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PRIMARY RESEARCH PAPER

Hidden diversity of cestode *Nippotaenia* in a widely distributed invasive fish *Perccottus glenii*

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Abstract Biodiversity is important for the sustainability of aquatic ecosystems, however, the microevolutionary evidences of such diversity have often escaped the attention of researchers. We assessed the genetic diversity of the cestode *Nippotaenia* from several river systems in Europe, Siberia, and the Far East. Individuals of this parasite were obtained from two host species of odontobutid fishes, *Perccottus glenii* (European and Asian Russia, China) and *Odontobutis obscurus* (Japan). Molecular data on the NADH dehydrogenase subunit I (*nad1*) mitochondrial gene and the second internal transcribed spacer (ITS2) region of the nuclear rDNA were analyzed. We identified five phylogenetic lineages of *Nippotaenia* from *P. glenii* and one lineage (probably *N. mogurndae s. str.*) from *O. obscurus.* The presented *Nippotaenia* lineages from *P. glenii* show high genetic differentiation from *Nippotaenia ex O. obscurus* and from each other, comparable to interspecific differences in other cestodes. All five lineages were found in *P. glenii* from its native range, and only two from its invaded range. The revealed complex genetic structure of *Nippotaenia*, which parasitize in odontobutid fishes and are used as biological tag, provides new perspectives for the analysis of invasion pathways of their fish hosts.

Keywords Haplotype diversity · Invaded geographic range · Molecular operational taxonomic unit · Native geographic range · *Nippotaenia mogurndae* · *Nippotaenia perccotti*

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Introduction

Taxonomic diversity is important for the sustainability of aquatic ecosystems (Giller et al., 2004; Costantini & Rossi, 2010). Correct taxonomic identification of aquatic organisms, especially those belonging to closely related taxa, is crucial for

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understanding their ecological roles (Chiesa et al., 2015; Makhrov et al., 2023). There are examples on speciation, due to multiple host switches, biogeographic patterns, etc. (e.g., D'Bastiani et al., 2023). However, in the diversity of parasites of aquatic organisms, such as fish, it has often been observed that two or more congeneric species can parasitize the same host species (e.g., Šimková & Morand, 2008). The speciation mechanisms underlying this pattern of diversity under constant conditions are not entirely clear. In our view, they are hidden in microevolutionary processes that can be recovered by estimating intraspecific genetic variation and population genetic structure.

In this study, we explored the intra-host diversity of congeneric nippotaeniid tapeworms. Nippotaeniidae Yamaguti, 1939 is a small family of higher cestodes belonging to the monotypic order Nippotaeniidea Yamaguti, 1939. *Nippotaenia* Yamaguti, 1939, the only genus of the Nippotaeniidae, includes six nominal species: *Nippotaenia chaenogobii* Yamaguti, 1939, *Nippotaenia contorta* Hine, 1977, *Nippotaenia decidua* (Hine, 1977), *Nippotaenia fragilis* Hine, 1977, *Nippotaenia mogurndae* Yamaguti & Miyata, 1940, and *Nippotaenia perccotti* (Achmerov, 1941) (Yamaguti, 1939; Akhmerov, 1941; Hine, 1977). Several authors have suggested dividing the nippotaeniids into two genera, *Nippotaenia* and *Amurotaenia* Achmerov, 1941 (Akhmerov, 1941; Hine, 1977; Bray, 1994; Kvach et al., 2016), but this idea has not been validated by phylogenetic reconstructions (Scholz et al., 2017; Sokolov et al., 2018). The life cycle of *Nippotaenia* spp. involves an intermediate host (copepods) and the definitive host (freshwater fishes) (Yamaguti, 1951; Demshin, 1985).

Nippotaenia perccotti is the most taxonomically problematic species of the genus. It was originally described by Akhmerov (1941) based on specimens from the fish *Perccottus glenii* Dybowski, 1877 (Odontobutidae) caught in the Amur Basin during the Amur parasitological expedition (1937–1939). The type locality of this species was not given in the original description (Akhmerov, 1941). However, we found out that the type locality of the species (Lake Bolon, Khabarovsk Krai, Russia) is clearly indicated in the expedition report compiled by V. Dogiel and A. Akhmerov in 1949. The report is stored at the Saint Petersburg branch of the Russian Federal Research Institute of Fisheries and Oceanography.

Perccottus glenii is an invasive fish species that has expanded its range during the 20th and the early twenty-first centuries and now occurs over large areas in Europe and Asia (Reshetnikov, 2010; Reshetnikov & Schliewen, 2013; Pihlström et al., 2022; Šmejkal et al., 2024). Its native range is restricted to the basin of the Middle and the Lower Amur, the Far East of Eurasia, and several neighboring water systems (Reshetnikov, 2010). *Nippotaenia*, co-introduced with *P. glenii*, is present in many of its European and Siberian invasive populations (Sokolov et al. 2014; Sokolov & Reshetnikov, 2020), where its prevalence, abundance, and aggregation are considerably higher than in the native range (Sokolov et al., 2023). This parasite has been successfully used as a biological tag to analyze the pathways and even vectors of invasion of its host (Reshetnikov et al., 2011, 2017). Therefore, the assessment of possible taxonomic uniformity or heterogeneity of this parasite is important for a deeper understanding of the invasion processes of its host.

Dubinina (1962) suggested that *Nippotaenia* ex *P. glenii*, recognized by Akhmerov (1941) as *N. perccotti*, was in fact conspecific with *N. mogurndae* and consistently defended this point of view (Dubinina, 1971, 1987). *Nippotaenia mogurndae* was originally described from the fish *Odontobutis obscurus* (Temminck & Schlegel, 1845) in Japan (Yamaguti & Miyata, 1940), which is closely related to *P. glenii* (Li et al., 2018). Hine (1977) and Kvach et al. (2016) rejected Dubinina's concept of *N. mogurndae s. lato* based on the differences between *N. perccotti* and *N. mogurndae s. str.* in strobila length (0.9–3.6 mm *vs* 2.8–5.3 mm) and the number of proglottids (2–11 *vs* 25–45). It should be noted, however, that these differences only become apparent when comparing the original description (Yamaguti & Miyata, 1940; Akhmerov, 1941); they are smoothed out when considering Dubinina's (1971) data on specimens from *P. glenii.* Dubinina's opinion about conspecificity of *N. perccotti* and *N. mogurndae* had dominated until recently (Sokolov et al., 2014; Sokolov & Reshetnikov, 2020).

Dubinina's hypothesis was tested by Scholz et al. (2017) and Sokolov et al. (2018) using data on 18S and/or 28S rDNA markers obtained from single individuals collected in the non-native range of the host. However, the conclusions of these two studies were opposite. It is apparent that 18S and 28S rDNA markers are not suitable for this task, and the analysis of

the evolutionary events that led to the formation of the late diverging lineages of *Nippotaenia* spp. must be based on more variable molecular genetic markers and include more geographical isolates.

