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Integrative Taxonomy of the Khanka Spiny Bitterling *Acheilognathus chankaensis* (Acheilognathidae) in the Russian Far East: Cryptic Diversity and Phylogeographic Patterns

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Abstract—We present the first molecular-genetic analyses of *Acheilognathus chankaensis* free embryos floodplain lake of the Razdolnaya River basin (Primorsky region, Russia). Examination of 115 *Nodularia douglasiae* individuals revealed 24 infested bivalves hosting 219 free embryos (length 3–5 mm). Genetic analysis of 23 specimens via *cox1* mtDNA sequencing confirmed identity with *A. chankaensis* populations from Northeast China and South Korea. Phylogenetic reconstruction (Bayesian inference, maximum parsimony, maximum likelihood) of six Acheilognathinae genera resolved nine generic groups and two distinct lineages of *A. chankaensis*: sensu stricto (11 haplotypes in haplogroups A1 (Primorye–Korea–Heilongjiang) and A2 (Yangtze basin)) and a polyphyletic *A. chankaensis* sensu lato group. Our results demonstrate possible postglacial dispersal patterns via connected drainages (haplogroup A1), ancient vicariance in South China (haplogroup A2), and taxonomic discordance requiring revision of *A. chankaensis* s.l.

Keywords: Razdolnaya River, Unionidae, host mussels, Acheilognathidae, *Acheilognathus*, phylogenetic reconstruction, *cox 1* mtDNA

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INTRODUCTION

Taxonomic revisions of the Acheilognathidae remain an ongoing challenge in ichthyology, particularly for East Asian lineages where molecular phylogenies often conflict with traditional morphology-based classifications. Previous studies have substantially advanced our understanding of bitterling systematics, yet critical uncertainties persist in generic delineation and species boundaries within *Acheilognathus* and related genera.

The systematics of Acheilognathidae has undergone significant reorganization. Chang et al. (2014) utilized the mitochondrial *Cyt b* gene to demonstrate that species traditionally classified under *Tanakia* comprised three distinct lineages, leading to the recognition of *Tanakia* sensu stricto, recovering of *Pseudorhodeus* and the establishment of new genus *Paratanakia*. These findings were corroborated by subsequent studies (Cheng et al., 2014; Kawamura et al., 2014), reinforcing the need for comprehensive taxonomic revisions. Turanov et al. (2019) further contributed to this effort through a phylogenetic analysis of eight *Acheilognathus* species from Khanka Lake (Primorsky region, Russia), identifying a distinct lineage forming a monophyletic clade with *A. asmussii* and *A. macropterus*.

Despite these advances, taxonomic consensus remains elusive. The World Register of Marine Species (WoRMS:https://www.marinespecies.org/aphia.php?p=taxdetails&id=154156, Version 08/2025) reflects competing classifications: one recognizing Acheilognathinae as a subfamily within Cyprinidae, and another elevating it to family rank (Acheilognathidae) under Cypriniformes. This discrepancy underscores the ongoing debate between morphological and molecular phylogenetic evidence and highlights the need for integrative taxonomic approaches.

Molecular identification has proven particularly valuable in resolving complexes of cryptic species. In the Russian Far East, genetic data have revealed misidentifications in early life stages, as demonstrated when free embryo from floodplain lake (Razdolnaya River), initially assigned to *Rhodeus sericeus* (Vainutis

and Bogatov, 2024), were subsequently re-identified using molecular methods (Maximov et al., 2024). The Khanka spiny bitterling *Acheilognathus chankaensis* exemplifies the taxonomic confusion within the group, with mitogenome sequences deposited in GenBank under both *Acanthorhodeus chankaensis* (Lv et al., 2014; Xu et al., 2020) and *Acheilognathus chankaensis* (Tan et al., 2019), while WoRMS treats *Acanthorhodeus* as a synonym of *Acheilognathus*.

This study aims to address key taxonomic uncertainties in Acheilognathidae through a comprehensive phylogenetic analysis, with particular emphasis on the *Acheilognathus chankaensis* complex. Using mitochondrial *cox 1* sequences from Primorsky region populations combined with GenBank data, we seek to clarify species boundaries, evaluate genetic divergence between lineages, and contribute to resolving the systematic relationships within this ecologically significant group.

