AKADÉMIAI KIADÓ

Acta Veterinaria Hungarica

70 (2022) 4, 274–281

DOI:
10.1556/004.2022.00031

RESEARCH ARTICLE

New record of *Tylodelphys* metacercariae (Diplostomidae) from *Perccottus glenii* (Odontobutidae) and their phylogenetic assessment

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Received: 26 May 2022 • Accepted: 15 September 2022
Published online: 12 October 2022

ABSTRACT

Metacercariae of *Tylodelphys* sp. were found in the abdominal cavity of the Chinese sleeper (*Perccottus glenii*) collected in Liaoning Province and Inner Mongolia Autonomous Region of China. The sequences of the mitochondrial *cox*1 gene and ribosomal ITS1–5.8S rDNA–ITS2 region were obtained and used for molecular identification and phylogenetic assessment of this parasite species. Results of phylogenetic analyses based on ITS and *cox*1 markers showed that the metacercariae of *Tylodelphys* sp. ex *P. glenii* from China were conspecific with specimens of *Tylodelphys* sp. collected by Sokolov et al. (2013) from the same fish-host species captured earlier in West Siberia, Russia. The examined *Tylodelphys* sp. ex. *P. glenii* is the only member of the genus whose metacercariae parasitise the abdominal cavity of fish in northern Eurasia. *Tylodelphys* sp. ex *P. glenii* clustered with *T. darbyi, T. immer, T. podicipina,* and *Tylodelphys* sp. of Soldánová et al., 2017 based on mitochondrial DNA markers, and with *T. darbyi, T. immer, T. kuerepus,* and *T. schreuringi* using nuclear DNA markers.

KEYWORDS

Chinese sleeper, abdominal cavity, *cox1* gene, ITS1–5.8S–ITS2 rDNA, Liaoning Province, Inner Mongolia Autonomous Region

INTRODUCTION

Metacercariae of trematodes of the genus *Tylodelphys* Diesing, 1850 are widespread parasites of freshwater vertebrates, mainly fish. Adult *Tylodelphys* spp. parasitise fish- and amphibian-eating birds, rarely mammals (Dubois, 1970; Niewiadomska, 2002; Blasco-Costa et al., 2017; Achatz et al., 2022a). In a previous study, we detected *Tylodelphys* sp. metacercariae in the abdominal cavity of the Chinese sleeper, *Perccottus glenii* Dybowski, 1877 (Actinopterygii: Odontobutidae) from Lake Zhiloye, West Siberia (Sokolov et al., 2013). We described its morphology and reconstructed the phylogeny of this *Tylodelphys* sp. using the ITS1–5.8S–ITS2 rDNA region (Sokolov et al., 2013). We found that *Tylodelphys* sp. ex *P. glenii* was a sister species to North American *Tylodelphys scheuringi* (Hughes, 1929). Later, these parasites were found in *P. glenii* in Lake Dolgoe, the Trans-Baikal Region of Russia (Sokolov et al., 2016). *Perccottus glenii* is an invasive fish species in these two regions, whose extensive introduced range encompasses various water bodies of Siberia and Central and Eastern Europe (Reshetnikov, 2010; Reshetnikov and Schliewen, 2013; Horvatić et al., 2022). Its native range is confined to the drainage basin of the Middle and the Lower Amur and several neighbouring water systems, including the Liao River catchment (Reshetnikov, 2010).