Given the apparent geographical isolation of continental isolates of *N. mogurndae s. l.* from those restricted to *O. obscurus* in Japan, we hypothesized that the cestode *N. mogurndae s. l.* is in fact a species complex or at least comprises different genetic lineages. During a parasitological studies of the fishes *O. obscurus* from Japan and *P. glenii* from different localities in northern Eurasia, we collected material on *Nippotaenia* spp. The cestode specimens from both fish species were initially identified as *N. mogurndae s. l.* according to Dubinina (1962, 1971, 1987). Later we began to consider specimens from *P. glenii* within the concept of the molecular operational taxonomic units (MOTUs) (Floyd et al., 2002; Blaxter, 2004), as we do in this study. The specimens from *O. obscurus* are considered as probable *N. mogurndae s. str*. basing on data of their host, locality, and the number of proglottids in strobila (27–32). The aim of our current study was to explore the genetic diversity of *Nippotaenia* collected from two host species from different localities in Eurasia (Japan, China, the Far East, Siberia, European part of Russia), including both the native and the invaded parts of the current range of *P. glenii*.

Materials and methods

Study area and sampling

Specimens of *Nippotaenia* spp. were collected from the intestine of *P. glenii* caught in China and in Russia as well as from the intestine of *O. obscurus* caught in Japan. In total, the samples were collected from 26 localities (Table 1). In most localities, cestode samples were collected as follows: immediately after capture, the host fish individuals were fixed in absolute ethanol, transported to the laboratory for further study, and dissected soon shortly thereafter; cestodes were pipetted into a watch glass, washed, sorted, counted, and then re-fixed in 96% ethanol. *Nippotaenia* specimens from *O. obscurus* as well as from *P. glenii* from European Russia, Siberia, and partly from the Far East (China, Zabaykalsky Krai, Khabarovsky Krai and some locations of Primorsky Krai of Russia) were adults with segmented strobila, while all the specimens from many other locations of Primorsky Krai of Russia were juvenile metacestode-like individuals. Unfortunately, the usage of the above-mentioned method of material collection resulted in severe deformation (twisting, compression) of the cestode bodies, preventing their detailed morphological study; therefore, in this study, *Nippotaenia* spp. were characterized only molecularly.

DNA extraction, amplification, and sequencing

Total DNA was extracted from individual tapeworms using the DNA-sorb B kit according to the manufacturer's protocols (Central Research Institute of Epidemiology, Russia). For reconstruction of phylogenetic relationships of *Nippotaenia* spp., the partial sequences of the NADH dehydrogenase subunit I (*nad1*) mitochondrial gene and the second internal transcribed spacer (ITS2) nuclear rDNA region were used. PCR amplification of the *nad1* gene fragment was conducted using the Cyclo_Nad1F (forward, 5′-GGNTATTSTCARTNTCGTAAGGG-3′) and Cyclo_trnNR (reverse, 5′-TTCYTGAAGTTAACA GCATCA-3′) (c. 850 bp) following Littlewood et al. (2008). Cycling conditions for the fragment of the *nad1* gene were as in Littlewood et al. (2008) with a few modifications: 95 °C for 5 min; 34 cycles of 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 60 s and a final extension at 72 °C for 5 min. ITS2 region was amplified using NC-6 (5′-ATCGACATCTTGAAC GCACATTGC-3′) and NC-2 (5′-TTAGTTTCTTTT CCTCCGCT-3′) (c. 493 655 bp) primers (Gasser & Chilton, 1995; Crosbie et al., 1998). The cycling conditions were as follows: 95 °C for 5 min; 32 cycles of 95 °C for 20 s, 52 °C for 20 s, and 72 °C for 60 s and a final extension at 72 °C for 5 min. Double-stranded DNA was amplified using the BioMaster HS-Taq PCR-Color (2×) kit (Biolabmix, Novosibirsk, Russia) according to the manufacturer's instructions. PCRs were 50 µl in volume and contained 25-µL BioMaster HS-Taq PCR-Color reaction mix, 0.2 µmol/l of each primer, 20 µl of sterile water, and 3 µl of total DNA was used as template. The PCR products were purified and sequenced in Eurogen (Moscow, Russia). The chromatograms of the amplicon sequences were evaluated based on the sharpness and clearness of each peak for each nucleotide. Sites with multiple peaks corresponding to the same nucleotide, located

Table 1 Sequences and accession numbers of *Nippotaenia* spp. specimens from *P. glenii* and *O. obscurus* used in the present studies according to their localities

Clade	Sample code	Sampling locality	Geographical latitude	Geographical longitude	*nad1* haplotype in median networks	*nad1* GenBank acc. no	ITS2 GenBank acc. no
Nippotaenia MOTU1							
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H1.1	PP768260	PP738330
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H1.5	PP768261	PP738331
	Khabarovsk_1	Basin of River Amur, Khabarovsky Krai, Russia	48.52	134.92	H1.2	PP768262	PP738332
	Khabarovsk_1	Basin of River Amur, Khabarovsky Krai, Russia	48.52	134.92	H1.2	PP768263	PP738333
	Kedrovaya	River Kedrovaya, Primorsky Krai, Russia	43.08	131.61	H1.3	PP768264	–
	Shenyang	Basin of River Daliao, Liaoning Province, China	42.13	123.38	H1.4	PP768265	PP738334
	Panjin	Basin of River Liaohe, Liaoning Province, China	41.26	121.64	H1.6	PP768266	PP738335
	Ilevskiy	Ilevsky fish farm, Nizhny Novgorod Region, Russia	54.95	43.03	H1.2	PP768267	PP738336
	Hulunbuir	River Yimin, Inner Mongolia Autonomous Region, China	49.26	119.72	H1.7	PP768268	PP738337
	Ulan-Ude	Lake Rotanikha, Republic of Buryatia, Russia	51.72	107.47	H1.8	PP768269	PP738338
	Zimnyak	Lake Zimyak, Novosibirsk Region, Russia	55.47	82.87	H1.9	PP768270	PP738339
	Zimnyak	Lake Zimyak, Novosibirsk Region, Russia	55.47	82.87	H1.9	PP768271	PP738340
	Zimnyak	Lake Zimyak, Novosibirsk Region, Russia	55.47	82.87	H1.9	PP768272	PP738341

Table 1 (continued)

Clade	Sample code	Sampling locality	Geographical latitude	Geographical longitude	*nad1* haplotype in median networks	*nad1* GenBank acc. no	ITS2 GenBank acc. no
	Zimnyak	Lake Zimyak, Novosibirsk Region, Russia	55.47	82.87	H1.9	PP768273	PP738342
	Zimnyak	Lake Zimyak, Novosibirsk Region, Russia	55.47	82.87	H1.9	PP768274	PP738343
	Biysk	Lake Dikoe, Altaisky Krai, Russia	52.56	85.32	H1.9	PP768275	PP738344
	Pyshma	Basin of River Pyshma, Sverdlovsk Region, Russia	64.28	56.98	H.10	PP768276	PP738345
	Khmeli	Basin of River Yurchim, Permsky Krai, Russia	56.03	57.96	H1.10	PP768277	PP738346
	Dolgoe	Lake Dolgoe, Zabaykalsky Krai, Russia	51.61	114.28	H1.11	PP768278	PP738347
	Lobnya	Basin of River Klyazma, Moscow Region, Russia	56.02	37.47	H1.10	PP768279	PP738348
	Tobolsk	Basin of River Irtysh, Tyumen Region, Russia	58.29	68.23	H.10	PP768280	PP738349
	Komissarovka	River Komissarovka, Primorsky Krai, Russia	44.9	131.59	H1.12	PP768281	PP738350
Nippotaenia MOTU2							
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H2.1	PP768282	PP738351
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H2.7	PP768283	PP738352
	Sivakonka_2	Basin of Lake Khanka, Primorsky Krai, Russia	44.73	132.4	H2.1	PP768284	PP738353
	Khabarovsk_1	Basin of River Amur, Primorsky Krai, Russia	48.52	134.92	H2.2	PP768285	PP738354
	Khabarovsk_1	Basin of River Amur, Primorsky Krai, Russia	48.52	134.92	H2.3	PP768286	PP738355
	Khabarovsk_1	Basin of River Amur, Primorsky Krai, Russia	48.52	134.92	H2.4	PP768287	PP738356