MATERIALS AND METHODS

Material Collection

Specimens of *Nodularia douglasiae* were collected in mid-May 2023 and 2025 from shallow waters (<1 m depth) of a 600-m long artificial floodplain lake within a former sand quarry (the Razdolnaya River basin, Nadezhdinsky District, Primorsky region, Russia (Fig. 1; Supplement 1). This site experiences periodic flooding, most recently in August 2023 following Typhoon Hanun (https://www.forbes.ru/society/494526-samoe-sil-noe-navodnenie-za-10-let-kak-vygladit-zatoplennyj-ussurijsk. Version 08/2025.). The collected mollusks (shell length 3–5 cm) were distributed in sandy substrates across a 60 m sampling area. For parasitological examination, we selected 15 mussels in 2023 and 100 mussels in 2025. The free embryos of bitterlings were not detected in the majority of mussels. From these collections, we isolated 219 free embryos (prelarvae) of the Khanka spiny bitterling *Acheilognathus chankaensis* from the gills of 24 host mussels, preserving 23 prelarvae in 96% ethanol for molecular genetic analysis.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from 23 samples of prelarvae of *Acheilognathus chankaensis* using alkaline lysis method HotShot (Truett, 2006). The folmer fragment of the cytochrome c oxidase subunit I mtDNA gene was amplified with DreamTaq Green PCR Master Mix (Thermo Scientific, USA) and universal primers: forward LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). The PCR mixture contained 2× DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 0.5 μL forward and reverse primers and 5 μL templates in total volume of 20 μL. The amplification protocol for *cox 1* mtDNA: 1 min denaturation hold at 94°C, 30 cycles of 15 s at 94°C, 30 s at 50°C, 2 min at 72°C, and a 7 min extension hold at 72°C. Each PCR reaction included a negative and positive control, using both primers to detect possible contamination. PCR products were directly sequenced using the primers LCO1490 and HCO2198 and the Brilliant Dye Terminator (v3.1) Cycle Sequencing Kit (Nimagen, Netherlands) as instructed by the manufacturer. The PCR products were read with an ABI Prism 3500 Genetic Analyzer (Applied Biosystems, USA) at the Resource Sharing Centre of the National Scientific Center of Marine Biology FEB RAS (Vladivostok, Russia).

Alignment and Phylogenetic Analyses

In total, 23 partial *cox 1* mtDNA sequences obtained in this study were assembled with MEGA12 software (Kumar et al., 2024) and aligned using ClustalW. New *cox1* gene sequences of *Acheilognathus chankaensis* were submitted to GenBank under accession numbers PV820667–PV820689. For comparative and phylogenetic analyses, we used 118 *cox 1* Folmer fragment sequences of 48 Acheilognathidae species from the NCBI database, along with three *Tinca tinca* sequences as the rooting outgroup. Genetic divergence was estimated using the *p*-distance model with 1000 bootstrap replicates in the MEGA 12 software; *p*-value was lower than 0.05. Phylogenetic analyses were carried out with Bayesian inference (BI) algorithm (Huelsenbeck et al., 2001) with the TPM3uf + G model selected in jModeltest v. 2.1.5 software as the best (Darriba et al., 2012). The Markov chain Monte Carlo (MCMC) algorithm was performed using two independent runs and 500000 generations (the average standard deviation of split frequencies was less than 0.01), and 25% of the generations were discarded as burn-in in MrBayes v. 3.1.2 software (Huelsenbeck et al., 2001). A supplementary phylogenetic trees were reconstructed using the maximum parsimony (MP) and maximum likelihood (ML) methods with 1000 bootstrap replicates in the MEGA 12 software.

Haplotype Diversity Examination

Genetic divergence was estimated using genetic *p*-distance values including all substitutions in MEGA 12. Nucleotide sequences were translated to amino-acid sequences using Vertebrate Mitochondrial code 21. Haplotype data were generated for *cox 1* fragment using DnaSP v. 6.12.03 (Rozas et al., 2017). Minimum spanning network was drawn for two haplogroups of *Acheilognathus chankaensis* from different localities using population genetics software PopART 1.7. (Leigh and Bryant, 2015). We used complete sequences of *cox 1* of other *Acheilognathus* spp. to clearly analyze haplotype diversity in the certain sites of *Acheilognathus chankaensis*: AP012986,

Fig. 1. Artificial floodplain lake of the Razdolnaya River basin near the village Razdolnoye, (●) the collection site of *Nodularia douglasiae* mollusks.

MN683735, KF695385, MK380726, NC_023101, MZ334545.

RESULTS

Taxonomic Summary of Acheilognathus chankaensis

Synonyms. *Devario chankaensis* Dybowski, 1872 (original name); *Acanthorhodeus asmussi sungariensis* Berg, 1931; *Acanthorhodeus atranalis* Günther, 1873; *Acanthorhodeus gracilis* Regan, 1908; *Acanthorhodeus tokunagai* Mori, 1934; *Acanthorhodeus wangi* Tchang, 1930; *Acanthorhodeus chankaensis* (Dybowski, 1872); *Acheilognathus bleekeri* (Berg, 1907); *Achilognathus chankaensis* (Dybowski, 1872); *Paracheilognathus bleekeri* Berg, 1907; *Parachilognathus imberbis* Bleeker, 1871.

