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Systematics and life cycles of four avian schistosomatids from Southern Cone of South America

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Abstract

Relative to the numerous studies focused on mammalian schistosomes, fewer include avian schistosomatids particularly in the southern hemisphere. This is changing and current research emerging from the Neotropics shows a remarkable diversity of endemic taxa. To contribute to this effort, nine ducks (*Spatula cyanoptera*, *S. versicolor*, *Netta peposaca*), 12 swans (*Cygnus melancoryphus*) and 1,400 *Physa* spp. snails from Chile and Argentina were collected for adults and larval schistosomatids, respectively. Isolated schistosomatids were preserved for morphological and molecular analyses (*28S* and *COI* genes). Four different schistosomatid taxa were retrieved from birds: *Trichobilharzia* sp. in *N. peposaca* and *S. cyanoptera* that formed a clade; *S. cyanoptera* and *S. versicolor* hosted *Trichobilharzia querquedulae*; *Cygnus melancoryphus* hosted the nasal schistosomatid, *Nasusbilharzia melancorhypha*; and one visceral, Schistosomatidae gen. sp., which formed a clade with furcocercariae from Argentina and Chile from previous work. Of the physid snails, only one from Argentina had schistosomatid furcocercariae that based on molecular analyses grouped with *T. querquedulae*. This study represents the first description of adult schistosomatids from Chile as well as the elucidation of the life cycles of *N. melancorhypha* and *T. querquedulae* in Chile and Neotropics, respectively. Without well-preserved adults, the putative new genus Schistosomatidae gen. sp. could not be described, but its life cycle involves *Chilina* spp. and *C. melancoryphus*. Scanning electron microscopy of *T. querquedulae* revealed additional, undescribed morphological traits, highlighting its diagnostic importance. Authors stress the need for additional surveys of avian schistosomatids from the Neotropics to better understand their evolutionary history.

Introduction

Schistosomatids (Digenea: Schistosomatidae) are digenetic flukes inhabiting the venous and arterial systems of birds and mammals, with the medically noteworthy genus *Schistosoma* Weinland, 1858 as the most well studied taxon. This family is currently composed of 17 genera, 13 of which are found parasitising different orders of freshwater and marine birds (Gibson *et al.*, 2002; Horák et al., 2015; Flores *et al.*, 2021; Lorenti et al., 2022). As part of the phylogenetic clade "Derived avian schistosomes", hereafter clade DAS (*sensu* Brant & Loker, 2013), the genus *Trichobilharzia* Skrjabin & Zakharow, 1920 is considered the most speciose with about 40 species, mostly parasitising birds of order Anseriformes, and is globally distributed (Brant & Loker, 2009, 2013; Horák et al., 2015; Ebbs *et al.*, 2022).

The life cycle of avian schistosomatids is not as complex compared to other digeneans because it requires only one intermediate host, an aquatic snail, rather than two or three. The snail hosts become infected with schistosomatids when miracidia in feces or nasal secretions of the infected bird hosts are released into the water. In the snail, sporocysts and then furcocercariae will develop, the latter is the infective stage to the birds (Horák & Kolářová, 2011; Horák et al., 2012). Once the furcocercaria penetrates the skin of the new bird host, it transforms into a schistosomula and migrates through the bloodstream to reach sexual maturation in the nasal, arterial, or venous system depending on the schistosomatid species. However, when these furcocercariae accidentally penetrate humans, it causes a zoonotic allergic condition of a cutaneous manifestation called cercarial dermatitis or swimmer's itch (Horák et al., 2015). The zoonotic cercarial dermatitis has been reported in both Argentina and Chile (Flores *et al.*, 2015; Oyarzún-Ruiz *et al.*, 2022).

Host specificity of avian schistosomatids to their mollusk intermediate host has been shown to be somewhat narrow but often not restricted to a single species (Horák & Kolářová, 2011; Horák et al., 2015). Unlike the mollusk hosts, reports suggest that the specificity of schistosomatids to their avian hosts is not as restricted to a single species of host or host group (Brant & Loker, 2009; Ebbs *et al.*, 2016). The appearance of schistosomatid host specificity in the absence of experimental work can be due to consistent ecological and behavioral traits of their avian hosts (Ebbs *et al.*, 2016). Although more studies are needed, there are examples of species that only have been found in a particular host group such as *Allobilharzia visceralis* Kolářová, Rudolfová, Hampl & Skírnisson, 2006 parasitising only swans in the Northern hemisphere (Kolářová *et al.*, 2006; Brant, 2007; Hayashi *et al.*, 2017), *Anserobilharzia brantae* (Farr & Blankemeyer, 1956) Brant, Jouet, Ferte & Loker, 2013 in geese, *Trichobilharzia physellae* (Talbot, 1936) McMullen & Beaver, 1945 in diving ducks and *Trichobilharzia querquedulae* McLeod, 1937 in blue-winged ducks (Brant & Loker 2009, 2013; Ebbs *et al.*, 2016). Although these species represent a few examples, the continued use of molecular tools and phylogenetic analyses can help establish if this phenomenon is more or less widespread in the avian schistosomes (Brant & Loker, 2009, 2013).

Flores *et al.* (2015) summarised the schistosomatid taxa reported in South America but in the past decade since that summary, there have been several more investigations into the systematics of schistosomatids parasitising aquatic birds (see Ebbs *et al.*, 2016; Pinto *et al.*, 2017, 2022; Flores *et al.*, 2021; Lorenti et al., 2022) revealing considerable new diversity. The focus of this work will be on waterfowl hosts (Anseriformes) in the Neotropics because much of the discovered diversity has come from this group of avian hosts. These hosts are also the host most likely to overlap with people in recreating or working aquatic areas; thus, the zoonotic potential to transmit cercarial dermatitis.

In Neotropical waterfowl there have been six species of avian host reported to have schistosomatids; white-cheeked pintail (*Anas bahamensis* Linnaeus), yellow-billed pintail (*Anas georgica* Gmelin), blue-winged teal [*Spatula discors* (Linnaeus)], silver teal [*Spatula versicolor* (Vieillot)], muscovy duck [*Cairina moschata* (Linnaeus)], and the black-necked swan [*Cygnus melancoryphus* (Molina)] from Argentina (Szidat, 1951; Flores *et al.*, 2015, 2021; Ebbs *et al.*, 2016), Brazil (Travassos et al., 1969; Freitas & Costa, 1972; Leite et al., 1978, 1979; Pinto *et al.*, 2017), Chile (Oyarzún-Ruiz *et al.*, 2019), and Cuba (Sánchez *et al.*, 2018).