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A lot of new data on *Tylodelphys* spp. have been published in recent years (Chibwana et al., 2013, 2015; Otachi et al., 2015; García-Varela et al., 2016; Blasco-Costa et al., 2017; Chaudhary et al., 2017a,b,c; Soldánová et al., 2017; Locke et al., 2018; Pelegrini et al., 2019; Sereno-Uribe et al., 2019; Heneberg and Sitko, 2021; Achatz et al., 2022a,b). It has been shown that *Tylodelphys* is a paraphyletic taxon within the monophyletic *Tylodelphys* spp. + *Austrodiplostomum* spp. group (Pelegrini et al., 2019; Achatz et al., 2022a,b). Several authors have included *Tylodelphys* sp. ex *P. glenii* in the phylogenetic analyses of *Tylodelphys* based on ITS datasets (Blasco-Costa et al., 2017; Chaudhary et al., 2017a,b,c; Heneberg and Sitko, 2021). According to the recent phylogenetic reconstruction (Heneberg and Sitko, 2021), the ITS2 sequences of *Tylodelphys* sp. ex *P. glenii* were identical to those of *Tylodelphys immer* (Dubois, 1961) and *T. podicipina* Kozicka & Niewiadomska, 1960. Based on these data, the authors concluded that *T. immer* and *T. podicipina* were conspecific. However, the taxonomic relationships of these nominal species with *Tylodelphys* sp. ex *P. glenii* were not discussed in that study. Moreover, the taxonomic concept of Heneberg and Sitko (2021) is based on an inaccurate comparison of ribosomal data (Achatz et al., 2022b). In addition, it has been found that the cytochrome c oxidase subunit 1 (*cox*1) gene provides a better resolution of the phylogeny of diplostomids at the interspecific level than the ITS1–5.8S–ITS2 region, and *cox*1 can be used to separate cryptic species (Moszczynska et al., 2009; Selbach et al., 2015; Locke et al., 2015, 2018; Hoogendoorn et al., 2020; Faltýnková et al., 2022).

In the present study, we analysed the phylogenetic position of *Tylodelphys* sp. ex *P. glenii* originating from a new geographical location, based on the *cox*1 gene and ITS1–5.8S–ITS2 rDNA data.

MATERIALS AND METHODS

Sampling

Twenty-two specimens of *P. glenii* were collected between 10 December 2021 and 12 January 2022 in Liaoning Province and the Inner Mongolia Autonomous Region of China (Fig. 1). In Liaoning Province, 12 fish (total length, TL 10.1–11.5 cm) were caught in an unnamed water body in Latahu Village, Shenbei District, Shenyang City (42°7′32″ N, 123°22′57″ E). In the Inner Mongolia Autonomous Region, 10 fish (TL 8.8–11.4 cm) were caught in the lower reaches of River Yimin, Hulunbuir City (49°15′26″ N, 119°43′23″ E). Immediately after capture, the fish specimens were fixed in absolute ethanol, transported to the laboratory for further study and dissected soon after that. Metacercariae were pipetted into a watch glass, washed, sorted, counted, then refixed in 96% ethanol.

Fig. 1. Map showing localities where metacercariae of *Tylodelphys* sp. were found in the abdominal cavity of *Perccottus glenii*. Triangles – this study, stars – previous studies (Sokolov et al., 2013, 2016)

DNA amplification and sequencing

Genomic DNA was isolated from one ethanol-fixed metacercaria specimen per fish (six in total, three metacercariae from each locality in China) using DNA-Extran kits (Synthol, Moscow). For all the six larvae, we amplified a fragment of the mtDNA *cox*1 gene using the primers Cox1_schist_5′ (5′-TCT TTR GAT CAT AAG CG-3′) and Cox1_schist_3′ (5′-TAA TGC ATM GGA AAA AAA CA-3′) of Lockyer et al. (2003). This fragment is further referred to as Fragment 1. For two of these larvae (one from each locality), another fragment of the *cox*1 gene (labelled below as Fragment 2) was additionally amplified with primers JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and JB4.5 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′) described by Bowles et al. (1992), for comparison with the data of Heneberg and Sitko (2021). These fragments overlap with less than 100 bp.

For five metacercariae specimens, the ITS1–5.8S–ITS2 rDNA region was amplified using primers D1 (F) (5′-AGG AAT TCC TGG TAA GTG GCA AG-3′) and D2 (R) (5′-CGT TAC TGA GGG GAA TCC TGG T-3′) (Galazzo et al., 2002). Additionally, Fragment 1 of *cox*1 was sequenced for the two metacercaria samples of *Tylodelphys* sp. ex *P. glenii* from Lake Zhiloye, whose ITS1–5.8S–ITS2 sequences (KF477191, KF477192) had been published previously (Sokolov et al., 2013).

PCR assay was carried out in 25 µl of reaction mixture containing 10 ng of total DNA, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 5 mM $MgCl_2$, 0.25 mM of each dNTP, 1.5 pmol of each primer, and 0.6–0.7 U Taq DNA polymerase (Thermo Fisher Scientific, Lithuania) and using an annealing temperature of 56 °C for nuclear rDNA amplifications (Galazzo et al., 2002) and 50 °C for *cox*1 amplifications (Lockyer et al., 2003; Heneberg and Sitko, 2021). The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen Ltd, UK) following the manufacturer's instructions, then sequenced directly with the automatic sequencing system ABI PRISM 3100-Avant (Applied Biosystems Inc., Foster City, CA, USA). Consensus sequences were assembled in MEGA v. 10 (Kumar et al., 2018).