Table 1 (continued)

Clade	Sample code	Sampling locality	Geographical latitude	Geographical longitude	*nad1* haplotype in median networks	*nad1* GenBank acc. no	ITS2 GenBank acc. no
	Khabarovsk_1	Basin of River Amur, Primorsky Krai, Russia	48.52	134.92	H2.5	PP768288	PP738357
	Khabarovsk_2	Basin of River Amur, Primorsky Krai, Russia	48.45	135.21	–	–	PP738358
	Khabarovsk_2	Basin of River Amur, Primorsky Krai, Russia	48.45	135.21	H2.6	PP768289	PP738359
	Berdsk	Berdsky pond, Novosibirsk Region, Russia	54.73	83.12	H2.8	PP768290	PP738360
	Berdsk	Berdsky pond, Novosibirsk Region, Russia	54.73	83.12	H2.9	PP768291	PP738361
	Razdolnaya	Basin of River Razdolnaya, Primorsky Krai, Russia	43.42	131.73	H2.11	PP768292	PP738362
	Tongjiang	Tongjiang City, China	47.65	132.49	H2.10	NC066810	–
	Tongjiang	Tongjiang City, China	47.65	132.49	H2.10	ON640728	–
Nippotaenia MOTU3							
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H3.1	PP768293	PP738363
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H3.2	PP768294	PP738364
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H3.3	PP768295	PP738365
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H3.4	PP768296	–
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H3.4	PP768297	–
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	H3.2	PP768298	PP738366
	Sivakovka_2	Basin of Lake Khanka, Primorsky Krai, Russia	44.47	132.35	H3.5	PP768299	PP738367
	Cherepashye	Lake Cherepashye, Primorsky Krai, Russia	43.29	132.29	H3.6	PP768300	–

Table 1 (continued)

Clade	Sample code	Sampling locality	Geographical latitude	Geographical longitude	*nad1* haplotype in median networks	*nad1* GenBank acc. no	ITS2 GenBank acc. no
	Cherepashye	Lake Cherepashye, Primorsky Krai, Russia	43.29	132.29	H3.6	PP768301	–
Nippotaenia MOTU4							
	Sivakovka_1	Lake Khanka, Primorsky Krai, Russia	44.53	132.34	–	PP768302	–
	Astrakhanka	Lake Khanka, Primorsky Krai, Russia	44.7	132.07	–	PP768303	PP738368
	Ivnyanka	Lake Ivnyanka, Primorsky Krai, Russia	43.44	132.3	–	PP768304	–
	Ivnyanka	Lake Ivnyanka, Primorsky Krai, Russia	43.44	132,3	–	PP768305	–
	Komissarovka	River Komissarovka, Primorsky Krai, Russia	44.9	131.59	–	PP768306	–
	Komissarovka	River Komissarovka, Primorsky Krai, Russia	44.9	131.59	–	–	PP738371
	Komissarovka	River Komissarovka, Primorsky Krai, Russia	44.9	131.59	–	PP768307	–
	Kievka	River Kievka, Primorsky Krai, Russia	42.9	133.66	–	–	PP738372
	Kievka	River Kievka, Primorsky Krai, Russia	42.9	133.66	–	–	PP738373
	Kievka	River Kievka, Primorsky Krai, Russia	42.9	133.66	–	–	PP738374
	Kievka	River Kievka, Primorsky Krai, Russia	42.9	133.66	–	–	PP738375
Nippotaenia MOTU5							
	Khabarovsk_1	Basin of River Amur, Khabarovsky Krai, Russia	48.52	134.92	–	PP768308	–
	Khabarovsk_1	Basin of River Amur, Khabarovsky Krai, Russia	48.52	134.92	–	PP768309	PP738376
Nippotaenia mogurndae s. str							

Table 1 (continued)

Clade	Sample code	Sampling locality	Geographical latitude	Geographical longitude	*nad1* haplotype in median networks	*nad1* GenBank acc. no	ITS2 GenBank acc. no
	Nippotaenia1	River Nukui, Japan	32.4	132.69	–	PP768310	PP738377
	Nippotaenia2	River Nukui, Japan	32.4	132.69	–	PP768311	PP738378

at the edges of the sequence, were excluded from the analysis or designated as 'N' otherwise. All sequences were aligned, edited, and sequences of *nad1* checked for unexpected stop codons in MEGA 7 (Kumar et al., 2016). All gaps and poorly aligned positions (hypervariable loci) in alignment of the ITS2 region were identified and removed using Gblocks (online version 0.91b, http://www.phylogeny.fr; Castresana, 2000). The following parameters were applied: minimum Number of sequences for a Conserved Position—26; minimum number of sequences for a flanking position—42; maximum number of contiguous nonconserved positions—8; minimum length of a block—5; and allowed gap positions—none. Newly obtained sequences (see Table 1) were deposited into GenBank (NCBI).

Phylogenetic analysis

Phylogenetic reconstructions of *Nippotaenia* spp. were performed using the maximum likelihood (ML) and Bayesian inference (BI) approaches. The optimal substitution model was determined using jModelTest 2.1.1 (Darriba et al., 2012). For the *nad1* gene, we employed the dHKY + G + I model of nucleotide substitutions, while for the ITS2 region, we applied the dHKY + G model. Maximum likelihood (ML) searches were conducted using the IQ-TREE 2 software (Minh et al., 2020). Statistical support of the phylogeny test was provided using an ultrafast bootstrap approximation with 10,000 replications (Hoang et al., 2018). Bayesian analysis was performed with MrBayes v.3.2.1 using the same models as in the previous approach (Ronquist et al., 2012). Two simultaneous runs with four Markov chains each were performed for 1×10^7 generations and sampled every 500 generations. The first 25% of generations were discarded as burn-in. The sequences of *Arostrilepis macrocirrosa* Makarikov, Gulyaev & Kontrimavichus, 2011 (Hymenolepididae) with accession nos. MN732519 (*nad1* gene tree) and MN019655 (ITS2 region tree) from GenBank were included in the phylogenetic analyses as an outgroup based on the findings of Cao et al. (2022). The following sequences from GenBank were also used in the phylogenetic analysis: ON640728 and NC066810 of *N. mogurndae s. lato* and JQ268550 of *N. chaenogobii*.