New locality. Russian Federation, Primorsky region, Nadezhdinsky district, Razdolnaya River basin, artificial floodplain lake near Razdolnoe village (43°34′39.8″ N, 131°53′58.7″ E).

Registration. GenBank: new sequence data for *A. chankaensis* were submitted to GenBank database under accession numbers PV820667–PV820689; ZooBank: urn:lsid:zoobank.org:act:733A2CD0-D21A-4FA0-8E39-B4A958953D7F.

Voucher material. The specimens with number ACH are the part of Zoological collection in the laboratory for Monitoring of Aquatic Bioresource Invasions of the Water Bioresources and Aquaculture department at the Far Eastern State Technical Fisheries University.

Genetic Analysis Based on cox 1 Sequencing

We obtained 23 new nucleotide sequences (714 bp) of the *cox 1* mtDNA gene (Folmer fragment) for *Acheilognathus chankaensis*. BI analysis of a 612 bp fragment (Fig. 2) resolved 50 Acheilognathidae species into three major subclades. The *A. chankaensis* complex was distributed across two distinct lineages: *A. chankaensis* sensu lato (s.l.) (including *A. macrop-*

terus, *A. taenianalis*, *Acheilognathus* sp. 1, *Acheilognathus* sp. 2, and misidentified *R. sericeus*) and *A. chankaensis* sensu stricto (s.s.), separated by 7.52–10.62% genetic divergence. The s.s. group further subdivided into Ahaplogroups A1 (0.16–0.49% divergence) and A2 (0–1.8% divergence), with inter-haplogroup divergence of 3.27–4.25% (Table 1).

MP and ML analyses (Supplements 2, 3) showed congruent resolution of terminal clades despite topological differences in deeper nodes compared to the BI tree. All methods consistently supported the relationships between *A. chankaensis* s.l., s.s., and the *A. typus* + *A. longipinnis* sister pair: with statistical support values of 1/70/96 (BI/MP/ML) for divergence between *A. chankaensis* s.s. and *A. typus* + *A. longipinnis*; 1/29/70 for terminal groups and 1/95/100 for A1/A2 haplogroup divergence.

Genetic divergence between the separate lineages of *Rhodeus* designated here as s.s., s.l. I, s.l. II, and s.l. III were as follows: 15.2–17.3% (*Rhodeus* s.s.—*Rhodeus* s.l. I); 11.9–17.7% (*Rhodeus* s.s.—*Rhodeus* s.l. II); 12.4–15.2% (*Rhodeus* s.s.—*Rhodeus* s.l. III); 13.7–19.3% (*Rhodeus* s.l. I—*Rhodeus* s.l. II); 14.5–16.2% (*Rhodeus* s.l. I—*Rhodeus* s.l. III); 12.1–14.1% (*Rhodeus* s.l. II—*Rhodeus* s.l. III) (Supplement 4).

To re-evaluate the phylogenetic relationships of *Acheilognathus* s.l., we estimated *p*-distances between *Acheilognathus striatus* and other generic groups (Supplements 5, 6). *Acheilognathus striatus* differed from other genera by the following values: 15.5–16.9% (*Acheilognathus* s.s.); 13.6–15.5% (*Rhodeus* s.s.); 13.9% (*Pseudorhodeus*); 14.4–15.4% (*Paratanakia*); 12.8–15.2% (*Tanakia*); 12.6% (*Sinorhodeus*); 13.9–15.9% (*Rhodeus* s.l. I). These values overlapped the intergeneric ranges between other genera: 12.3–13.9% (*Pseudorhodeus*–*Paratanakia*), 10.8–13.7% (*Tanakia*–*Pseudorhodeus*), 12.8–15.4% (*Tanakia*–*Paratanakia*), 12.6–14.7% (*Rhodeus* s.s.–*Pseudorhodeus*), 12.9–16% (*Rhodeus* s.s.–*Paratanakia*), 11.9–15.2% (*Rhodeus* s.s.–*Sinorhodeus*), 12.6–16.3% (*Rhodeus* s.s.–*Tanakia*).

Haplotype Diversity of Acheilognathus chankaensis

The minimum spanning haplotype network had a star-like conformation and revealed genetic relationships among 19 haplotypes (H1–H19) of the *Acheilognathus* fishes, sampled across East Asia (China, South Korea, Japan, and Russia's Primorye) (Fig. 3).

