacific (0.048%). There was no evidence that host species influenced population genetic structure.

Correlation between geographic distance and uncorrected$p$- distance was moderate (ρ= 0.473) indicating a relationship between genetic diversity and spatial separation. Geographic distances were not correlated with pairwise Φ_{ST} values (ρ= -0.025).

3.3.3 TRICHOBILHARZIA SP. A: Since TA was only recovered from two flyways a hierarchal AMOVA was not performed. Pairwise Φ_{ST} between the two flyways sampled (Pacific and Central) was significant (Φ_{ST}=0.3283, p<0.001). Genetic divergence within the Central flyway was higher (1.4%) than in the Pacific (0.36%) with an average$p$- distance of 1.38% between the two flyways. Due to limited geographic sampling a Spearmen rank test was not performed.

3.4 Genetic diversity and demographic analyses

Haplotype diversity was similar across TP, TQ and TA, however nucleotide diversity (π) and were substantially different across the three species (Table 2).

Figure 4 shows estimates of *Ne* over time (kya) for TP, TQ, and TA based on a 4% change/million years (Morgan et al., 2005; Jones et al., 2020), a lower rate of 2% was also tested (Additional File 5). For TP and TQ estimates of *Ne* were double under the 4% rate (and triple under 2% rate). Both TQ and TP indicate a recent population expansion, further supported by significant positive Tajima's D values (Table 2). Over coalescent time the effective size of TQ was approximately 3X greater than TP*,* suggesting the former species has a history of maintaining larger genetically diverse populations. TA exhibited the smallest effective population size of the three species with no evidence for notable historical demographic changes.

4. DISCUSSION

This study contrasted phylogeography and genetic structure of populations of three *Trichobilharzia* species,

revealing microevolutionary patterns within complex and widespread host-helminth systems. In multi-host-parasite systems, little is known of determinants of *Ne* , π and (Criscione et al., 2005b; Gorton, Kasl, Detwiler, & Criscione, 2012), which are fundamental parameters in disease ecology and parasite control. For example, movement of alleles (e.g. resistance alleles) within and between populations, which ultimately shapes the adaptive potential of a species (Avise, 2000), depend on these parameters. We encountered substantial differences in estimates of *Ne* , π and of three *Trichobilharzia* species, most notably between TQ and TP. Herein, we discuss features of the three host-*Trichobilharzia* relationships that may influence these values, and their implications from a microevolutionary and disease-ecology perspective.

Definitive Host Migration and Ecology

Comparative population genetic analyses of TQ, TP and TA revealed marked differences in their population structure and demographic histories (summarized in Tables 2-5). The plausibility of the population genetic structure (or relative lack thereof in TQ) observed in our study is supported by geographically widespread sampling of parasite species. Spatially limited sampling may fail to reveal structure in parasites that are widely distributed in birds (Louhi, Karvonen, Rellstab & Jokela, 2010), with patterns emerging mainly intercontinentally in markers such as CO1 (Locke et al. 2021). In TQ, a common and genetically diverse schistosome of *Spatula* spp. across four continents (Ebbs et al. 2016; this study), population co-structure was only weakly associated with duck migratory ranges, specifically with latitude as a proxy for breeding and non-breeding range. No phylogeographic structure was found within TQ despite its global distribution, and occurrence in non-migratory Southern Hemisphere *Spatula* spp. Historical effective population sizes (*Ne*) of TQ were at least three times greater than TP and TA. Contemporary regional measures of π and were high across the range of TQ, including in Southern Hemisphere populations, and regional values largely matched species wide and infra-population measures, suggesting high gene flow across the range of this species. In contrast, TP maintains lower species-wide π and . Phylogenetic analysis suggests the existence of two clades of TP, possibly separated into the Eastern and Western parts of the overall range. To our knowledge, the phylogeographic structure of North American *Aythya* spp. has not been characterized, and therefore it is unknown if the structure we observed within TP reflects that occurring among host populations. In Eurasia, *Aythya* spp. display moderate to high female philopatry, minimal structure between Europe and Asia and extensive admixture within the wintering range (Liu, Keller, & Heckel, 2011; Liu, Keller, & Heckel, 2012). Within TP, hierarchal AMOVA (Table 4) and pairwise Φ_{ST} values (Table 3) indicates geographic structuring potentially in association with migratory flyways, a result congruent with an east/west division. Overall, TP populations exhibit greater genetic structure relative to TQ, possibly indicating the importance of ecological behaviors specific to their duck hosts (i.e. distribution, philopatry, microhabitat preferences, diving rather than dabbling).