Phylogenetic analyses

The identity of the newly generated sequences was checked with the Basic Local Alignment Search Tool (BLASTn) (www.ncbi.nih.gov/BLAST/). The novel sequences (956 bp for Fragment 1, 319 bp for Fragment 2 of *cox1*, and 1,286 bp for ITS1-5.8S-ITS2) were aligned with representatives of the

family Diplostomidae in MEGA v. 10 (Kumar et al., 2018) and trimmed to the shortest length.

Three alignments (two for the different *cox*1 fragments and one for the ITS region) were prepared. The *cox*1 gene alignment for Fragment 1 (402 bp) comprised 46 sequence representatives of Diplostomidae. The *cox*1 Fragment 2 alignment (319 bp) consisted of 26 sequences of Diplostomidae family members. The ITS alignment (832 bp) included 37 sequences of diplostomids. The available sequences of members of different diplostomoid genera (*Diplostomum* von Nordmann, 1832, *Posthodiplostomum* Dubois, 1936 or *Cardiocephaloides* Sudarikov, 1959) were used as the outgroup.

To assess the phylogenetic relationships of *Tylodelphys* spp. and *Austrodiplostomum* spp., we applied Bayesian inference analysis to all datasets. Prior to the analyses, the best-fitting model was estimated with jModelTest v2.1.2 (Darriba et al., 2012); TN93 + I + G for *cox*1 Fragment 1 and GTR + G for *cox*1 Fragment 2, and HKY + G for the ITS alignment. Bayesian inference analyses were conducted using MrBayes (v3.2.3) (Ronquist et al., 2012). Markov chain Monte Carlo (MCMC) simulations were run for 3,000,000 generations, log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus tree. Posterior probability was calculated to estimate nodal support. FigTree v1.4 (Rambaut, 2012) was used to visualise the trees.

The genetic divergence (as pairwise distances) among taxa was estimated with the bootstrap method (1,000 replicates) and with nucleotide substitution (transition + transversion) uniform rate using MEGA v. 10 (Kumar et al., 2018).

RESULTS

General observations

All the examined specimens of *P. glenii* from the River Yimin and an unnamed water body in Latahu Village (Fig. 1) were infected with metacercariae of *Tylodelphys* sp., with an intensity of 2–15 parasites per host in both localities (the mean intensity was 9.3 and 8.9 individuals, respectively). The locations of the sampling area, where morphologically similar metacercariae had been found previously in the abdominal cavity of *P. glenii* in the Asian part of Russia (Lakes Zhiloye and Dolgoe), are also shown in Fig. 1. Non-encysted metacercariae were localised in the abdominal cavity of the fish hosts.

Phylogenetic analysis

We obtained 956-bp-long sequences of the *cox*1 Fragment 1 for eight *Tylodelphys* sp. specimens collected from *P. glenii* from Russia (Lake Zhiloye) and China (River Yimin and an unnamed water body in Latahu Village), and 319-bp-long sequences of Fragment 2 for two specimens from each locality in China. ITS sequences (1,286 bp) were obtained for five specimens collected only in China (River Yimin and an unnamed water body in Latahu Village). All sequences were deposited in GenBank with accession numbers ON426801–ON426805 for the ITS1–5.8S–ITS2 region, ON469982–ON469989 for *cox*1 Fragment 1 and ON470006–ON470007 for *cox*1 Fragment 2.