If the species richness of Neotropical waterfowl is considered, only a small fraction of these have been reported as definitive hosts of avian schistosomatids. Therefore, it is not a case of low species richness of these schistosomatids in birds, but rather a minimal effort in the study of their helminth fauna, particularly for schistosomatids (Agüero et al., 2016; Oyarzún-Ruiz & González-Acuña, 2021). To date, despite only a small proportion of anseriform host diversity having been examined, there are already five species belonging to three genera of avian schistosomatids that have been described: *Trichobilharzia*, *Nasusbilharzia* Flores, Viozzi, Casalins, Loker & Brant, 2021 and *Dendritobilharzia* Skrjabin & Zakharov, 1920 (Leite et al., 1978; Ebbs *et al.*, 2016; Flores *et al.*, 2015), with *Nasusbilharzia* restricted to the Neotropics (Flores *et al.*, 2021). Only two of the five species, both from Argentina, have been molecularly characterised: *T. querquedulae* and *Nasusbilharzia melancorhypha* Flores, Viozzi, Casalins, Loker & Brant, 2021 (Ebbs *et al.*, 2016; Flores *et al.*, 2021). In Chile there are two previous records of avian schistosomatids parasitising aquatic birds (i.e., Chilean flamingo [*Phoenicopterus chilensis* Molina] and *C. melancoryphus*), but none of these studies included a morphological or molecular characterisation, only the histopathological discoveries from the infected birds (Paré & Black, 1999; Oyarzún-Ruiz *et al.*, 2019). In Argentina and Brazil, studies have found avian schistosomatids in gulls and penguins (Brant *et al.*, 2017; Vanstreels et al., 2018; Lorenti et al., 2022).

To continue to advance our impoverished understanding of the evolutionary ecology and life history of avian schistosomatids in the Neotropics, this study aimed to use both morphological and molecular features to characterise the avian schistosomatids parasitising waterfowl and freshwater snails in Chile and Argentina.

Material and methods

Sampling and necropsy of waterfowl

The sampling of waterfowl from Chile (Ñuble region, Biobío region, and Los Ríos region) and Argentina (La Pelada, Corrientes province) was performed between January 2019 and January 2021. Five carcasses of *C. melancoryphus* (died by natural causes) from the Ramsar site Carlos Anwandter Nature Sanctuary, Los Ríos region, Chile, were retrieved following an official permission for research by the state organisation CONAF (Permission number 1201020). Seven wild black-necked swans from different localities from Ñuble and Biobío regions, which were euthanised for humanitarian reasons at Centro de Rehabilitación de Fauna Silvestre, Universidad de Concepción, Chillán, Chile, were also included in this study. In addition, the carcasses of six wild cinnamon teal [*Spatula cyanoptera* (Vieillot)] from Ñuble region hunted by certified hunters, following the hunting state law Ley de Caza no. 19.473 (SAG, 2018), were also included. For Argentina, one rosy-billed pochard [*Netta peposaca* (Vieillot)] from La Pelada, Corrientes province, collected during 2013, and two *S. versicolor* from Estancia Santa Rita, Buenos Aires province, and Gualeguaychú, Entre Ríos province, both collected during 2014, were included as part of this study (Figure 1).

For the necropsy, the nasal mucosa and turbinate, heart and associated blood vessels, mesenteric blood vessels and its ramifications, liver, lungs, and kidneys were examined for the presence of avian schistosomatids following Lutz *et al.* (2017). The isolated trematodes were relaxed in citrated saline and fixed and preserved in 80% ethanol. Worms for molecular analyses were preserved in absolute ethanol and kept at -20°C (Christiansen *et al.*, 2016; Kolářová *et al.*, 2010; Horák et al., 2012). An additional pool of worms was preserved in 80% ethanol for scanning electron microscopy (SEM) (Oyarzún-Ruiz *et al.*, 2022). Small fragments of worms aimed for SEM were previously sliced and preserved for molecular analyses. Parasitological descriptors such as prevalence (P), mean intensity ($_{M}$I) and mean abundance ($_{M}$A) were estimated and interpreted following Bush *et al.* (1997).

Cercarial emergence and dissection of snails

In an attempt to elucidate the life cycle of the avian schistosomatids belonging to clade Q, *sensu* Brant & Loker (2009), a total of 1,390 *Physa* cf. *acuta* Draparnaud (Physidae) snails were collected between 2019 and 2020 in different freshwater bodies from Ñuble region (n = 351), Biobío region (n = 819), and Los Ríos region (n = 220) in Chile. In addition, during 2015, 10 *Physa* sp. snails were collected from Laguna Fantasma, Río Negro province, Argentina (Figure 1).

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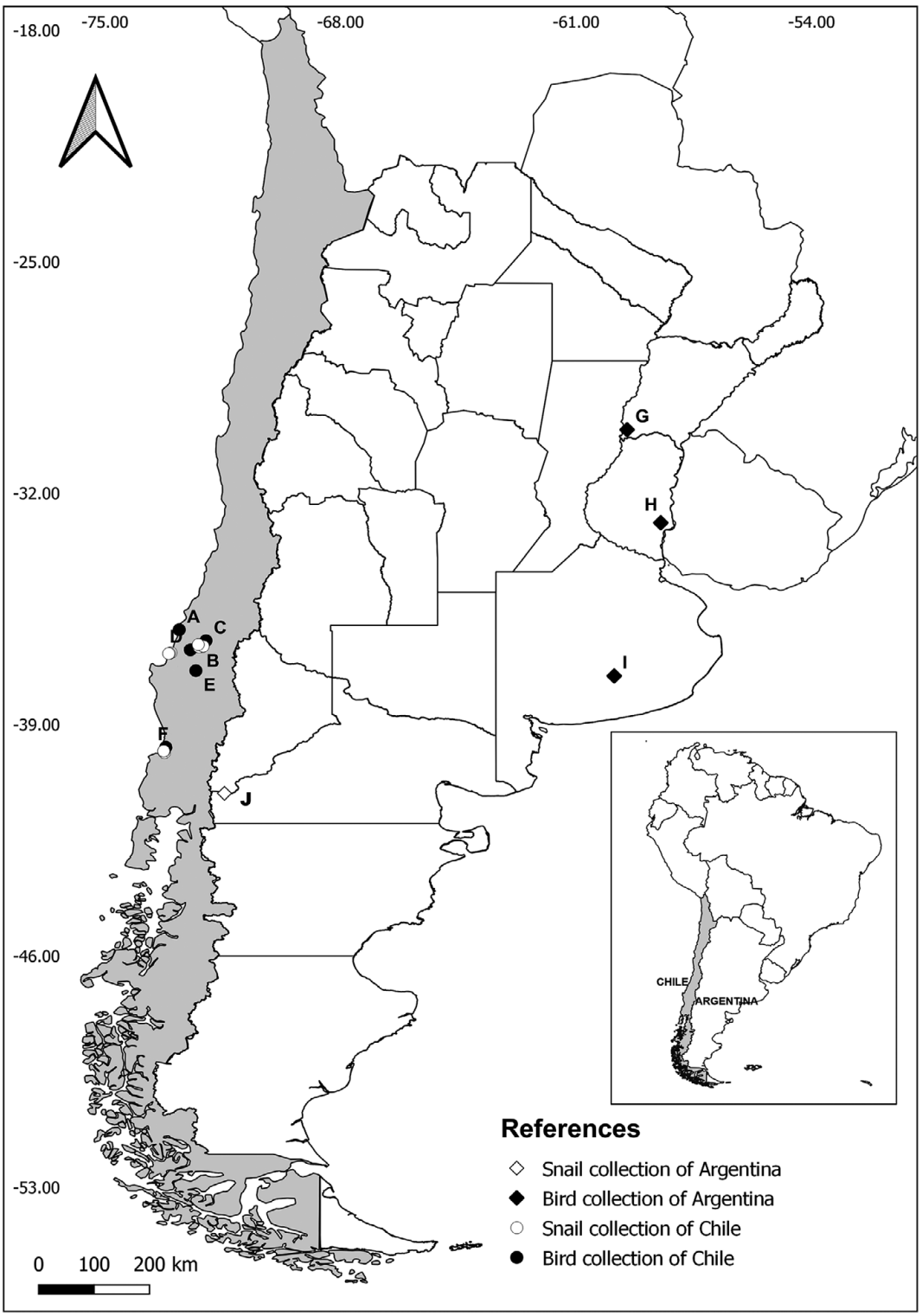