The central (core) haplotypes H16 and H9 of *A. chankaensis* s.s. formed two haplogroups A1 and A2, respectively. On average, one mutational event (me) separated the central haplotypes from the surrounding haplotypes of the respective haplogroup. The central haplotypes of *A. chankaensis* were distant from each other with 19 me. *Acheilognathus tonkinensis* (H1) and *A. typus* (H8) were chosen as outgroup taxa to visualise the interspecific divergence of *Acheilognathus*. H16 of *A. chankaensis* s.s. was significantly distant from H7 of *A. chankaensis* s.l.—46 me. The haplotypes H17 (s.s.) and H7 (s.l.) both indicated as *Rhodeus sericeus* from the same river Heilongjiang were separated with 45 me.

The haplotype network of *Acheilognathus chankaensis* s.s. reconstructed using the Minimum spanning network method (43 sequences; 623 bp *cox 1* fragment) (Fig. 4) revealed a star-like topology with two distinct haplogroups: haplogroup A1 centered on H16 (21 samples), flanked by haplotypes H13–H19 (1–2 mutational steps distant); haplogroup A2 anchored by H9 with radiating haplotypes H10–H12 (1–9 mutational steps). Strong geographic segregation: 20 mutational steps separated the central haplotypes H16 (A1) *vs* H9 (A2), reflecting deep divergence and long-term isolation between northern (A1; Primorye–Korea–Heilongjiang)) and southern (A2; Yangtze basin) lineages. Hap16 (A1) represents the most frequent haplotype, suggesting a founder effect or recent expansion in Northeast Asia, while singleton haplotypes (e.g., H13, H15, H17–H19) indicate higher genetic diversity in northern populations. Unique haplotypes in the Razdolnaya River highlight the region's evolutionary significance (Table 2).

DISCUSSION

The Phylogeny and Taxonomy of the Acheilognathidae

Our phylogenetic analysis supports the recognition of Acheilognathidae as a distinct family within Cypriniformes, consistent with recent studies (Chang et al., 2014; Stout et al., 2016; Betancur-R et al., 2017; Esmaeili et al., 2020). Despite relatively low generic diversity compared to Leuciscidae and Cyprinidae, bitterlings have undergone extensive taxonomic reorganization, reflecting their complex evolutionary history. The phylogenetic reconstruction (Fig. 2) reveals several taxonomic uncertainties requiring revision. Specifically, we question the identification of *Rhodeus sericeus* reported from the Razdolnaya River basin (Sayenko and Palatov, 2023; Vainutis and Bogatov, 2024; Sayenko, 2025), as our genetic data suggest possible misidentification. Current systematic frameworks inadequately resolve several key groups, including *Acheilognathus* s.l., *Rhodeus* s.s., *Rhodeus* s. l., and the *Rhodeus cyanorostris* + *Sinorhodeus* complex.

Fig. 2. Phylogenetic Bayesian tree reconstructed with the extended *cox 1* mtDNA sequence data of six genera of the Acheilognathidae. Original sequence data of the Khanka spiny bitterling *Acheilognathus chankaensis* are in bold. Branch numbers are posterior probabilities of the Bayesian Inference algorithm. Different colors are used to distinguish between valid genera and the taxa sensu lato stemming from separate ancestral nodes, which are considered as potential independent genera.