On the phylogenetic tree of *Tylodelphys* spp. and *Austrodiplostomum* spp. *cox*1 Fragment 1 showed samples of *Tylodelphys* sp. ex *P. glenii* from Russia and China clustered together in one strongly supported clade (Fig. 2). Sequences from these metacercariae demonstrated a low level of differentiation (P = 0–0.01%), and no patterns of intraspecific polymorphism related to geographic location could be identified. The sister clade of *Tylodelphys* sp. ex *P. glenii* was formed by *T. darbyi* Presswell & Blasco-Costa, 2020 and the well-supported sub-clade of *T. immer* and *Tylodelphys* sp. of Soldánová et al. (2017) [KY513215]. The clade of *Tylodelphys* sp. ex *P. glenii, T. immer, T. darbyi,* and *Tylodelphys* sp. of Soldánová et al. (2017) was nested into a larger group of *Tylodelphys* spp. with low nodal support, and contained *Tylodelphys kuerepus* Sereno-Uribe et al., 2019, *T. conifera* (Mehlis, 1846), *T. scheuringi, T. podicipina robrauschi* Dubois, 1969, *T. variabilis* (Chandler, 1932), and *T. aztecae* García-Varela Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz & Pérez-Ponce de León, 2016. This large group was a sister clade of *Austrodiplostomum* spp. and several Neotropic and Nearctic *Tylodelphys* spp. The type species of *Tylodelphys* – *Tylodelphys clavata* (von Nordmann, 1832) – was included in the group, and placed in the basal position compared to all the above-mentioned trematodes.

The phylogenetic analysis of the ITS region (Fig. 3) showed that the newly obtained sequences of metacercariae collected from *P. glenii* from China were 100% identical to the two sequences of *Tylodelphys* sp. 1 DIL-2013 collected previously from the same host species in Lake Zhiloye (KF477191, KF477192). *Tylodelphys* sp. ex *P. glenii* clustered in one moderately supported clade together with *T. darbyi, T. immer, T. kuerepus*, and *T. scheuringi*. However, the exact positions within the clade of *Tylodelphys* containing the species mentioned above were poorly resolved.

The use of *cox*1 Fragment 2 (Fig. 4) allowed us to involve in the phylogenetic reconstruction a number of Eurasian species, for which no other molecular markers were available, namely, *Tylodelphys circibuteonis* Odening, 1962, *T. nigriciconis* Heneberg & Sitko, 2021, and *T. podicipina*. Here, the *cox*1 Fragment 2 sequences of *Tylodelphys* sp. ex *P. glenii* samples from the two examined locations in China appeared as a poorly supported sister clade to the well-supported *T. podicipina* + *T. darbyi* group. In turn, the *Tylodelphys* sp. ex *P. glenii* + (*T. podicipina* + *T. darbyi*) clade was a poorly supported sister group to *T. immer*.

DISCUSSION

Most of the *Tylodelphys* spp. have a strict localisation in the body of the second intermediate host at the metacercarial stage (Sudarikov, 1971, 1974; Blasco-Costa et al., 2017), although some authors believed that metacercariae of the same *Tylodelphys* sp. were found in different organs of the