Analysis of pairwise genetic distances (p-distances) within and between phylogenetic lineages was conducted in MEGA 7 (Kumar et al., 2016). Standard error of p-distances was estimated using bootstrap methods with 1000 replications. The number of haplotypes and levels of DNA polymorphism were calculated for the clades obtained from our phylogenetic analysis using the program DNASP 6 (Rozas et al., 2017). Popart 1.7 software (https://popart.otago.ac.nz) was used to calculate and visualize the median-joining network of phylogenetic relationships among haplotypes (Bandelt et al., 1999). The distribution of genetic variation was examined using analysis of molecular variance (AMOVA) as implemented in ARLEQUIN 3.5.2.2 (Excoffier & Lischer, 2010), with significance tests based on 1000 permutations.

In addition, Assemble Species by Automatic Partitioning (ASAP) method was used for delimitation of *Nippotaenia* lineages (Puillandre et al., 2021). We also employed ASAP to identification of the haplogroups while analyzing the median-joining network. For this purpose, subsets of each of the three *Noppotaenia* MOTUs ex *P. glenii* that exhibit haplotype diversity were analysed. ASAP analyses were conducted using the program's web interface (https://bioinfo.mnhn.fr/abi/public/asap) with the Kimura 2-Parameter substitution model (Kimura, 1980). This model was chosen because it is the one closest to dHKY + G + I and dHKY + G, which was selected for the phylogenetic analysis. We selected the five best partitions based on the highest ASAP scores.

Results

Genetic differentiation and phylogenetic analyses

The fragments of the *nad1* gene (697 bp each) and the ITS2 region (341 bp each) were amplified and sequenced from 52 to 47 cestode specimens, respectively.

Bayesian and ML phylogenetic analyses based on the *nad1* gene sequence dataset showed that all *Nippotaenia* specimens grouped into seven lineages. One of these lineages was associated with *Chaenogobius urotaenia* (Hilgendorf, 1879) from Japan (*N. chaenogobii*), one with *O. obscurus* from the same region (*N. mogurndae s. str.*) and five lineages with *P. glenii* from continental Eurasia (Figs. 1 and 2). Genetic p-distance values between these seven lineages ranged from 7.2 ± 0.96 to $18 \pm 1.41\%$ and the values within the lineages ranged from 0 to $4.6 \pm 0.79\%$. AMOVA analysis of *nad1* gene fragment variability performed for all *Nippotaenia* isolates included in our analysis showed that the values of molecular variance, associated with differentiation of the isolates among the seven phylogenetic lineages, were significantly higher than those due to variability within each lineage (Table 2). The lineages of *Nippotaenia* from *P. glenii* were treated as MOTUs. Since we have no specimens of *Nippotaenia* from Lake Bolon, the type locality of *N. perccotti*, it remains unclear which of the MOTU, parasitizing *P. glenii*, corresponds to

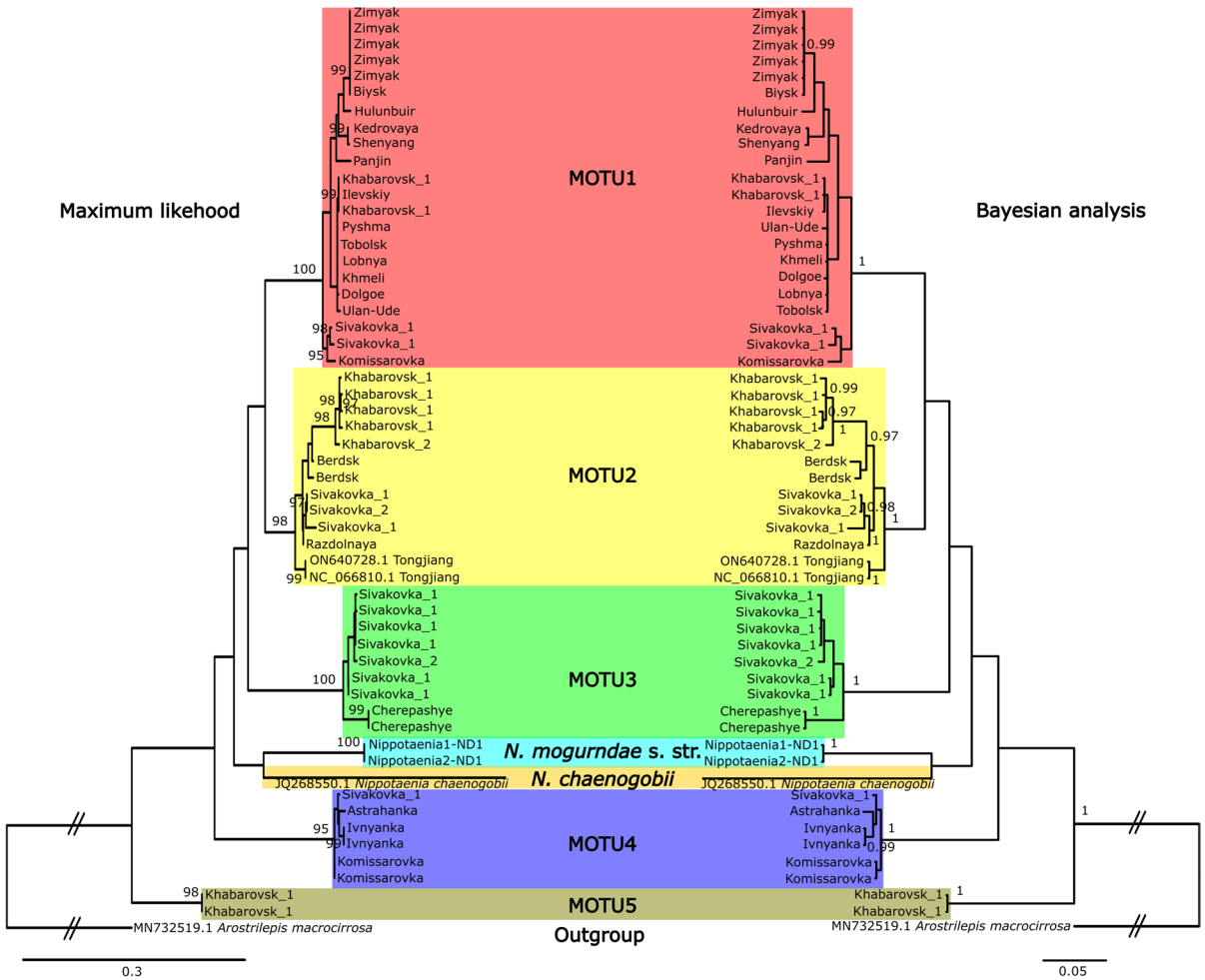

Fig. 1 Phylogenetic relationships of *Nippotaenia* spp. reconstructed by analyses of *nad1* gene sequences. Sample codes correspond to those in Table 1