We report a 63% difference in the prevalence of TQ and TP across all hosts, and a 64% prevalence difference between the single most common host of each species (*Spatula discors* and *Aythya affinis* respectively), suggesting a stark difference in probability of definitive host infection. Both TP and TQ both utilize *Physa* spp. (Physidae) as their snail intermediate host, which are more abundant in the shallow littoral zone. The higher prevalence of TQ in *Spatula* spp. may be related to its feeding ecology (dabbling in shallow waters and marshes) relative to the diving habits in deeper water of *Aythya* spp., which were less commonly infected with TP. *Physa* spp. are abundant in different habitats across North America, and parts of South America (Wethington & Lydeard, 2007). One species, *P. acuta* is globally invasive occurring on 6 of the 7 continents (Bousset, Pointier, David, & Jarne, 2014; Ebbs, Loker, & Brant, 2018). *Physa* spp. are ubiquitous and can be found in large densities in a diversity of freshwater habitats (Vinarski, 2017). While experimental infections are necessary to rule out inherent differences in snail and duck host susceptibility, these results may imply that certain combinations of hosts and microhabitats favor parasite populations, facilitating larger effective sizes and accordingly higher parasite genetic diversity.

Infrapopulation Diversity and Within-Host Recruitment

Infrapopulation diversity of TQ was equal to overall component population diversity and the number of unique haplotypes within infrapopulations increased linearly with the number of individuals sampled. This suggests that infrapopulation recruitment (transmission) is random, likely occurring across time and space and a well-mixed component population. Suggesting that ducks are not recruiting *Trichobilharzia* in clonal packets but accumulate unique haplotypes, similar results have been demonstrated *Diplostomum pseudospathaceum* (Rauch, Kalbe & Resuch, 2005). Notably, the majority of worms sampled were males due to the difficulty of recovering females from the duck host, as commonly reported in prior *Trichobilharzia*surveys (McLeod, 1937; McMullen & Beaver 1945; Blair & Islam 1983; Brant & Loker 2009). Female worms migrate to and remain in the intestinal serosa to lay their eggs, thus greatly increasing screening time per bird as well as reducing probability of locating and sampling them for genetics. The high level of infrapopulation diversity observed suggests that duck hosts sampled maintain a genetically diverse pool of male worms. Sex specific population genetic structure has been reported in the related human schistosome, *Schistosoma mansoni* (Prugnolle, 2005), where male genotypes were found to be more randomly distributed relative to female genotypes, and therefore sex-related differences cannot be excluded as a potential confounder in this study.

In contrast to TQ, infrapopulations of TP appear to be less heterogeneous (Figure 2, Table 2) possibly suggesting that recruitment is structured geographically, perhaps associated with the overall greater population subdivision observed within TP relative to TQ. Deeper sampling of TP infrapopulations across regions is needed to clarify the extent and cause of this pattern, which could indicate differential transmission dynamics and ultimately local adaption among subpopulations (Lively, 2017).

Multiple infrapopulations of TA were sequenced from American wigeon (*Mareca americana*), which hosts the highest *Trichobilharzia* lineage diversity. Our data suggest that *M. americana* maintain at least 5 distinct *Trichobilharzia* lineages, 4 of which are undescribed (Brant and Loker 2009, Ebbs et al. 2016).*Mareca americana* is considered one of the most generalist species of dabbling duck (Johnson & Grier, 1988) in terms of its seasonal habitat preferences and this habitat variability may increase the probability of encountering different snail species and their associated parasites.