Figure 1. Map of sampled localities in Chile and Argentina. Circles indicate collections from Chile (A-F). Ñuble region: A = Cobquecura; B = Nebuco; C = Ninquihue; D = Quillón; Biobío region: E = Santa Clara; Los Ríos region: F = Carlos Anwandter Nature Sanctuary. Diamonds indicate collections from Argentina (G-J). G = La Pelada Lodge; H = Gualeguaychú; I = Estancia Santa Rita; J = Laguna Fantasma.

To examine snails for infections, they were pooled in cell culture plates (maximum five snails per well) under artificial light for a 12-hour light:12-hour night period during three consecutive days. Once a well was found with furcocercariae, the snails were arranged in individual wells to identify the ones that were parasitised. When the cercarial emergence period completed, all snails were dissected under stereomicroscope to look for prepatent infections (i.e., presence of sporocysts in the tissues) (Oyarzún-Ruiz *et al.*, 2022). The cercariae were identified morphologically following Schell (1985) and Ostrowski de Núñez (1992).

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Because just one snail from Argentina released too few cercariae, all these cercariae had to be preserved in absolute ethanol at -20°C for molecular analyses. Snail shells from Chile were deposited at Museo de Zoología, Universidad de Concepción, Concepción, Chile (accession number MZUC-UCCC 47890). The whole *Physa* sp. snail from Argentina was deposited at the Museum of Southwestern Biology Division of Parasites, New Mexico, USA (catalog number MSB:Host:21642).

Morphological identification of avian schistosomatids

Adult worms were stained with Alum carmine, dehydrated in increasing concentrations of ethanol (70%-100%), cleared in oil clove, and mounted in Canada balsam (Lutz *et al.*, 2017). Avian schistosomatids were photographed and measured using the software Motic Images Plus 2.0 associated with the light microscope MOTIC BA310. Morphological traits and measurements of these worms were compared with the taxonomic keys and descriptions by McLeod (1937), McLeod & Little (1942), Schell (1985), Gibson *et al.* (2002), Kolářová *et al.* (2006), Brant & Loker (2009), and Flores *et al.* (2021). SEM was performed with the scanning electron microscope HITACHI SU 3500 following Oyarzún-Ruiz *et al.* (2022). Worms were disposed in an ionic solution for a maximum of 4 hours to achieve an appropriate conduction of electrons in the SEM equipment. Because of the equipment specifications, there was no need for bathing the worms in gold. The stub with worms was frozen at -30°C with vacuum sealing inside the cool stage. Values for spot values, variable pressure (VP-SEM), kilovolts (Kv), and pascals (Pa) were stated.

Slides and vouchers of avian schistosomatids were deposited at Museo de Zoología, Universidad de Concepción, Concepción, Chile (accession numbers MZUC-UCCC 47891-47897). Fragments of the avian schistosomatid collected from *N. peposaca* and *Physa* sp. from Argentina were deposited at the Museum of Southwestern Biology Division of Parasites (catalog number MSB:Para:23182 and MSB:Para:25363, respectively) (Supplementary Table S1).

DNA extraction, polymerase chain reaction, and phylogenetic analyses

Genomic DNA was extracted using DNeasy blood and animal tissue kit (QIAGEN, Germany) following the manufacturer's instructions. The quantity and quality of DNA for each extracted sample was measured with a spectrophotometer Epoch™ Microplate. Samples with values between 1.6 and 2.0 and an absorbency proportion A260/A280 were considered pure and suitable for amplification through polymerase chain reaction (PCR) (Khare et al., 2014). Extracted DNA was kept at -20°C until molecular analyses were performed.

A Touchdown PCR was performed to amplify partial sequences of the cytochrome c oxidase subunit I gene (hereafter *COI*; expected length of band 600-1,000 bp) and 28S rDNA gene (hereafter *28S*; expected length of band 1,500 bp) (Brant & Loker, 2009; Oyarzún-Ruiz *et al.*, 2022). Primers were the following: for *COI* Cox1_schis'_5' and Cox1_schis'_3' (Lockyer *et al.*, 2003), and CO1F15, CO1R15, and CO1RH3R internal (Brant & Loker, 2009); for *28S* U178, L1642, DIG12 internal, and ECD2 internal (Tkach et al., 2000; Lockyer *et al.*, 2003; Olson *et al.*, 2003). PCR was performed following Dvořák et al. (2002) and Horák et al. (2012): 3 µL of template DNA was added into a mix of 0.3 µL DreamTaq Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.5 µL dNTPs (0.2 mM), 2.5 µL DreamTaq Buffer, 1 µL of each primer (10 pmol), and 16.7 µL of ultra-pure water to achieve a final volume of 25 µL.

PCR thermal conditions were different according to each locus, for *COI* there were 15 hybridisation cycles at 50°C, 49°C, 48°C, 47°C, and 46°C with three cycles each for 30 seconds. Then 20 cycles of hybridisation at 45°C for 30 seconds. For *28S* the protocol considered 15 hybridisation cycles at 55°C, 54°C, 53°C, 52°C, and 51°C with three cycles each for 30 seconds. Then 20 hybridisation cycles at 50°C for 30 seconds. For both loci, extension temperature was at 72°C and denaturation was at 95°C. Amplicons were submitted to electrophoresis in 2% agarose gel, stained with GelRed® (Biotum, Tehran, Iran), and visualised in an ENDURO™ GDS UV transilluminator. Amplicons of expected size were purified and sequenced in both directions at Macrogen (South Korea).

The obtained sequences were verified and edited with Geneious Prime® v. 2021.2.2 to get the consensus sequences. A basic local alignment search was performed with BLASTn tool (https://blast.ncbi.nlm.nih.gov) and similar sequences were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) to build multiple alignments with the former software using the MAFFT algorithm (Katoh & Standley, 2013). Phylogenetic analyses were performed using Bayesian Inference (BI) with the software BEAST v2.5 and substitution model rates were estimated with BEAUTi v2.6.7 (Bouckaert et al., 2019).