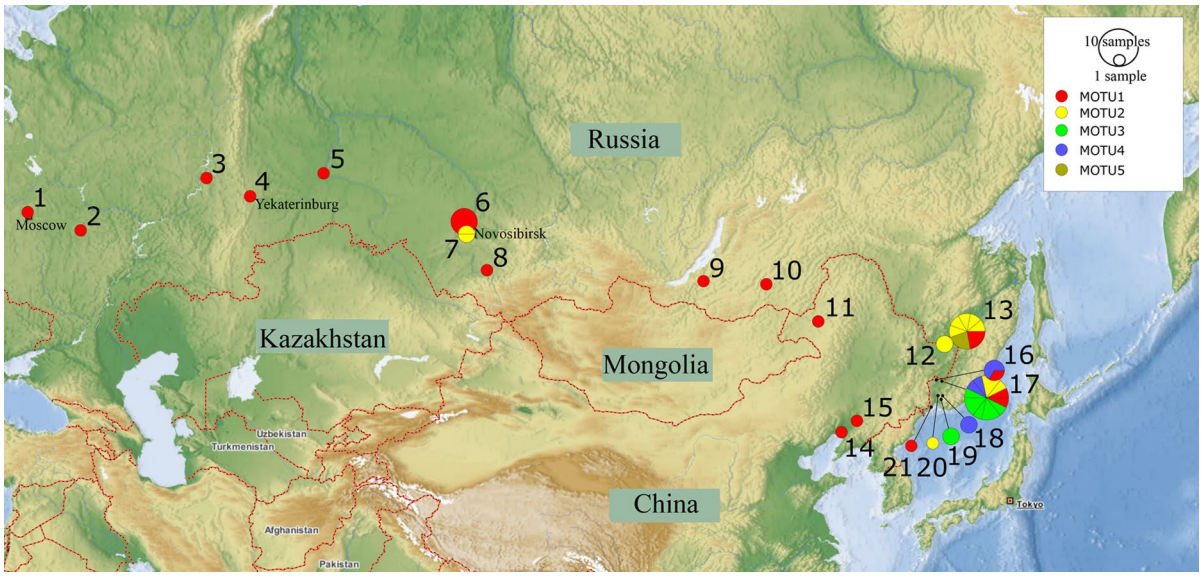

Fig. 2 Geographical distribution of *Nippotaenia* MOTUs ex *P. glenii*. Cestode specimens from different MOTUs are marked in different colors. Sampling localities (sample codes correspond to those in Table 1): 1. Lobnya; 2. Ilevskiy; 3. Khmeli; 4. Pyshma; 5. Tobolsk; 6. Zimnyak; 7 Berdsk; 8. Biysk; 9. Ulan-Ude; 10. Dolgoe; 11. Hulunbuir; 12. Tongjiang; 13. Khabarovsk_1 and Khabarovsk_2; 14. Panjin; 15. Shenyang; 16. Komissarovka; 17. Sivakovka_1, Sivakovka_2 and Astrakhanka; 18. Kievka; 19. Cherepashye and Ivnyanka; 20. Razdolnaya; and 21. Kedrovaya

Table 2 Analysis of molecular variance (AMOVA) of *nad1* gene fragments in *Nippotaenia* isolates considering their differentiation among seven phylogenetic lineages

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	Fixation index (F_{ST}) and significance level (P value)
Between phylogenetic lineages	6	1420.617	33.78826	83.24	
Within phylogenetic lineages	48	326.619	6.80457	16.76	F_{ST}: 0.83237 P value < 0.01
Total	54	1747.236	40.59283		

N. perccotti. *Nippotaenia* MOTU1 included specimens from *P. glenii* caught in China (three localities), European Russia (three localities), and Asian Russia (several localities in Siberia and the Far East). *Nippotaenia* MOTU2 was composed of specimens from China (one locality) and Asian Russian (one locality in Siberia and several localities in the Far East). *Nippotaenia* MOTU3, *Nippotaenia* MOTU4, and *Nippotaenia* MOTU5 were formed by specimens from the Russian Far East. All five *Nippotaenia* MOTUs from *P. glenii* and the clade of *N. mogurndae s. str.* specimens were highly supported, with ultrafast bootstrap and posterior probability values ≥ 95% and 0.95, respectively.

In both analyses utilizing the *nad1* marker, *Nippotaenia* MOTU1 was observed as a poorly supported sister lineage to *Nippotaenia* MOTU2. The *Nippotaenia* MOTU1 + *Nippotaenia* MOTU2 clade was an equally poorly supported sister to *Nippotaenia* MOTU3. *Nippotaenia chaenogobii* appeared as a poorly supported sister to *N. mogurndae s. str.* In turn, the *N. chaenogobii* + *N. mogurndae s. str.* clade had a poorly supported sister relationship with the *Nippotaenia* MOTU3 + (*Nippotaenia* MOTU1 + *Nippotaenia* MOTU2) clade. The *Nippotaenia* MOTU4 clade was a poorly supported sister to the large *Nippotaenia* clade, including all the six lineages listed above, while *Nippotaenia* MOTU5 was basal to all

the other MOTUs, as well as *N. chaenogobii* and *N. mogurndae s. str*.

Phylogenetic analyses based on the ITS2 region sorted all *Nippotaenia* specimens into five lineages, four of which corresponded to those described above, namely, *Nippotaenia* MOTU3, *Nippotaenia* MOTU4, *Nippotaenia* MOTU5, and *N. mogurndae s. str*. *Nippotaenia* MOTU1 formed polytomy with isolates related to *Nippotaenia* MOTU2 in *nad1* trees, while *N. chaenogobii* was absent in the ITS2 dataset. Phylogenetic relationships among the five lineages in the ITS2-based trees were poorly resolved (Fig. 3).

ASAP calculated five possible partitioning schemes for the *nad1* and the ITS2 datasets. One scheme partitioning of the *nad1* dataset obtained in this analysis suggested sequences differentiation between the seven lineages mentioned above (Table 3, #3). For the ITS2 dataset, none of the five partitioning schemes implies the differentiation of any of the lineages identified in the phylogenetic analyses based on this marker presented above.