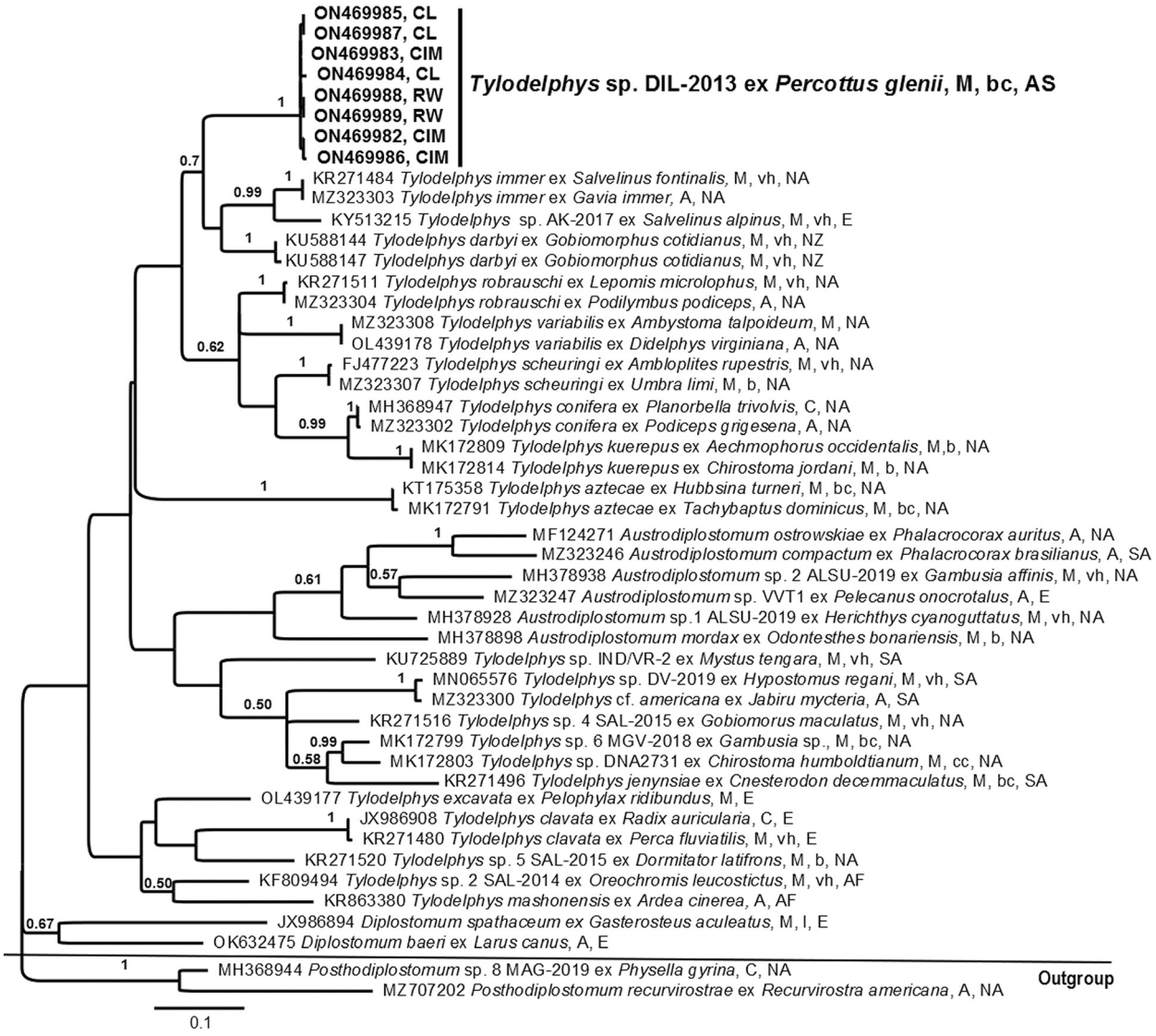

Fig. 2. Phylogenetic tree of *Tylodelphys* spp. and *Austrodiplostomum* spp. based on Fragment 1 of *cox*1 gene sequences (402 bp) inferred using Bayesian inference analysis. Posterior probability values lower than 0.5 are not shown. Samples from the present study are labelled bold. Abbreviations: A, adult; C, cercaria; M, metacercaria; b, brain; bc, body cavity; cc, cranial cavity; l, eye lens; vh, vitreous humour in the eye; AF, Africa, AS, Asia; E, Europe; NA, North America; SA, South America; NZ, New Zealand; CL, Liaoning Province of China; CIM, Inner Mongolia Autonomous Region of China; RW, West Siberia of Russia

same fish individual (Haderlie, 1953; Pandey, 1970; Pelegrini et al., 2019; Achatz et al., 2022b). Of these, the conspecificity of *Tylodelphys* metacercariae with different localisations (*T. scheuringi* from the brain and the eye) was confirmed by reliable (molecular) methods only in a single case (Achatz et al., 2022b).

Tylodelphys sp. ex *P. glenii* examined in our study is not the only species of this genus reported from *P. glenii*. Metacercariae of *T. clavata* have been found in the eye vitreous humour of this fish species (in non-native populations) in Nizhniy Novgorod