The genetic distances between the sequences generated in the present study and sequences from GenBank were estimated using MEGA7 (Kumar *et al.*, 2016). The sequences obtained in the present study were deposited in the NCBI GenBank database (accession numbers: PP333189-PP333197 for the *28S* gene; PP333615-PP333623, PP334484-PP334485 for the *COI* gene).

Results

Four distinct adult schistosomatid taxa, one nasal and three visceral, were isolated from the anatid hosts examined. From Argentina, *N. peposaca* (P = 1/1) and *S. versicolor* (P = 2/2), only a few fragments of visceral schistosomatids were retrieved from their liver, precluding a formal morphological assessment. From Chile, in *S. cyanoptera*, two taxa were recovered, both visceral; one was a pool of unidentifiable juvenile avian schistosomatids (P = 1/6; $_{M}$I = 5; $_{M}$A = 0.83) isolated from liver, and the second was *T. querquedulae* (P = 6/6; $_{M}$I, $_{M}$A = 68.83) that was isolated mostly from the mesenteric vessels of small intestine and viscera, but also the liver and kidneys, heart, caeca and colon mucosa. Morphology and morphometric data of the recovered *T. querquedulae* (Supplementary Table S2) agreed with the description of McLeod (1937), McLeod & Little (1942), and Brant & Loker (2009). The SEM images of *T. querquedulae* showed additional putative diagnostic characters that are not included in the generic or specific diagnosis of this *T. querquedulae*: tegument covered by small spines that alternate with papillae-like structures of greater diameter distributed from the oral sucker to posterior end, irregularly altern on the tegument. On the lateral border of the gynaecophoric canal, papillae-like structures were observed, with several distributed around the genital papilla. The tegument of the ventral surface of the gynaecophoric canal area is corrugated with deep folds over its complete extension, then resumes its spinous surface on body surface. Oral sucker showed triangular spines on the anterior border of oral aperture, which are directed internally, and several circumoral papillae were seen on the anterior border of oral sucker (Figure 2).

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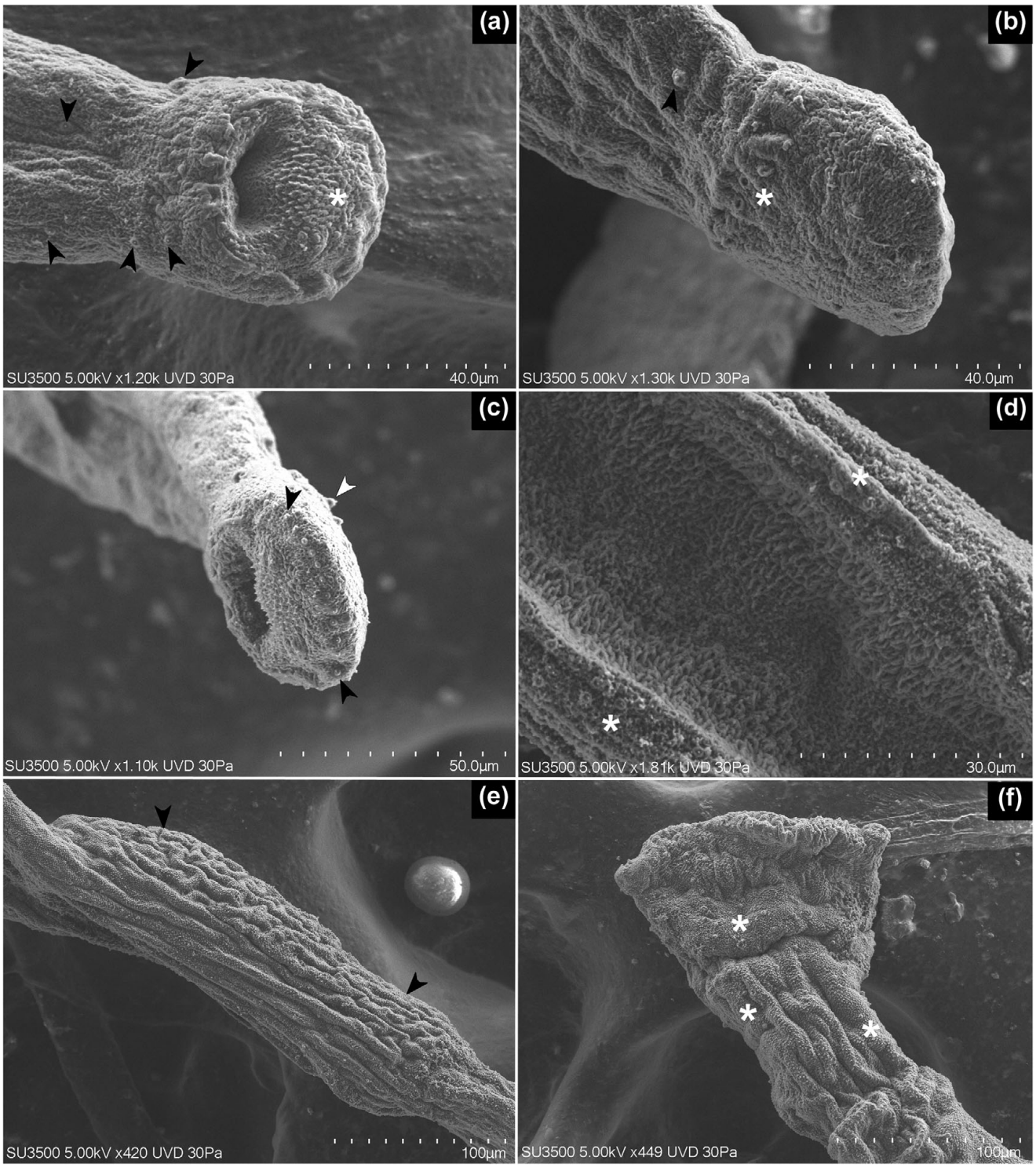

Figure 2. Scanning electron microscopy images of *Trichobilharzia querquedulae* isolated from *Spatula cyanoptera* from Chile. (A) Oral sucker with small triangular spines on its ventral surface, which are also directed to its aperture (*). Note the small papillae immediately in the posterior border of oral sucker (arrowheads). (B) Dorsal surface of oral sucker with evident papillae at its base (*) as well as on the tegument, immediately posterior to it (arrowhead). (C) Presence of circumoral papillae slightly anterior-dorsal to the border of oral sucker (black arrowheads), also note a pair of dorsal papillae (white arrowhead). (D) Detail of gynaecophoric canal, which is densely covered by small fine spines. Also note the presence of small papillae covering the border of gynaecophoric canal (*). (E) Dorsal surface of gynaecophoric canal densely covered by small, rounded spines and characterised by a notorious folded tegument (arrowheads). (F) Spatulated posterior end with its tegument densely covered by small, rounded spines and papillae randomly distributed on its surface (*).