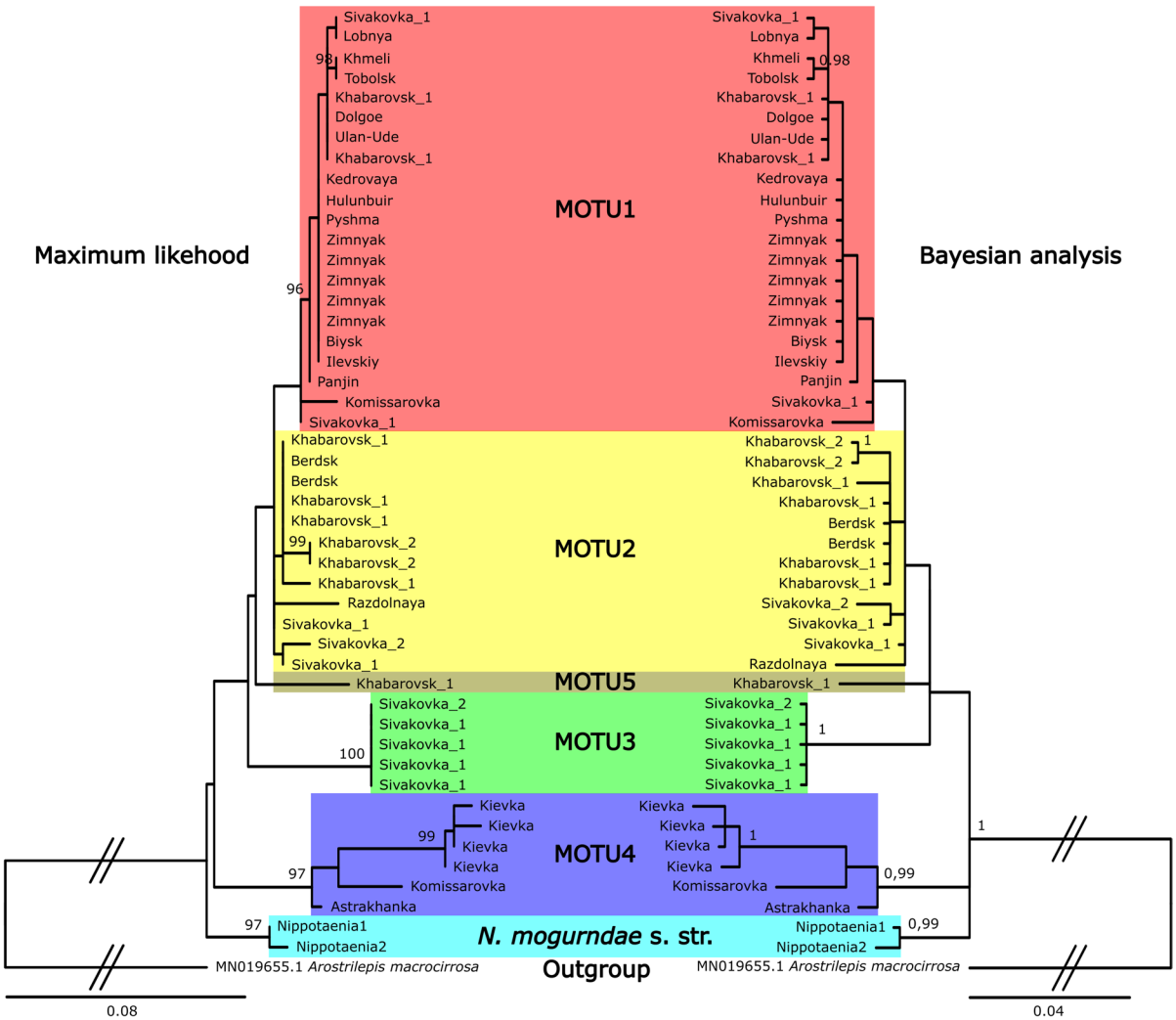

Fig. 3 Phylogenetic relationships of *Nippotaenia* spp. reconstructed by analyses of ITS2 region sequences. Sample codes correspond to those in Table 1

Table 3 ASAP analysis of *Nippotaenia* isolates based on *nad1* sequences gene dataset

Subsets number	asap-Score	*P* value	W Rank	Threshold Distance
15	2.5	0.0195	0.000426	0.013782
22	3.5	0.639	0.000609	0.008653
7	6.5	0.75	0.000441	0.053943
23	7	0.723	0.000406	0.007203
25	7.5	0.491	0.0003	0.005037

Mitochondrial *nad1* gene-based haplotype network analysis

Within the *Nippotaenia* MOTU1 clade, 12 *nad1* haplotypes with 49 polymorphic sites were revealed (intra-group p-distances were 0–4.2 ± 0.76%). The haplotype diversity and nucleotide diversity for this clade were 0.896 ± 0.045 and 0.02065 ± 0.00207, respectively. The *Nippotaenia* MOTU2 clade also included 11 *nad1* haplotypes with 53 polymorphic sites (intra-group p-distances were 0–4.6 ± 0.79%). The haplotype diversity and nucleotide diversity for this clade were 0.974 ± 0.039 and 0.02691 ± 0.00216, respectively. The lineages *Nippotaenia* MOTU3, *Nippotaenia* MOTU4, *Nippotaenia* MOTU5, *N. mogurndae s. str.*, and *N. chaenogobii* were represented by 6, 4, one, one, and one *nad1* haplotypes, respectively. The haplotype diversity and the nucleotide diversity for *Nippotaenia* MOTU3 were 0.917 ± 0.073 and 0.01714 ± 0.00479, respectively, with 30 polymorphic sites (intra-group p-distances were 0–3.7 ± 0.71%). The haplotype diversity and the nucleotide diversity for *Nippotaenia* MOTU4 were 0.867 ± 0.129 and 0.0087 ± 0.00147, respectively, with 13 polymorphic sites (intra-group p-distances were 0–1.3 ± 0.43%). No dominant haplotype and no star-shaped figures were noted for any of the clades (Figs. 4, 5 and 6). At the same time, all the clades were characterized by numerous substitutions between haplotypes.

Geographically, specific haplogroups were found in *Nippotaenia* MOTU1, *Nippotaenia* MOTU2, and *Nippotaenia* MOTU3 (Figs. 4, 5 and 6). In the haplotype network of *Nippotaenia* MOTU1, we noted two separate haplogroups from China (Fig. 4; haplogroups 1.1 and 1.2), Primorsky–Liaoning haplogroup (Fig. 4; haplogroup 1.3), a haplogroup from Western Siberia (Fig. 4; haplogroup 1.4), a haplogroup with a wide distribution in European and Asian parts of Russia (Fig. 4, haplogroup 1.5), and

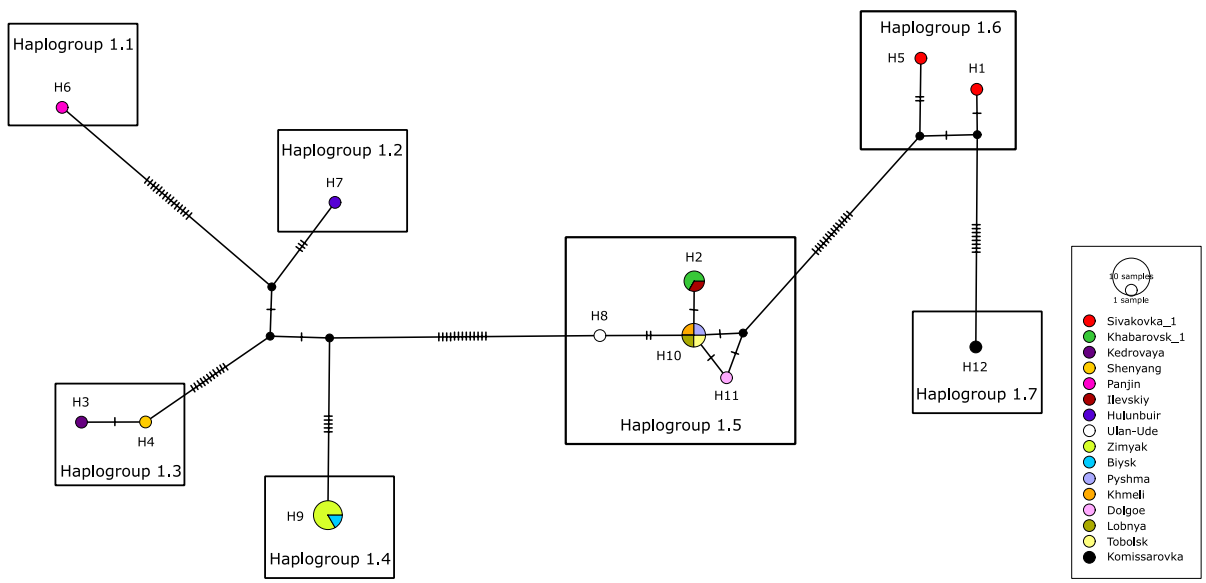

Fig. 4 Median network of haplotypes (*nad1* gene) of *Nippotaenia* MOTU1 clade. Haplogroups 1.1 and 1.2 are composed of haplotypes from China; haplogroup 1.3—haplotypes from China and Primorsky Krai of Russia; haplogroup 1.4—haplotypes from Western Siberia; haplogroup 1.5—haplotypes from European and Asian parts of Russia; and haplogroups 1.6 and 1.7 — haplotypes from Primorsky Krai of Russia. Sample codes correspond to those in Table 1 and Fig. 2