Province and Tyumen Province of Russia (Sokolov et al., 2014; Sokolov and Reshetnikov, 2020). The examined *Tylodelphys* sp. is the only member of this genus whose metacercariae parasitise the abdominal cavity of fish in northern Eurasia. Although morphometrics were not recorded in the present study (because of the fixative used), the general morphology of the examined metacercariae was in accordance with the previous description by Sokolov et al. (2013). *Tylodelphys* sp ex. *P. glenii* is characterised by the following morphometric features as described by Sokolov et al. (2013): the body is 901–1,116 × 261–307 µm, the oral sucker is 55–61 × 40–49 µm, the pharynx is 31–34 × 18–21 µm, the ventral sucker is 58–67 × 49–64 µm, the holdfast organ is 159–172 × 52–74 µm; the distance from the anterior end of the body to midlevel of the ventral sucker is 57–62% of the body length (Sokolov et al., 2013). Six other *Tylodelphys* spp. whose metacercariae have been found in fish and amphibians of Eurasia – *T. attenuata* (Baryscheva in Sudarikov, 1974), *T. cerebralis* (Chakrabarti, 1968), *T. clavata* (von Nordmann, 1832), *T. craniaria* (Diesing, 1858), *T. excavata* (Rudolphi, 1803), and *T. podicipina* Kozicka & Niewiadomska, 1960 — are localised in