For the swan host, *C. melancoryphus*, from Chile, two taxa were recovered. The first was *N. melancorhypha* (P = 50%, 6/12; $_{M}$I = 15; $_{M}$A = 7.5) in the nasal blood vessels of swans from the Ramsar site Carlos Anwandter wetland (Los Ríos) and San Fabián commune (Ñuble); small fragments of this same species (based on genetic data) were retrieved from the kidney of one bird (A92R; Figure 3), which could represent a migrating worm to the nasal blood vessels. The morphology and morphometric

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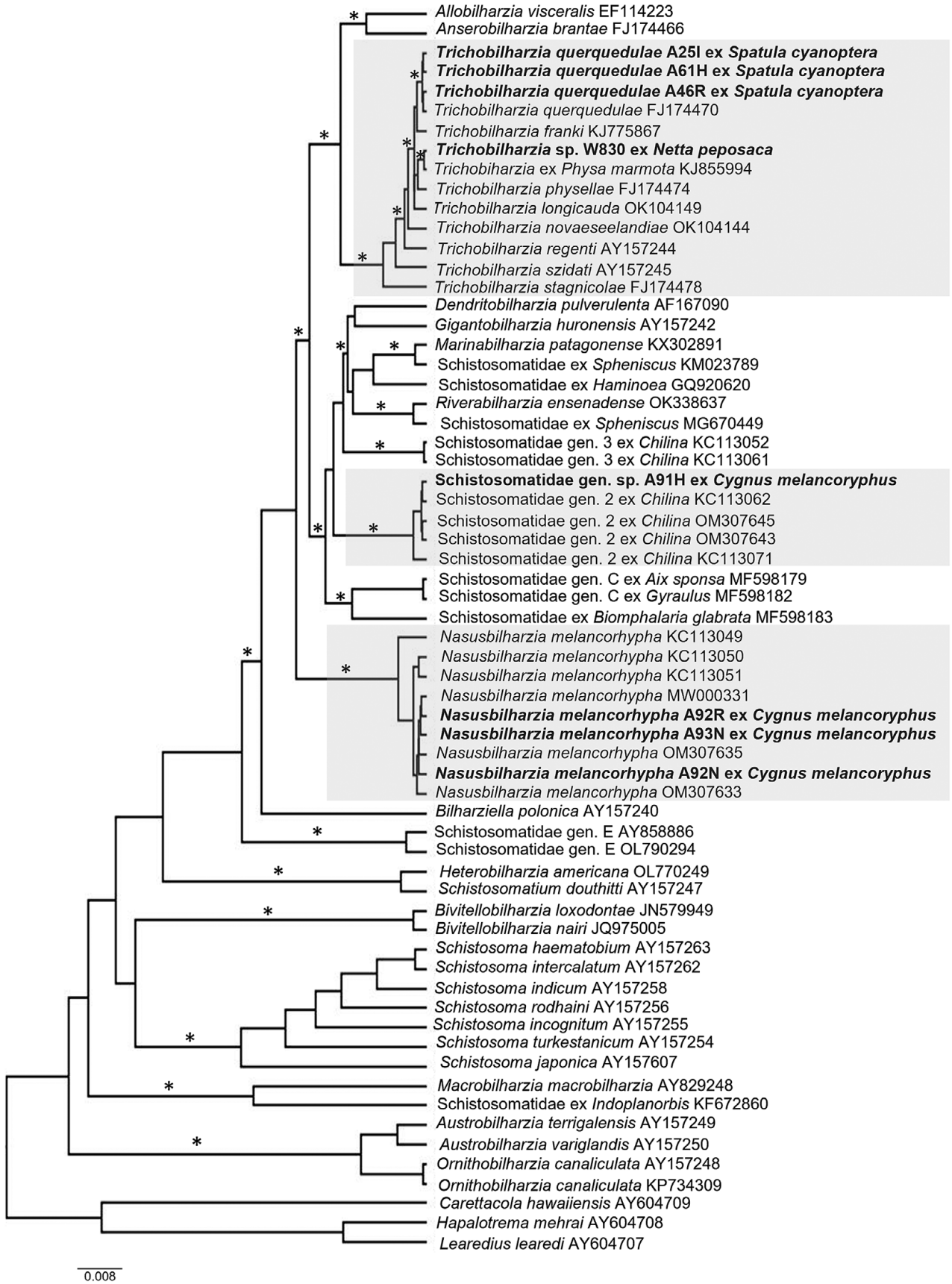

Figure 3. Phylogenetic *28S* gene tree placing the new taxa among the available sequences of avian schistosomatid taxa in GenBank. Specimens from this study are in bold and the clades containing the new taxa are highlighted in gray boxes. The "*" represent significant posterior probability support for the Bayesian analysis, values lower than 0.95 are not indicated. GenBank accession numbers follow the taxon names. To generate the file for BEAST, BEAUTi v2.6.7 was used with Substitution Model GTR rates estimated, substitution rate estimated, for Priors Yule Model default, MCMC chain length 10,000,000, starting tree random, to generate the xml file for BEAST v2.6.4 (Bouckaert *et al.*, 2019). The tree was visualised in FigTree v1.4.4.