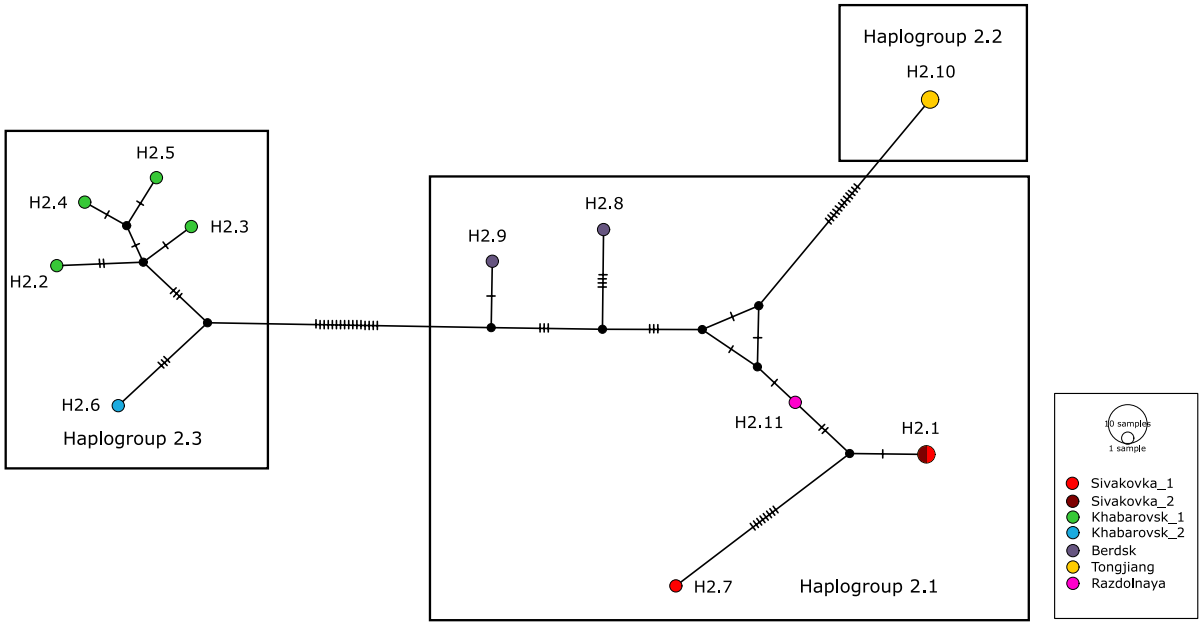

Fig. 5 Median network of haplotypes (*nad1* gene) of *Nippotaenia* MOTU2 clade. Haplogroups 2.1 is composed of haplotypes from Primorsky Krai and Novosibirsk Region of of Russia, respectively; haplogroup 2.2—haplotypes from China; and haplogroup 2.3—haplotypes from Khabarovskiy Kray of Russia. Sample codes correspond to those in Table 1 and Fig. 2

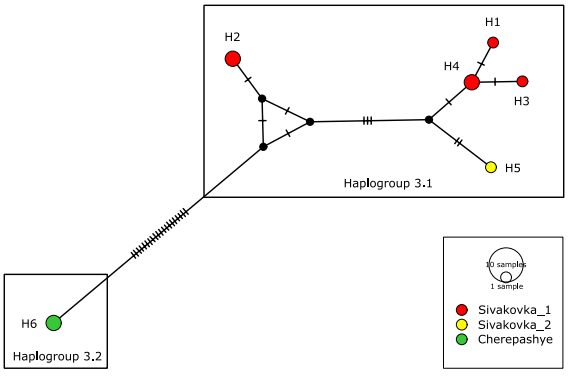

Fig. 6 Median networks of haplotypes (*nad1* gene) of *Nippotaenia* MOTU3. Haplogroups 3.1 and 3.2 are composed of haplotypes from two distant locations in Primorsky Krai of Russia. Sample codes correspond to those in Table 1 and Fig. 2

two haplogroups from Primorsky Krai of Russia (Fig. 4, haplogroups 1.6, 1.7). *Nippotaenia* MOTU2 was represented by three haplogroups, namely haplogroup from Primorsky Krai and Novosibirsk Region of Russia (Fig. 5, haplogroup 2.1), haplogroup from China (Fig. 5, haplogroup 2.2), and haplogroup from Khabarovsky Krai of Russia (Fig. 5, haplogroup 2.3). *Nippotaenia* MOTU3 contained two haplogroups from Primorsky Krai of Russia (Fig. 6, haplogroups 3.1, 3.2).

AMOVA analysis of the variability of the *nad1* gene fragments within each of the three MOTUs showed that the values of molecular variance associated with the geographic differentiation of haplogroups were significantly higher than those due to variability within the haplogroups (Table 4).

Discussion

Our results, based on the mitochondrial DNA marker (*nad1* gene), showed a genetic segregation of the entire array of *Nippotaenia* isolates into seven lineages, five of which (*Nippotaenia* MOTU1—*Nippotaenia* MOTU5) were recorded in one fish species, *P. glenii*. Two of these listed lineages are presumed to be the generally accepted nominal species *N. chaenogobii* and *N. mogurndae s. str.* (Hine, 1977; Scholz et al., 2017). We have already discussed above the convention of identifying the cestode specimens we found in *O. obscurus* as *N. mogurndae s. str.* The reliability of the species identification of the specimen reported as *N. chaenogobii* in Waeschenbach

Table 4 Analysis of molecular variance (AMOVA) of *nad1* gene fragments in *Nippotaenia* MOTU1, *Nippotaenia* MOTU2, and *Nippotaenia* MOTU3 considering geographical patterns of haplogroups

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	Fixation index (F_{ST}) and significance level (P value)
MOTU1					
Between haplogroups	6	143.97	8.71985	94.81	
Within haplogroups	15	7.167	0.47778	5.19	F_{ST}: 0.94805
Total	21	151.136	9.19763		P value < 0.01
MOTU2					
Between haplogroups	2	78.295	8.90769	71.70	
Within haplogroups	10	35.167	3.51667	28.30	F_{ST}: 0.71695
Total	12	113.462	12.42436		P value < 0.01
MOTU3					
Between haplogroups	1	35.492	10.84402	86.07	
Within haplogroups	7	12.286	1.75510	13.93	F_{ST}: 0.86070
Total	8	47.778	12.59913		P value = 0.0264 ± 0.0048
MOTU4					
Between haplogroups	2	12.5	2.92424	76.69	
Within haplogroups	3	2.667	0.88889	23.31	F_{ST}: 0.76689
Total	5	15.167	3.81313		P value = 0.0166 ± 0.0037