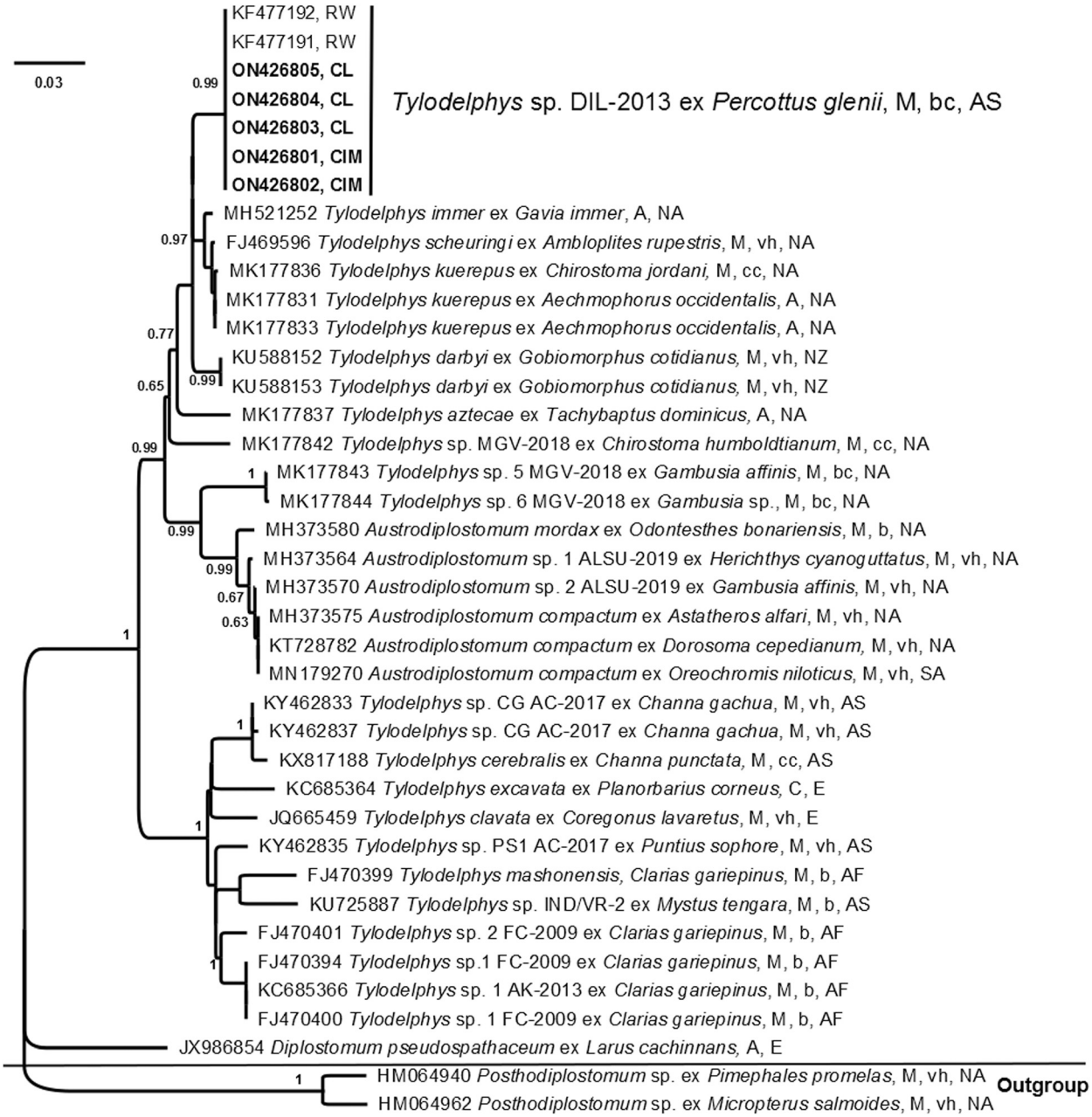

Fig. 3. Phylogenetic tree of *Tylodelphys* spp. and *Austrodiplostomum* spp. based on ITS1–5.8S rDNA–ITS2 nuclear DNA region sequences (832 bp) inferred using Bayesian inference analysis. Posterior probability values lower than 0.5 are not shown. Samples from the present study are labelled bold. Abbreviations: A, adult; C, cercaria; M, metacercaria; b, brain; bc, body cavity; cc, cranial cavity; vh, eye vitreous humour; AF, Africa, AS, Asia; E, Europe; NA, North America; SA, South America; NZ, New Zealand; CL, Liaoning Province of China; CIM, Inner Mongolia Autonomous Region of China; RW, West Siberia of Russia

the eyes, cranial cavity, or cerebrospinal canal of their second intermediate hosts (Sudarikov et al., 2002; Niewiadomska, 2010).

The metacercariae of *Tylodelphys* spp. localised in the fish body cavity have been recorded in the United States (Haderlie, 1953; Locke et al., 2015), Mexico (Moreno-Navarrete and Aguilar-Aguilar, 2013; García-Varela et al., 2016; Sereno-Uribe et al., 2019), Argentina (Szidat, 1969; Locke et al., 2015), Brazil (Pelegrini et al., 2019), and India (Pandey, 1970). However, molecular data are available only for three species with such localisation: *Tylodelphys* sp. 6 MGV-2018 of Locke et al. (2015) (ITS region for GenBank acc. numbers MK177844 in Fig. 3, and *cox1* for MK172799 in Fig. 2), *T. aztecae* and *T. jenynsiae* Szidat, 1969 (see Locke et al., 2015; García-Varela et al., 2016).

Interestingly, the examined *Tylodelphys* sp. ex *P. glenii* sample has no close phylogenetic relationship with any of the species from the fish body cavity mentioned above. It clustered with species from the vitreous humour (*T. darbyi, T. immer, T. podicipina, Tylodelphys* sp. of Soldánová et al., 2017) by mitochondrial DNA markers, or from the vitreous humour and the cranial cavity (*T. darbyi,*