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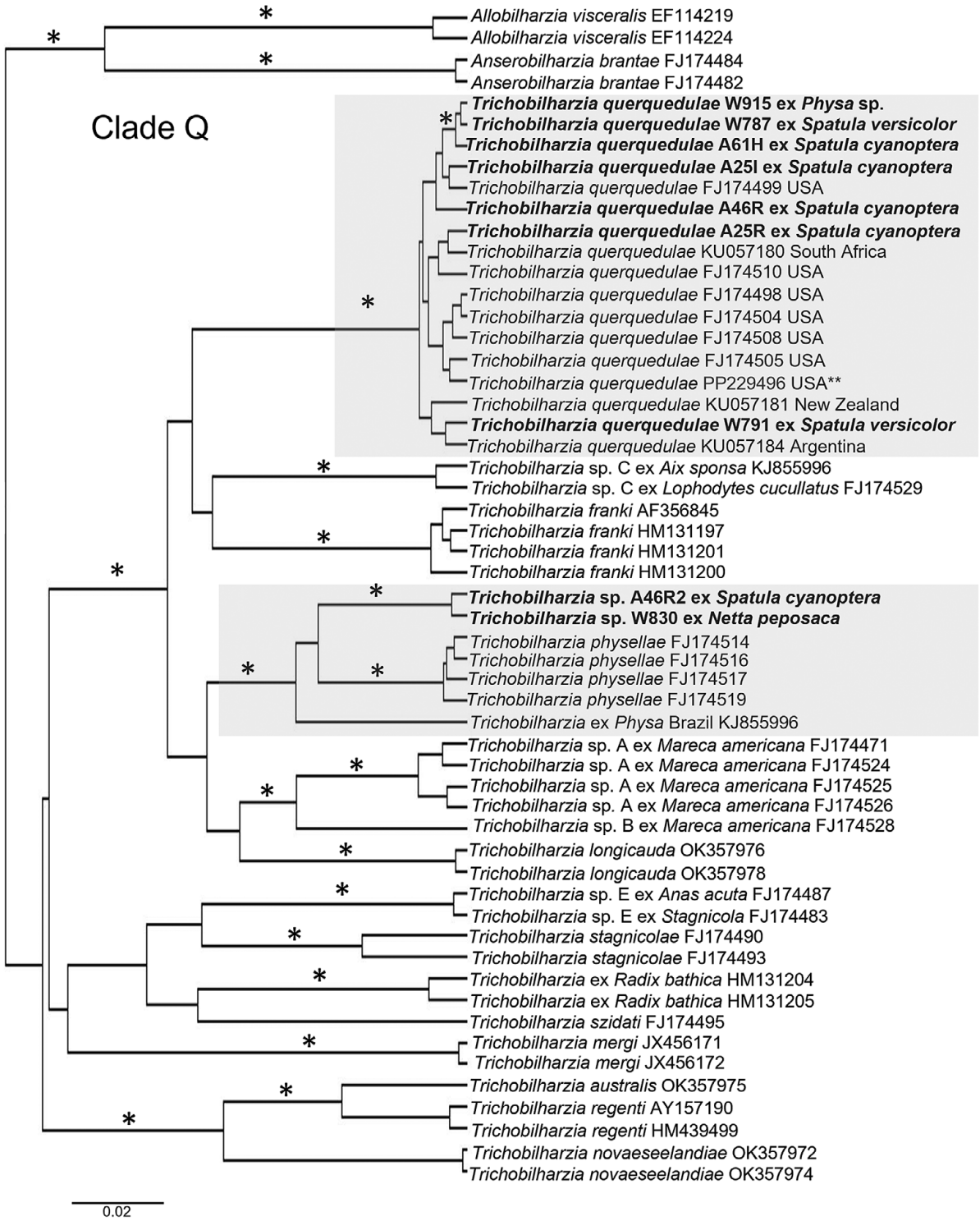

Figure 4. Phylogenetic *COI* gene tree placing the new taxa among the available sequences of avian schistosomatid taxa in GenBank. Specimens from this study are in bold and the clades containing the new taxa are highlighted in gray boxes. The "*" represent significant posterior probability support for the Bayesian analysis, values lower than 0.95 are not indicated. GenBank accession numbers follow the taxon names. The "**" denotes a sequence from a specimen that was also identified morphologically and is vouchered at Museum of Southwestern Biology (MSB:Para:181). Bayesian inference analysis performed in BEAST v2.6.4 (Bouckaert *et al*., 2019). To generate the file for BEAST, BEAUTi v2.6.7 was used with Substitution Model GTR rates estimated, substitution rate estimated, for Priors Yule Model default, MCMC chain length 10,000,000, starting tree random. Tree was visualised in FigTree v1.4.4.

data of recovered worms (Supplementary Table S3) agreed with the description by Flores *et al*. (2021). From the same locality as described previously, the second schistosomatid taxon was a visceral schistosomatid recovered from the liver of two swans. However, it could not be formally described because only fragments were retrieved from the previously frozen birds. No co-infections between these two avian schistosomatid taxa were recorded.

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All taxa recovered from the hosts belonged to the DAS clade of avian schistosomatids (*sensu* Brant & Loker, 2013; Ebbs *et al.*, 2022) with robust nodal support (Figure 3). Phylogenetic analysis of BI for both loci supported the morphological identification of *T. querquedulae* and the placement of *Trichobilharzia* sp. within clade Q (Figure 4). The worms of *T. querquedulae* isolated from *S. cyanoptera* grouped with other conspecific sequences with morphological confirmation and museum vouchers, as a monophyletic clade with robust nodal support for both loci (Figures 3-4). For COI analysis of the larvae and adult fragments, the furcocercariae from *Physa* sp. W915 from Laguna Fantasma, Argentina, fragments from *S. cyanoptera* sequence (A61H), and the sequences isolated in *S. versicolor* from Argentina (W787, W791) were also grouped within the *T. querquedulae* clade (Figure 4).

According to both *28S* and *COI* analyses, the unidentified avian schistosomatid fragments from *S. cyanoptera* and *N. peposaca* did not group with any currently available sequences of morphologically described *Trichobilharzia* sp. in GenBank. The phylogeny of *28S* placed those fragments of *Trichobilharzia* sp. from *N. peposaca* as part of a monophyletic clade with a furcocercaria isolated from *Stenophysa* (= *Physa*) *marmorata* (Guilding) snail from Brazil (KJ855994). Furthermore, for *COI*, the *Trichobilharzia* sp. from the *N. peposaca* formed a monophyletic clade with the pool of juvenile worms of *Trichobilharzia* sp. isolated in a *S. cyanoptera* from Chile, suggesting they might be conspecifics but await morphological identification (Figure 4). This was also supported by the genetic distances (0.5%; Supplementary Table S5). Unfortunately, we could not get a *28S* sequence for the *Trichobilharzia* sp. from *S. cyanoptera.*

The sequences of *N. melancorhypha* formed a monophyletic clade with previously morphologically (also vouchered in a museum) and genetically described individuals for *28S*, with a robust nodal support. This clade also includes the sequences of adult worms from the same avian host and furcocercariae of *Chilina gibbosa* Sowerby snails from Argentina (Flores *et al.*, 2015, 2021), and furcocercariae isolated from *Chilina dombeiana* (Bruguière) from Chile (Oyarzún-Ruiz *et al.*, 2022). This clade based on genetic data falls basal to the rest of clade DAS, as was shown by Flores *et al.* (2015, 2021) and Oyarzún-Ruiz *et al.* (2022).

In the *28S* phylogenetic analysis, the unidentified taxon, Schistosomatidae gen. sp. A91H (PP333192), isolated from a *C. melancoryphus*, formed an independent clade with sequences of an unidentified furcocercariae isolated from freshwater snails *Chilina* spp. from Argentina (Lineage 2) (Flores *et al.*, 2015) and Chile (Schistosomatidae gen. sp. Lineage II) (Oyarzún-Ruiz *et al.*, 2022). This finding represents the first molecular characterisation of the adult worm from its bird host and thus the life cycle is illuminated. Future collections should uncover more adults suitable for a formal morphological description.

A total of 20 furcocercariae morphologically identified to Schistosomatidae were isolated from one *Physa* sp. (P= 10%, 1/10) collected at Laguna Fantasma, Argentina. No *P.* cf. *acuta* from Chile was found parasitised by furcocercariae. Because of the small number of furcocercariae, no morphological characterisation was done because they were used for molecular analyses. The molecular phylogeny showed that these larvae were part of the clade Q (*sensu* Brant & Loker, 2009), particularly in the well-supported clade of *T. querquedulae* (Figure 4). The genetic distances for comparative purposes as proxies for how taxa are related, for each gene, are reported and detailed in Supplementary Tables S4-S5.