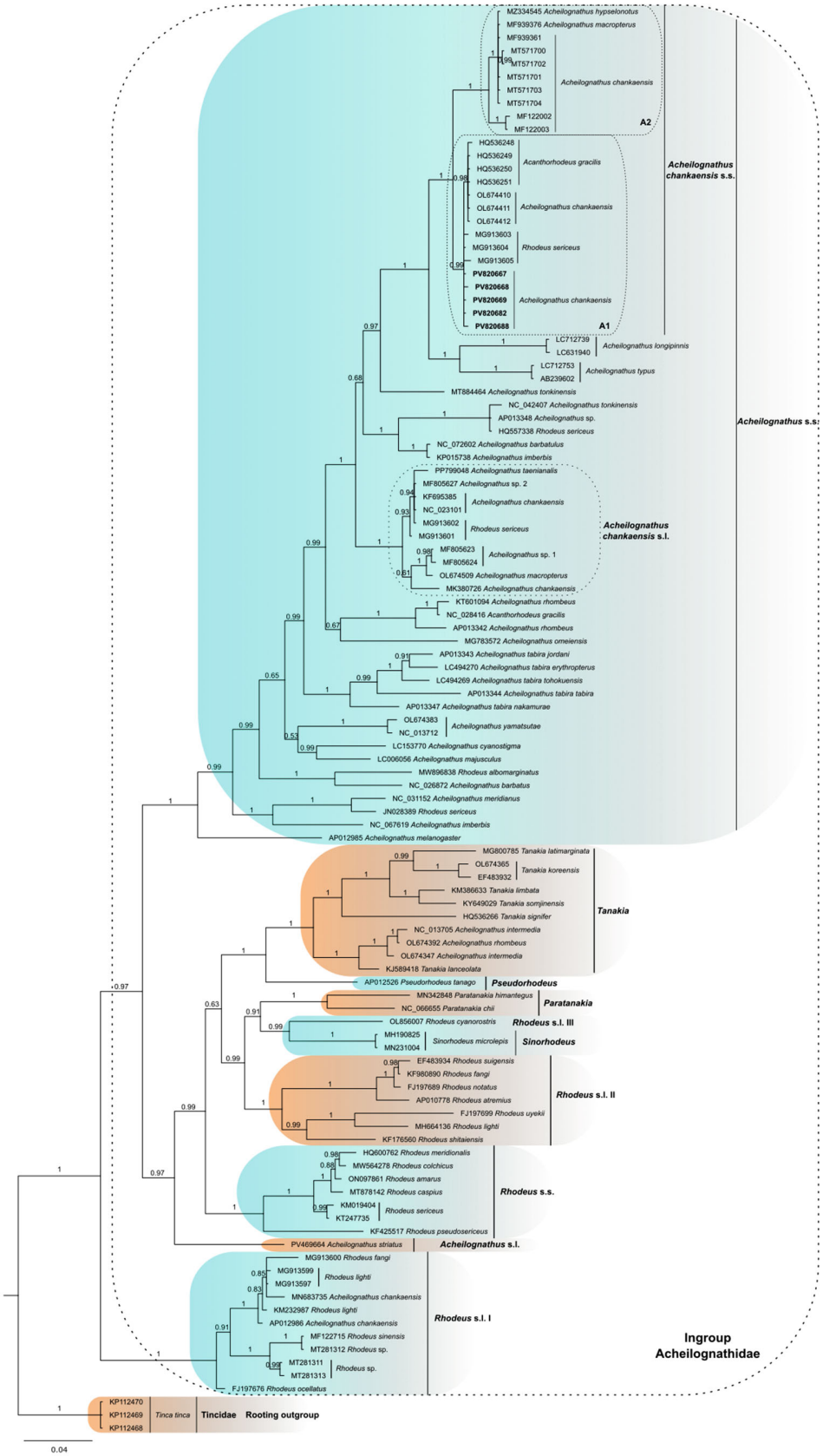
MZ334545 Acheilognathus hypselonotus
MF939376 Acheilognathus macropterus
MF939361
MT571700
MT571702
MT571701
MT571703
MT571704
Acheilognathus chankaensis
MF122002
MF122003
A2
HQ536248
HQ536249
HQ536250
HQ536251
Acanthorhodeus gracilis
OL674410
OL674411
OL674412
Acheilognathus chankaensis
MG913603
MG913604
MG913605
Rhodeus sericeus
PV820667
PV820668
PV820669
PV820682
PV820688
Acheilognathus chankaensis
A1
Acheilognathus chankaensis s.s.
LC712739
LC631940
Acheilognathus longipinnis
LC712753
AB239602
Acheilognathus typus
MT884464 Acheilognathus tonkinensis
NC_042407 Acheilognathus tonkinensis
AP013348 Acheilognathus sp.
HQ557338 Rhodeus sericeus
Acheilognathus s.s.
NC_072602 Acheilognathus barbatulus
KP015738 Acheilognathus imberbis
PP799048 Acheilognathus taenianalis
MF805627 Acheilognathus sp. 2
KF695385
NC_023101
Acheilognathus chankaensis
MG913602
MG913601
Rhodeus sericeus
Acheilognathus chankaensis s.l.
MF805623
MF805624
Acheilognathus sp. 1
OL674509 Acheilognathus macropterus
MK380726 Acheilognathus chankaensis
KT601094 Acheilognathus rhombeus
NC_028416 Acanthorhodeus gracilis
AP013342 Acheilognathus rhombeus
MG783572 Acheilognathus omeiensis
AP013343 Acheilognathus tabira jordani
LC494270 Acheilognathus tabira erythropterus
LC494269 Acheilognathus tabira tohokuensis
AP013344 Acheilognathus tabira tabira
AP013347 Acheilognathus tabira nakamurae
OL674383
NC_013712
Acheilognathus yamatsutae
LC153770 Acheilognathus cyanostigma
LC006056 Acheilognathus majusculus
MW896838 Rhodeus albomarginatus
NC_026872 Acheilognathus barbatus
NC_031152 Acheilognathus meridianus
JN028389 Rhodeus sericeus
NC_067619 Acheilognathus imberbis
AP012985 Acheilognathus melanogaster
MG800785 Tanakia latimarginata
OL674365
EF483932
Tanakia koreensis
KM386633 Tanakia limbata
KY649029 Tanakia somjinensis
HQ536266 Tanakia signifer
Tanakia
NC_013705 Acheilognathus intermedia
OL674392 Acheilognathus rhombeus
OL674347 Acheilognathus intermedia
KJ589418 Tanakia lanceolata
AP012526 Pseudorhodeus tanago
Pseudorhodeus
MN342848 Paratanakia himantegus
NC_066655 Paratanakia chii
Paratanakia
OL856007 Rhodeus cyanorostris
Rhodeus s.l. III
MH190825
MN231004
Sinorhodeus microlepis
Sinorhodeus
EF483934 Rhodeus suigensis
KF980890 Rhodeus fangi
FJ197689 Rhodeus notatus
AP010778 Rhodeus atremius
FJ197699 Rhodeus uyekii
MH664136 Rhodeus lighti
KF176560 Rhodeus shitaiensis
Rhodeus s.l. II
HQ600762 Rhodeus meridionalis
MW564278 Rhodeus colchicus
ON097861 Rhodeus amarus
MT878142 Rhodeus caspius
KM019404
KT247735
Rhodeus sericeus
KF425517 Rhodeus pseudosericeus
Rhodeus s.s.
PV469664 Acheilognathus striatus
Acheilognathus s.l.
MG913600 Rhodeus fangi
MG913599
MG913597
Rhodeus lighti
MN683735 Acheilognathus chankaensis
KM232987 Rhodeus lighti
AP012986 Acheilognathus chankaensis
MF122715 Rhodeus sinensis
MT281312 Rhodeus sp.
MT281311
MT281313
Rhodeus sp.
FJ197676 Rhodeus ocellatus
Rhodeus s.l. I
Ingroup
Acheilognathidae
KP112470
KP112469
KP112468
Tinca tinca
Tincidae
Rooting outgroup
0.04