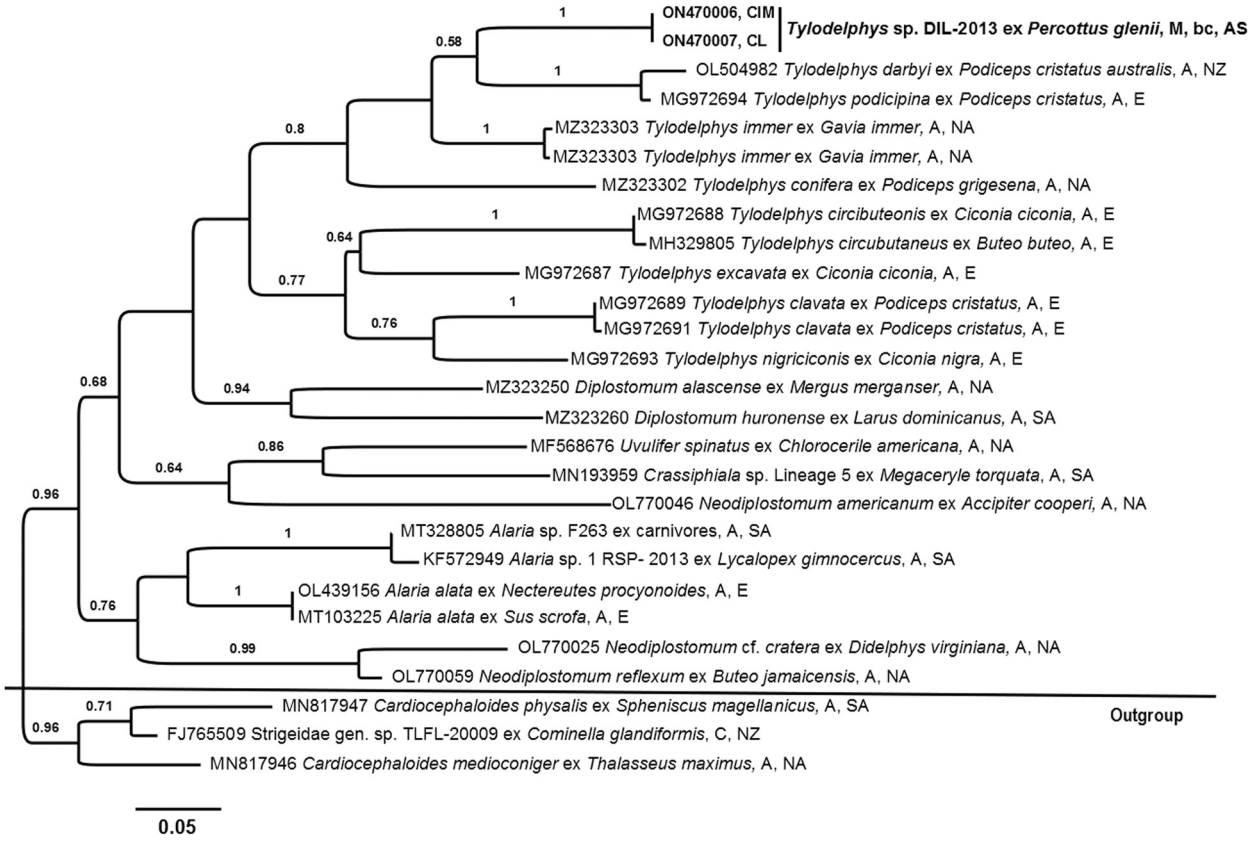

Fig. 4. Phylogenetic tree of diplostomids based on Fragment 2 of *cox*1 gene sequences (319 bp) inferred using Bayesian inference analysis. Posterior probability values lower than 0.5 are not shown. Samples from the present study are labelled bold. Abbreviations: A, adult; C, cercaria; M, metacercaria; bc, body cavity; AS, Asia; E, Europe; NA, North America; SA, South America; NZ, New Zealand; CL, Liaoning Province of China; CIM, Inner Mongolia Autonomous Region of China

T. immer, T. kuerepus, T. schreuringi) using the nuclear ITS rDNA marker. The obtained phylogenetic results do not support the hypothesis of Heneberg and Sitko (2021) about the conspecificity of *T. immer* to *T. podicipina*. Our data, like those of Achatz et al. (2022b), suggest that they are phylogenetically related but distinct species.

According to Pelegrini et al. (2019) and Achatz et al. (2022a,b), *Tylodelphys* is a paraphyletic taxon since the phylogenetic relationships of some of its Neotropic and Nearctic representatives are resolved in combination with *Austrodiplostomum* spp. Our data on *cox*1 gene Fragment 1 and the ITS regions are concordant with the findings of these authors. The data available at present suggest that there is no branching between the type species of *Tylodelphys* (i.e. *T. clavata*) and the members of the large clade comprising *T. aztecae, T. conifera, T. darbyi*, *T. immer, T. kuerepus, T. robraushi, T. scheuringi, T. variabilis,* as well as several undescribed *Tylodelphys* spp. including *Tylodelphys* sp. ex *P. glenii*. If this topology is confirmed in the future, the genus affiliation of *Tylodelphys* sp. ex *P. glenii* as well as other *Tylodelphys* spp. from this large clade will have to be revised.

Tylodelphys sp. has been detected both in the native (Liaoning Province of China) and in the non-native (Western Siberia and Trans-Baikal Region of Russia, Inner Mongolia Autonomous Region of China) populations of *P. glenii,* but only in the Asian part of the geographic range of this fish host. It is worth noting that the populations of this fish in China seem to be more heavily infected with *Tylodelphys* sp. than the ones in Russia (Sokolov et al., 2013, 2016; present study). Unfortunately, neither the intermediate nor the definitive host of *Tylodelphys* sp. ex *P. glenii* is known yet. Therefore, the causes of the different levels of infestation can only be speculated at present. It could be due to climatic factors affecting the frequency of hosts, size characteristics of the fish samples examined, or other, still unknown factors.

ACKNOWLEDGEMENTS

The current study was funded by the Russian Ministry of Science and Education: FFER-2021-0005 (SS) and FMEN-2022-0005 (DL). We thank Petr Sokolov, Elena Sokolova and Andrey Reshetnikov (Moscow, Russia) for their help in transporting the samples.

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