Discussion

This study represents the first morphological and molecular characterisation of adult avian schistosomatids from Chilean anseriform hosts. A high proportion of the bird species examined were found infected with the three visceral and one nasal schistosomatids. Based on our results in Chile, the geographical distribution of *T. querquedulae* is extended (from Canada to Patagonia in western hemisphere), *N. melancorhypha* was isolated in the swan, and both *S. cyanoptera* and *N. peposaca* are recorded as additional definitive hosts for species of *Trichobilharzia* in the Neotropics (see Flores *et al.*, 2015; Ebbs *et al.*, 2016).

Genetically the specimens of *T. querquedulae* isolated in *S. cyanoptera* grouped with those from Brant & Loker (2009) and Davis *et al.* (2022). Morphologically, the specimens were mostly similar to the descriptions of Brant & Loker (2009) and McLeod (1937) but there were subtle differences. The specimens from this paper had a longer gynaecophoric canal and seminal vesicle, a wider distance between acetabulum and gynaecophoric canal, and fewer testes (180 vs 210 testes) in comparison to the previously mentioned authors (see Supplementary Table S2). Notwithstanding, this morphological identification was supported by the phylogenetic analyses of both loci, placing our specimens in the *T. querquedulae* clade (that includes a sequence from a museum voucher that was morphologically identified, GenBank PP229496). Therefore, based on the known data, with published sequences and vouchers from the blue-winged ducks (*Spatula* spp.) clade such as *S. versicolor* from Argentina (Ebbs *et al.*, 2016), *S. discors* and *S. cyanoptera* from the USA (Brant & Loker, 2009; Garvon et al., 2011), and the Australian shoveler [*Spatula rhynchotis* (Latham)] from New Zealand (Davis *et al.*, 2022), they are most likely *T. querquedulae.* Interestingly, previous records from other species of *Spatula* from the USA (Brant & Loker, 2009), and the recent proposal by Ebbs *et al.* (2016), stated that *T. querquedulae* has a cosmopolitan distribution in *Spatula* spp. The specimens found here represent the first records from Chile, widening its known geographic distribution in the Southern Cone, plus representing the second record and an additional host species in the Neotropics. The high prevalence of *T. querquedulae* in *S. cyanoptera* (6/6) and intensity of infection ($_{M}$A = 68.83) are remarkable similar to what has been reported previously (Brant & Loker, 2009; Davis *et al.*, 2022).

Szidat (1951) recorded a visceral *Trichobilharzia* sp. from *S. versicolor* collected in Tapalqué, Argentina, which might represent the *T. querquedulae* mentioned here. However, no morphological description, images, and most importantly, no vouchers exist; thus, new collections are warranted to stablish its specific identity.

The SEM characterisation of *T. querquedulae* highlighted additional morphological traits that were not mentioned in the original description by McLeod (1937) and McLeod & Little (1942) or Gibson *et al.* (2002), but that will be useful in future diagnoses. There are only two publications that used SEM features for *Trichobilharzia* species characterisation. These SEM features include the presence of circumoral papillae in *Trichobilharzia australis* Blair & Islam, 1983, a nasal avian schistosomatid from Australia (Blair & Islam, 1983). These same papillae distributed over the tegument were also described by SEM images in *Trichobilharzia arcuata* Islam, 1986. These papillae in *T. arcuata* were restricted between the oral sucker and gynaecophoric canal and thus used as a distinguishing character from *T. australis* SEM (Islam, 1986). SEM is underused for the differentiation of avian schistosomatids; it provides additional diagnostic features to the existing keys (e.g. Blair &

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Islam, 1983; Islam, 1986; Flores *et al*., 2021; Lorenti et al., 2022; Oyarzún-Ruiz *et al*., 2022) for species with few features visible in light microscopy. The present study emphasises the importance to include features visible in SEM for the morphological description of both adult and larval schistosomatids, with an eventual amendment of *T. querquedulae* at least, but also the genus *Trichobilharzia,* based on SEM features.

In the *COI* phylogenetic tree (Figure 4), there are two unidentified clades, *Trichobilharzia* sp. Brazil KJ855996 (ex physid snail) and two immature specimens from this study (A46R ex *S. cyanoptera*, W830 ex *N. peposaca*) that group with but not within *T. physellae.* The North American *T. physellae* is most often found in an ecological group of diving ducks (usually *Aythya* spp.), and cycles through *Physa* spp. snails (Brant & Loker, 2009; Pinto *et al*., 2014), though a recent record from the snail *P. acuta* was found in Europe (Helmer *et al*., 2021). Thus far, no genetically or morphologically identified specimens have been found in South America that correspond to *T. physellae.* One of the specimens collected herein was from a diving duck, *N. peposaca*, but those worms were immature and did not form a clade with *T. physellae* or the physid specimen of *Trichobilharzia* from Brazil. Thus, more specimens from diving ducks are needed to understand the species diversity of *Trichobilharzia* in these ducks from South America. It would be interesting to show if the clade of *Trichobilharzia* found here, and that of *T. physellae*, are exclusive to the ecological clade of diving duck hosts (see Johnsgard, 2010), similar to what has been shown for *T. querquedulae* in *Spatula* spp.

Clade Q is composed of species of *Trichobilharzia* that are transmitted by aquatic snails in the families Physidae (*T. querquedulae* and *T. physellae*) and Lymnaeidae (*Trichobilharzia franki* Müller & Kimmig, 1994, *Trichobilharzia longicauda* [Macfarlane, 1944] Davis, 2006, *Trichobilharzia regenti* Horák, Kolářová & Dvořák, 1998, *Trichobilharzia novaeseelandiae* Davis & Brant, 2022, *Trichobilharzia* sp. A), with most taxa transmitted by lymnaeid snails (Brant & Loker, 2009; Jouet et al., 2010a, b; Brant *et al*., 2011; Horák et al., 2015; Ebbs *et al*., 2016; Ashrafi *et al*., 2021; Davis *et al*., 2022). In South America there are three previous reports of physid snails as intermediate hosts of avian schistosomatids, all of which have been recorded from *S. marmorata.* Those furcocercariae were identified as Cercaria I from Argentina (Ostrowski de Núñez, 1978), and *Trichobilharzia jequitibaensis* Leite, Costa & Costa, 1978 (Leite et al., 1979) and *Trichobilharzia* sp. from Brazil (Pinto *et al*., 2014). However, some caution with *T. jequitibaensis* should be taken as the authors report both a physid and lymnaeid serving as intermediate hosts. In Argentina and Chile there are four native species of Physidae, with *P. acuta* as the invasive species for both (Valdovinos, 2006; Rumi *et al*., 2008). *Physa acuta* has been recorded as an intermediate host of *T. physellae* in its natural distribution in North America (Brant & Loker, 2009; Brant *et al*., 2011), but also recently in Europe, where it is considered invasive (Ebbs *et al*., 2018; Helmer *et al*., 2021).