Table 1. Genetic *p*-distances estimated with 612 bp fragment of the *cox 1* mtDNA gene. The sampling included sequences of *Acheilognathus* sensu lato and sensu stricto groups

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	**PV820667 *Acheilognathus chankaensis***		*0.0017*	*0.0017*	*0.0023*	*0.0074*	*0.0074*	*0.0074*	*0.0074*	*0.0075*	*0.0082*	*0.0107*	*0.0108*	*0.0108*	*0.0109*	*0.0111*	*0.0110*	*0.0112*	*0.0115*	*0.0112*
2	OL674410 *Acheilognathus chankaensis*	0.16		*0.0024*	*0.0017*	*0.0076*	*0.0076*	*0.0076*	*0.0076*	*0.0077*	*0.0084*	*0.0108*	*0.0109*	*0.0109*	*0.0110*	*0.0113*	*0.0112*	*0.0113*	*0.0116*	*0.0113*
3	MG913603 *Rhodeus sericeus*	0.16	0.33		*0.0029*	*0.0076*	*0.0076*	*0.0076*	*0.0076*	*0.0077*	*0.0084*	*0.0107*	*0.0109*	*0.0109*	*0.0110*	*0.0112*	*0.0112*	*0.0113*	*0.0116*	*0.0112*
4	HQ536248 *Acanthorhodeus gracilis*	0.33	0.16	0.49		*0.0076*	*0.0076*	*0.0076*	*0.0076*	*0.0078*	*0.0084*	*0.0109*	*0.0110*	*0.0110*	*0.0111*	*0.0114*	*0.0113*	*0.0114*	*0.0117*	*0.0113*
5	MF939361 *Acheilognathus chankaensis*	3.27	3.43	3.43	3.59		*0*	*0*	*0*	*0.0016*	*0.0052*	*0.0113*	*0.0114*	*0.0114*	*0.0118*	*0.0121*	*0.0116*	*0.0120*	*0.0122*	*0.0110*
6	MT571701 *Acheilognathus chankaensis*	3.27	3.43	3.43	3.59	0		*0*	*0*	*0.0016*	*0.0052*	*0.0113*	*0.0114*	*0.0114*	*0.0118*	*0.0121*	*0.0116*	*0.0120*	*0.0122*	*0.0110*
7	MZ334545 *Acheilognathus hypselonotus*	3.27	3.43	3.43	3.59	0	0		*0*	*0.0016*	*0.0052*	*0.0113*	*0.0114*	*0.0114*	*0.0118*	*0.0121*	*0.0116*	*0.0120*	*0.0122*	*0.0110*
8	MF939376 *Acheilognathus macropterus*	3.27	3.43	3.43	3.59	0	0	0		*0.0016*	*0.0052*	*0.0113*	*0.0114*	*0.0114*	*0.0118*	*0.0121*	*0.0116*	*0.0120*	*0.0122*	*0.0110*
9	MT571700 *Acheilognathus chankaensis*	3.43	3.59	3.59	3.76	0.16	0.16	0.16	0.16		*0.0053*	*0.0114*	*0.0115*	*0.0115*	*0.0118*	*0.0122*	*0.0117*	*0.0121*	*0.0123*	*0.0112*
10	MF122002 *Acheilognathus chankaensis*	3.92	4.08	4.08	4.25	1.63	1.63	1.63	1.63	1.80		*0.0115*	*0.0116*	*0.0116*	*0.0121*	*0.0121*	*0.0117*	*0.0122*	*0.0123*	*0.0114*