et al. (2012), whose mitochondrial DNA sequences have been deposited in GenBank is not really clear. It remains unknown what characters other than host and locality were used by these authors to identify this *Nippotaenia* specimen. However species identification of all *Nippotaenia* MOTUs is much more problematic. *Nippotaenia* MOTU1, *Nippotaenia* MOTU2, and *Nippotaenia* MOTU5 were represented by adult specimens, whereas the other two *Nippotaenia* MOTUs were represented by juvenile, metacestode-like individuals. This means that at least representatives of *Nippotaenia* MOTU1, *Nippotaenia* MOTU2, and *Nippotaenia* MOTU5 use *P. glenii* as a definitive host. The role of this fish species in the life cycles of the members of *Nippotaenia* MOTU3 and *Nippotaenia* MOTU4 remains to be elucidated. Results of phylogenetic analyses based on the ITS2 region sequence dataset indicate that *Nippotaenia* MOTU1 and *Nippotaenia* MOTU2 represent a single lineage, whereas the other three *Nippotaenia* MOTUs were segregated similarly to the *nad1* sequence dataset. In some groups of parasitic flatworms, the ITS marker shows lower resolution in segregating phylogenetic lineages (e.g., Meinilä et al., 2002; Lebedeva et al., 2021; Sokolov et al., 2022). Our results may also be explained by this circumstance. At the same time, we do not exclude that the removed hypervariable fragments of the ITS region from the alignment after adjustment in Gblocks may act as molecular features differentiating *Nippotaenia* MOTU1 and *Nippotaenia* MOTU2. However, this issue is the subject of a separate study.

The p-distance values by *nad1* marker within each of the five *Nippotaenia* MOTUs ex *P. glenii* did not exceed 4.6 ± 0.8%, while those between each of the seven *Nippotaenia* lineages identified in our study exceeded 7.2 ± 1.0%. According to Vilas et al. (2005), p-distance values < 5% between *nad1* gene sequences in cestodes generally correspond to an intraspecific level of differences, while those > 5% correspond to an interspecific level. Thus, the p-distance values by this marker allow us to hypothesize that *Nippotaenia* MOTU1, *Nippotaenia* MOTU2, *Nippotaenia* MOTU3, *Nippotaenia* MOTU4, and *Nippotaenia* MOTU5 are separate species. According to ASAP, recognising each of the five *Nippotaenia* MOTUs ex *P. glenii* as a separate species may be considered as one of the realistic taxonomic options. At the same time, we underline that the recognition of these five *Nippotaenia* MOTUs as independent species needs further evidence in terms of morphology and ecology. In this regard, these *Nippotaenia* MOTUs cannot

yet be definitively interpreted in a taxonomic context. This is also suggested by the weak support for sister relationships between different lineages or their groups in our trees (Figs. 1 and 3). In the absence of a clear taxonomic interpretation of the MOTUs, we cannot definitively reject the Dubinina's (1962, 1971, 1987) hypothesis about the conspecificity of *Nippotaenia* from *P. glenii* and from *O. obscurus*, but our results cast considerable doubt on it.

The pattern of genetic diversity of *Nippotaenia* MOTUs revealed in our study raises the question of possible evolutionary scenarios of lineage differentiation of these cestodes. It is known that parasites can evolve together with their hosts (co-speciation) and independently of their hosts (intra-host speciation) (Hafner & Nadler, 1988; Paterson & Poulin, 1999). The genetic divergence of *Nippotaenia*, revealed in our study, seems to follow the second scenario. In its native part of the range, *P. glenii* exhibits high genetic differentiation among populations due to the Quaternary history of glacial refugia formation and inter-basin redistribution in the river systems of the Far East (Xu et al., 2014). Based on this hypothesis, we believe that different *Nippotaenia* MOTUs from *P. glenii* (or at least three MOTUs whose members actually use *P. glenii* as a definitive host) evolved during the Pleistocene glacial events accompanied by the isolation of some populations of this fish species. Using genetic data, we have shown that one to four *Nippotaenia* MOTUs can occur in a water body within the native range of *P. glenii*. Current biogeographic patterns appear to reflect secondary contacts between these populations.

All five MOTUs were found in *P. glenii* within its native range. Among them, *Nippotaenia* MOTU1 and *Nippotaenia* MOTU2 were detected within the invaded range of the host, the latter only locally (Berdsk pond, Novosibirsk Region, Russia). It is noteworthy that haplotypes of *Nippotaenia* MOTU1 from Moscow Region, Nizhny Novgorod Region, Permsky Krai, Sverdlovsk Region, Tyumen Region, Zabaykalsky Krai, and the Republic of Buryatia were identical or very similar to those from the vicinity of the city of Khabarovsk in Khabarovsky Krai, Russia. This result is consistent with the fact that one of the fish farms near Khabarovsk was a donor for non-deliberate spread of *P. glenii* outside its native range (Reshetnikov & Ficetola, 2011). Importantly, *Nippotaenia* MOTU1 haplotypes from the River Ob basin from our samples of Novosibirsk Region and Altai Krai of Russia have no direct relationships with the above-mentioned location in Khabarovsky Krai (Russian Far East) based on Median-Joining network analysis. Therefore, we hypothesize that the donor population of *P. glenii* for Novosibirsk Region and Altai Krai is located in Primorsky Krai, Russia. This hypothesis is supported by the fact that a haplotype of *Nippotaenia* MOTU2 from Berdsk Pond (Novosibirsk Region, Russia) is very close to that from the Razdolnaya River (Primorsky Krai, Russia).

Importantly, cestodes of the genus *Nippotaenia*, which are associated with *P. glenii*, are alien parasites to European, Siberian, and Upper-Amur water bodies, co-introduced together with the host, and have been successfully used to analyse the pathways and even vectors of the invasion of its host (Reshetnikov et al., 2011, 2017). Based on parasitological data, we have also suggested the existence of two different sources of *P. glenii* invasion in the upper part of the Amur river basin: the Selenga river basin and the lower section of the Amur River (Sokolov et al., 2016). In the current investigation, the registration of distinct haplotypes of *Nippotaenia* MOTU1 in two parts of the Upper-Amur invaded subrange (Ingoda basin in Zabaykalsky Krai, Russia, and Argun basin near Hulunbuir, China) is consistent with this hypothesis. Therefore, the data we obtained in this study will provide additional opportunities for such analyses.

Conclusion

We found an extensive genetic diversity of congeneric lineages of a cestode parasite in a single fish host species, *P. glenii*, from several river basins in the northern Eurasia. The five newly described *Nippotaenia* genetic lineages, associated with this fish host, differ from each other by p-distance values, calculated based on mitochondrial *nad1* sequence dataset, which are consistent with the level of interspecific differentiation in other cestodes based on the same molecular marker. A final conclusion on the species status of these five lineages can be made after detailed morphological studies of representatives of each lineage. The identification of several lineages of *Nippotaenia* opens new perspectives for the analysis of the invasion of *P. glenii*.

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Author contributions SGS and ANR contributed to conceptualization, sampling, and writing and editing. PGV contributed to sampling, molecular data, phylogenetic analysis, and writing and editing. DMA contributed to sampling, molecular data, and writing and editing. MU contributed to sampling and molecular data. GVI contributed to molecular data. PY and MBS contributed to sampling. MMS contributed to project administration, funding, and writing and editing.

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Data availability Newly obtained sequences were deposited into GenBank (NCBI). The other datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval The research protocol of this work has been approved by the Bioethics Commission of the Severtsov Institute of Ecology and Evolution and Russian Academy of Sciences (resolution no. 47 dated April 23, 2021) and complies with the current laws and regulations of Russia, China, and Japan.

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