The present study represents the first record of *T. querquedulae* recovered from a physid snail in the Neotropics. Unfortunately, no specific identification was achieved for the snail host, but it has been vouchered in a museum collection (Museum of Southwestern Biology, Division of Parasites, MSB:Host:21642) and is available for specific identification. Availability of physical and curated specimens is particularly important considering the high intraspecific variations for these snails (Cuezzo, 2009; Collado *et al*., 2019). Transmission dynamics of *Trichobilharzia* spp in physids is important, for example, to determine if *P. acuta* from Chile is refractory to the infection with the native avian schistosomatids from Clade Q (e.g. Stanicka et al., 2022), or if *Physa* sp. from Argentina is a native species, which might explain the infection despite the small sample size examined. In summary for Clade Q in South America *T. querquedulae* from *Spatula* spp. and Physidae, *Trichobilharzia* sp. (W830/A46R) from Argentina and Chile, together with *Trichobilharzia* sp. (KJ855994) from Brazil (Pinto *et al*., 2014; Ebbs *et al*., 2016; this study) have been reported.

In addition to the species lineages of *Trichobilharzia* recovered in Chile, two other taxa of avian schistosomatids were recovered, one nasal and one visceral schistosomatid from *C. melancoryphus.* The morphology and genetics of the nasal schistosomatid, *N. melancorhypha*, was in accordance with Flores *et al.* (2021), although with some minor differences such as a smaller acetabulum, smaller distance between oral sucker and acetabulum, longer seminal vesicle, and a slightly shorter gynaecophoric canal. The small size of certain structures could be due to how these worms were preserved before observation, for example, the specimens in Flores *et al.* (2021) were measured from hot 5% formaldehyde fixed material. Notwithstanding the previous information, both phylogenetic analyses of *28S* and *COI* supported the morphological diagnosis as *N. melancorhypha.* The confirmation of *N. melancorhypha* parasitising *C. melancoryphus*, together with the recent description of its furcocercariae from *C. dombeiana* (Oyarzún-Ruiz *et al*., 2022), make this taxon the first avian schistosomatid with its life cycle elucidated in Chile.

The finding of *N. melancorhypha* extends its geographic distribution, originally from Argentina (Flores *et al*., 2021), to Central and Southern Chile, maintaining its monotypic status. This is noteworthy because its intermediate host, snails of genus *Chilina* Gray, and its definitive host, *C. melancoryphus*, are both endemic to South America (Cuezzo, 2009; Fuentealba *et al*., 2010; Johnsgard, 2010), suggesting the life cycle is restricted to the distribution of *Chilina* spp. *Cygnus melancoryphus* is the only native swan in the Neotropics (Johnsgard, 2010), which might limit the possibility of finding *N. melancorhypha* in other anatids. This is in contrast with *Allobilharzia* Kolářová, Rudolfová, Hampl & Skírnisson, 2006, which exclusively parasitises swans (*Cygnus* spp.) in the Northern hemisphere, from North America, Europe, and Japan (Kolářová *et al*., 2006; Brant, 2007; Hayashi *et al*., 2017).

There was an unexpected finding of *N. melancorhypha* fragments in the kidneys of one of the swans (A92R), with the genetic identification confirmed through phylogenetic analysis. Although the migration route of juvenile worms for this nasal species remains unknown, this observation could represent a systemic migration of the worms. In contrast, *T. regenti*, another nasal schistosomatid, migrates exclusively through the nervous system before reaching the preferred nasal tissue (Horák et al., 2012; Prüter et al., 2017). The explanation of this finding herein requires additional samplings and experimental studies to trace the migration of the schistosomules.

In addition to the finding of the nasal schistosome in *C. melancoryphus*, a visceral avian schistosomatid was isolated; Schistosomatidae gen. sp. Unfortunately, because of the poor state of the worm fragments from the host, they could not be described properly. Until a morphological assessment is performed genetically, this undescribed visceral schistosomatid phylogenetically groups within the DAS clade. Interestingly, Schistosomatidae gen. sp. grouped in a clade with the undescribed Lineage 2/II of furcocercariae isolated from *Chilina fulgurata* Pilsbry, *Chilina perrieri* Mabille and *C. gibbosa* from Argentina (Flores *et al*., 2015), and *C. dombeiana* from Chile (Oyarzún-Ruiz *et al*., 2022). These results suggest they are likely conspecific and represent a new Neotropical

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visceral schistosomatid with *C. melancoryphus* as the definitive host for the furcocercariae identified as Lineage 2/II from Flores *et al.* (2015) and Oyarzún-Ruiz *et al.* (2022), representing another endemic taxon to the Neotropics.

Considering that most visceral schistosomatids are typically found in the venous system of birds, spreading through parenchymatous organs such as the liver and mesenteric vessels (Horák et al., 2015; Ashrafi *et al.*, 2021), the schistosomatids reported by Oyarzún-Ruiz *et al.* (2019) in *C. melancoryphus* from Southern Chile might also correspond to Schistosomatidae gen. sp. within Lineage 2/II (Figure 3). In the study herein, the specimens of Schistosomatidae gen. sp. were not ideal because of the poor state of preservation of the hosts, which disrupts the integrity of these digeneans for morphological assessment (Lutz *et al.*, 2017). The necropsy of swans recently salvaged or euthanised would offer better material for the morphological description of this undescribed schistosomatid.

Future efforts to classify avian schistosomatids from the Neotropics should consider not only collecting non-studied waterfowl but also other aquatic avian groups that have not been properly considered such as Suliformes, Charadriiformes, Phoenicopteriformes, and Passeriformes (especially those inhabiting wetlands) (Brant, 2007; Horák & Kolářová, 2011; Flores *et al.*, 2015; Horák et al., 2015; Lorenti et al., 2022). Even though these groups of birds have been reported as definitive hosts of avian schistosomatids, several of these records have no phylogenetic work, vouchers to reexamine, or genetic data (Horák & Kolářová, 2011). Using an integrative approach, Lorenti et al. (2022) described two new genera from two common South American gulls and discussed the entangled systematics of the globally, but poorly understood, schistosomatid genus *Gigantobilharzia* Odhner. Taking into consideration that Argentina and Brazil concentrate most of the knowledge regarding South American avian schistosomatid diversity (see Pinto *et al.*, 2014, 2017; Flores *et al.*, 2015, 2021; Brant *et al.*, 2017; Lorenti et al., 2022), there is a clear need to replicate these efforts in neighboring countries to enhance the understanding of these neglected parasites not only from the biodiversity point of view, but also for the whole understanding of their evolutionary relationships. In addition, we need researchers to deposit museum vouchers for re-examination that is critical to replicating past studies and clarifying authors hypotheses. Expanding collections to other southern hemisphere continents to look at how biogeography and host use has shaped the evolutionary history of these avian schistosomatids, relative to what we know about their distribution in the northern hemisphere (Pinto *et al.*, 2017, 2022) is critical.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X2400035X.

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