Table 1. (End.)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
11	MG913601 *Rhodeus sericeus*	7.52	7.68	7.68	7.84	8.99	8.99	8.99	8.99	9.15	8.66		*0.0016*	*0.0016*	*0.0124*	*0.0054*	*0.0040*	*0.0064*	*0.0050*	*0.0102*
12	KF695385 *Acheilognathus chankaensis*	7.68	7.84	7.84	8.01	9.15	9.15	9.15	9.15	9.31	8.82	0.16		*0*	*0.0126*	*0.0056*	*0.0037*	*0.0067*	*0.0052*	*0.0104*
13	MF805627 *Acheilognathus* sp. 2	7.68	7.84	7.84	8.01	9.15	9.15	9.15	9.15	9.31	8.82	0.16	0		*0.0126*	*0.0056*	*0.0037*	*0.0067*	*0.0052*	*0.0104*
14	LC712753 *Acheilognathus typus*	7.84	8.01	8.01	8.17	9.64	9.64	9.64	9.64	9.64	9.97	10.46	10.62	10.62		*0.0127*	*0.0127*	*0.0129*	*0.0127*	*0.0131*
15	OL674509 *Acheilognathus macropterus*	8.17	8.33	8.33	8.50	9.97	9.97	9.97	9.97	10.13	9.64	1.80	1.96	1.96	10.62		*0.0061*	*0.0066*	*0.0027*	*0.0106*
16	PP799048 *Acheilognathus taenianalis*	8.17	8.33	8.33	8.50	9.31	9.31	9.31	9.31	9.48	8.99	0.98	0.82	0.82	10.62	2.45		*0.0072*	*0.0058*	*0.0106*
17	MK380726 *Acheilognathus chankaensis*	8.66	8.82	8.82	8.99	10.46	10.46	10.46	10.46	10.62	10.13	2.61	2.78	2.78	11.44	2.78	3.27		*0.0068*	*0.0107*
18	MF805623 *Acheilognathus* sp. 1	8.66	8.82	8.82	8.99	10.13	10.13	10.13	10.13	10.29	9.80	1.63	1.80	1.80	10.46	0.49	2.29	2.94		*0.0107*
19	MT884464 *Acheilognathus tonkinensis*	8.66	8.82	8.82	8.99	8.66	8.66	8.66	8.66	8.82	9.31	7.19	7.35	7.35	11.60	7.35	7.68	7.52	7.52	

Original sequence of *Acheilognathus chankaensis* is in bold. Genetic distances below diagonal and standard error above diagonal in italics (p-value <0.05).