Definitive Host Habitat Predictability

The hosts of TQ in North America (*Spatula clypeata, S. discors*and *S. cyanoptera*), have a broad migratory range and select local habitats (Johnson & Grier, 1988), preferring shallow marsh habitat (Osborn, Hagy, Mcclanahan, Davis, & Gray, 2017). Site selection among North American dabbling ducks, such as *Spatula* spp., is highly variable (Humphreys, Murrow, Sullivan, & Prosser, 2019). Habitat quality is the primary determinant of site selection for these species; consequently ducks move locally to find optimal habitat, increasing their probability of encountering infected snails across the sampling different habitats (Johnson & Grier, 1988; Austin, O'Neil, & Warren, 2016) These behaviors result in*Spatula* spp. spending a majority of their time in*Physa* -rich habitat, increasing the likelihood of encountering*Trichobilharzia* spp. In contrast, North American *Aythya*spp. occupy larger and more stable water bodies and may not spend as much time in shallow areas, particularly on their wintering grounds.*Aythya* spp. are highly philopatric, as a result habitat quality is not a primary factor in site selection and consequently local movement of *Aythya* spp. is relatively limited compared to*Spatula*spp.(. These behaviors in combination decrease the likelihood of *Aythya* spp. to encounter locally available *Trichobilharzia,* corresponding to the low prevalence of TP observed in this study. It can also be predicted that philopatric host species are more likely to facilitate population genetic structure among their parasites (Prugnolle, 2005), which is consistent with patterns we observed in TP relative to TQ. Additionally, the timing of migration between North American*Spatula* spp. and *Aythya* spp. is markedly different (Anteau & Afton, 2004; Arzel, Elmberg, & Guillemain, 2006), potentially conferring temporal separation of ducks hosts and available*Trichobilharzia* spp. within shared water bodies. The life spans of parasites could also affect patterns observed. Adult TP may be less long lived than TQ, leading to a smaller standing crop, and lower prevalence as observed in our samples. Experimental work is necessary to fully elucidate adult life-history of the respective species, however it can be noted that anecdotally our survey data suggests the prevalence of TP appears stable over season and sampling year. Further, differences

in life span would not account for the substantial differences in estimates of π, , *Ne* and population genetic structure that we observe.

The host range of a parasite (i.e. host specificity) is determined by host-parasite *encounter* and host-parasite *compatibility* , which have been conceptualized as successive filters to establishing infections (Combes, 2001). Studies of the compatibility filter in schistosome-bird interactions are scant, owing to difficulties of rearing birds and parasites and of securing the necessary approvals. Older studies suggest that *Trichobilharzia* are compatible with anatids and some non-anatids. For example, McMullen and Beaver (1945)) demonstrated that TP could develop to patency in non-anatids (e.g canaries and pigeons). On balance, the encounter filter appears to dominate in schistosome-bird interactions in both older and more recent work. Extensive molecular surveys (Brant & Loker 2009, Ebbs et al. 2016, this study) suggest that many, if not most, *Trichobilharzia* species (Additional File 2) are predictably recovered from a specific duck species or ecological group, and present but rare in other likely compatible host species. Here, we found the *encounter* filter was narrowed by the ecology of the host species/ecological group. From our survey of 1,358 birds, 6 of the 9 (˜67%) *Trichobilharzia* lineages recovered with adequate sampling were found to have >90% of reported infections occur within a single duck species or ecological group (Table 1, Additional File 2).

Adaptive Potential among Trichobilharzia *species*

The parameter ($= 4N_e\mu$) may indicate a species' genetic flexibility, or ability to respond to environmental or demographic change(Criscione & Blouin, 2004; Nuismer & Gandon, 2009; Archie & Ezenwa, 2011) and as such it is often used as a measure of adaptive potential (Gorton et al., 2012). In the case of *Trichobilharzia* spp. it can be hypothesized that TQ, with higher estimates of , has a greater adaptive potential than TP. Under this view, populations of TQ may be more resilient to environmental change (e.g. host extinction, climate change) relative to TP.

Adaptive potential of metazoan parasites is in part a function of host demography (Prugnolle, 2005; Barrett et al., 2008), specifically host effective population size. In light of global changes in the population dynamics of North American waterfowl (Zhao, Silverman, Fleming & Boomer, 2016; Zhao, Boomer & Royle, 2019) understanding the genetic distribution and microevolutionary consequences of these complex host-parasite interactions is crucial. For example, lesser scaup (*Aythya affinis*) populations, a primary host for TP, have been in decline since the 1980's (Afton & Anderson, 2001; Koons et al., 2006), for reasons not fully understood, but undoubtedly exacerbated by mass die offs from infection of invasive trematodes; *Cyathocotyle bushiensis* and*Sphaeridiotrema globulus* (Herrmann & Sorensen 2011, England et al. 2018). The population dynamics of diving ducks such as *A. affinis* are more sensitive to environmental variability than dabblers, in both North America and Europe (Pöysä et al., 2016). How long-term host demographic changes will affect *Trichobilharzia* populations is unclear. Interestingly, since the late 1970's the sex ratio of lesser scaup has been skewed towards males (Afton & Anderson 2001), with an extreme ratio of 2.37 males to 1 female. We were unable to test for host sex bias in *Trichobilharzia* infections because sex was not always recorded. We hypothesize that because breeding females likely spend more time in transmission-rich sites while rearing offspring, they are probably exposed to more *Trichobilharzia* than males.

Conclusions

In sum, using both museum specimens and target field collections, we investigated population genetics of three *Trichobilharzia* species and found that estimates of π, ϑ, *Ne* and population structure varied greatly among species. Our data reveal markedly different patterns among species of *Trichobilharzia* , some of which can be plausibly attributed to host ecological traits, which may affect parasite gene flow. Our study highlights the need for experimental infections to 1) characterize the role of phylogenetically influenced compatibility barriers in definitive host specificity; 2) better characterize the course of infection in birds including lengths of prepatent and patent periods and lifespan of male and female worms; 3) test the likelihood that wild bird species can be reinfected with the same or different schistosome species. Additionally, this study highlights the utility, and in the case of most multi-host parasite systems, necessity of museum collections as commented on by other authors (Harmon, Littlewood & Wood, 2019; Thompson et al., 2021). *Trichobilharzia*

is becoming an increasingly important zoonotic parasite globally as outbreaks of HCD are becoming more frequent and widespread (Horak et al. 2015). Our data suggests that specific features of duck natural history and demographics affect *Trichobilharzia*transmission and population dynamics, and these features may have relevance for HCD outbreaks. For example, our data suggest that habitats frequented by dabbling ducks present more risk of HCD than those frequented by diving ducks.

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DATA ACCESSIBILITY

All sequence data generated is uploaded to GenBank and accessible through accession numbers XXXX-XXXX. All alignments and phylogenies are available on TreeBASE

All host and parasite records are publicly searchable through the Arctos Database and are curated by the Museum of Southwestern Biology, Parasites Division in Albuquerque New Mexico.

AUTHOR CONTRIBUTIONS

ETE: Contributed to all aspects of the study; Study conceptualization conceived, designed and carried out the research, analyzed and interpreted data, wrote the manuscript.

ESL: Provided samples, funding, discussed all aspects and contributed to the final manuscript.

DM: Contributed to sequence data collection

SAL: Contributed critical specimens, contributed to the final manuscript

ND: Contributed critical specimens

VVT: Contributed critical specimens

SVB: Contributed data and specimens and to the study design, performed research, provided funding, discussed all aspects and contributed to the final manuscript.

Captions for Figures:

Figure 1. Summary of TQ, TP and TA infections by duck host. Cladogram is based on Sun et al. 2017. TQ= orange, TP=green and TA=blue.

Figure 2. *cox*1 phylogeny. BI inference of TQ, TP and TA, only posterior probabilities [?].95 are presented. Branches are colored by species; TQ= orange, TP=green and TA=blue. Individual worms are labeled by the migratory flyway they were collected from, denoted by a colored box. The two TP clades are denoted as I and II.

Figure 3. Minimum spanning network of *cox*1 haplotypes.(A) TQ haplotypes, (B) TP and (C) TA. Haplotypes are colored by the migratory flyway the host was collected from.

Figure 4. Bayesian skyline plot of *cox*1 datasets.Estimates of *Ne* (y-axis) over time (kya, x-axis). Lines are colored by species; TQ= orange, TP=green and TA=blue.

Additional Files:

Additional File 1: Locality and database information for all samples included in this study. MFW= North American Migratory Flyway. (*) denotes sequences that were published in Brant & Loker 2009. (ˆ) denotes sequences that were published in Ebbs et al. 2016

Additional File 2: *Trichobilharzia* survey data.

Additional File 3: Pairwise genetic distances for *Trichobilharzia*.

Additional File 4. BI inference of TQ, TP and TA, only posterior probabilities [?].95 are presented. Branches are colored by species; TQ= orange, TP=green and TA=blue. Individual worms are labeled by the migratory flyway they were collected from, denoted by a colored box. **(A) *nad*4 Phylogeny:** average pairwise*p*- distances between TQ subclades are indicated. **(B)*ITS*1 Phylogeny.**

Additional File 5 . Minimum spanning network, showing relationships of haplotypes by colored by host genera. (A) TP, (B) TQ

Additional File 5. Bayesian skyline plot of *cox*1 datasets, all mutation rates. Estimates of *Ne* (y-axis) over time (kya, x-axis), using a 2%(dashed line) and 4% (solid line) rate of*cox* 1 change. TA populations were further subdivided by flyway as significant among flyway divergence was suspected. Lines are colored by species; TQ= orange, TP=green and TA=blue.

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