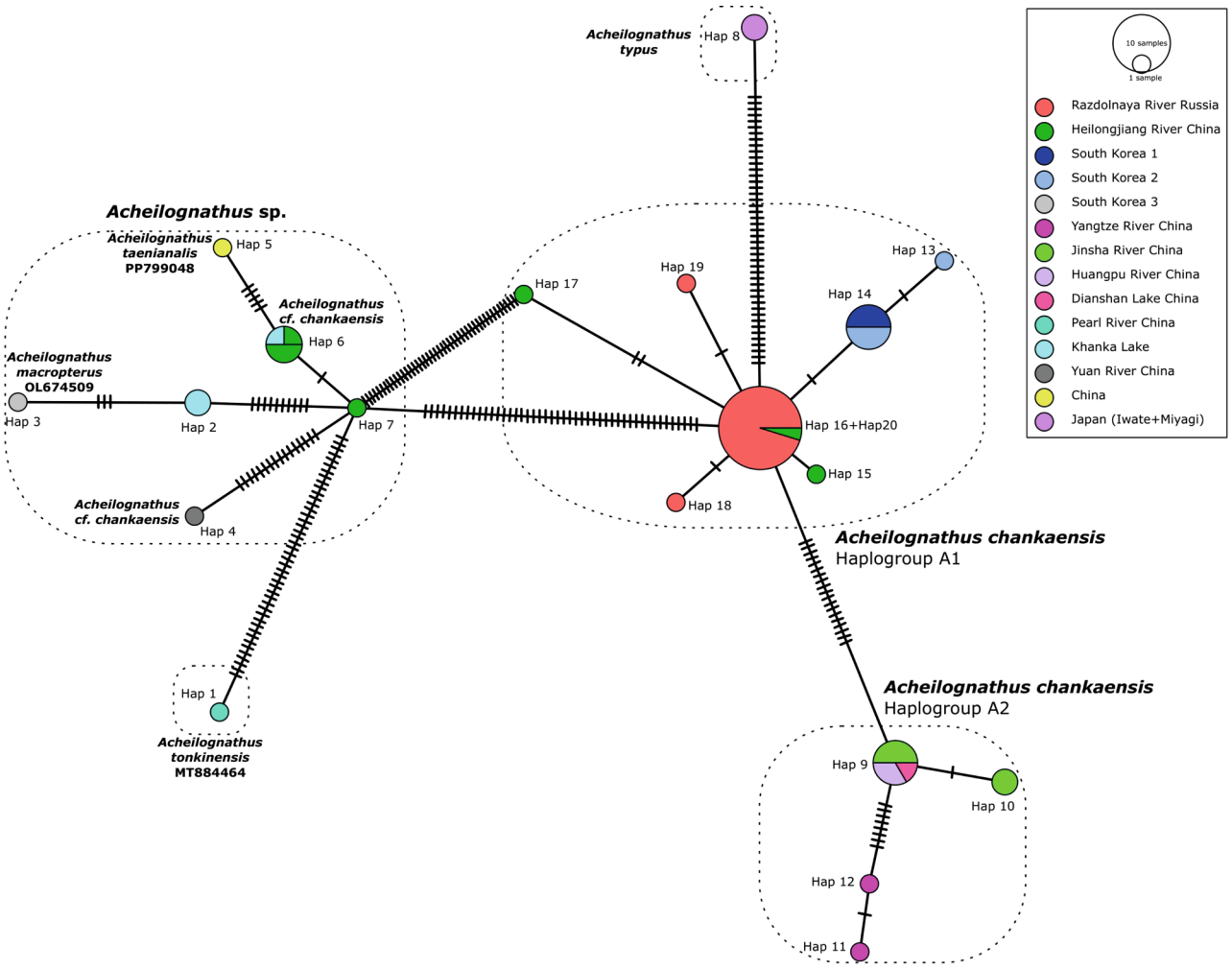

Fig. 3. Minimum spanning network of the Khanka spiny bitterling *Acheilognathus chankaensis* haplotypes based on *cox 1* mtDNA sequences. Here and in Fig. 4: circles represent haplotypes (Hap 1–Hap 19), with sizes proportional to sample counts (e.g., Hap 1 = 1 sample; Hap 2 = 2 samples).

Taxonomic Relationships and Polyphyly in the Genera Acheilognathus and Rhodeus

Our phylogenetic data confirm that the genera *Acheilognathus* and *Rhodeus*, in their current composition, are polyphyletic (Fig. 2). This is particularly evident for species such as *Acheilognathus rhombeus*, *Rhodeus lighti*, *R. fangi*, and *R. sericeus*, whose sequences are scattered across five different clades. Previous phylogenetic studies over the last decade, often based on limited data or focused on specific taxa, have largely supported a monophyletic concept for these genera (Uemura et al., 2018; Turanov et al., 2019), while others using mitogenomes (Tan et al., 2019; Xu et al., 2020) or more expanded phylogenies (Li et al., 2017; Esmaeili et al., 2020) have revealed greater complexity.

Species of the genus *Rhodeus* form four polyphyletic groups, intermixing with representatives of *Acheilognathus* and *Sinorhodeus*. Specifically, *R. cyanorostris* forms a common clade with *Sinorhodeus microlepis*, supported by shared morphological traits, such as

Table 2. Sites with unique nucleotide substitutions in three isolates of *Acheilognathus chankaensis* from floodplain lake of the Razdolnaya River

Site number	Isolate	GenBank Accn. no.	Haplotype	Mutation	Nucleotide in other specimens
121	ACH2	PV820668	H18	Adenine	Cytosine
265	ACH26	PV820688	H19	Adenine	Guanine
717	ACH16	PV820682	H20	Cytosine	Thymine

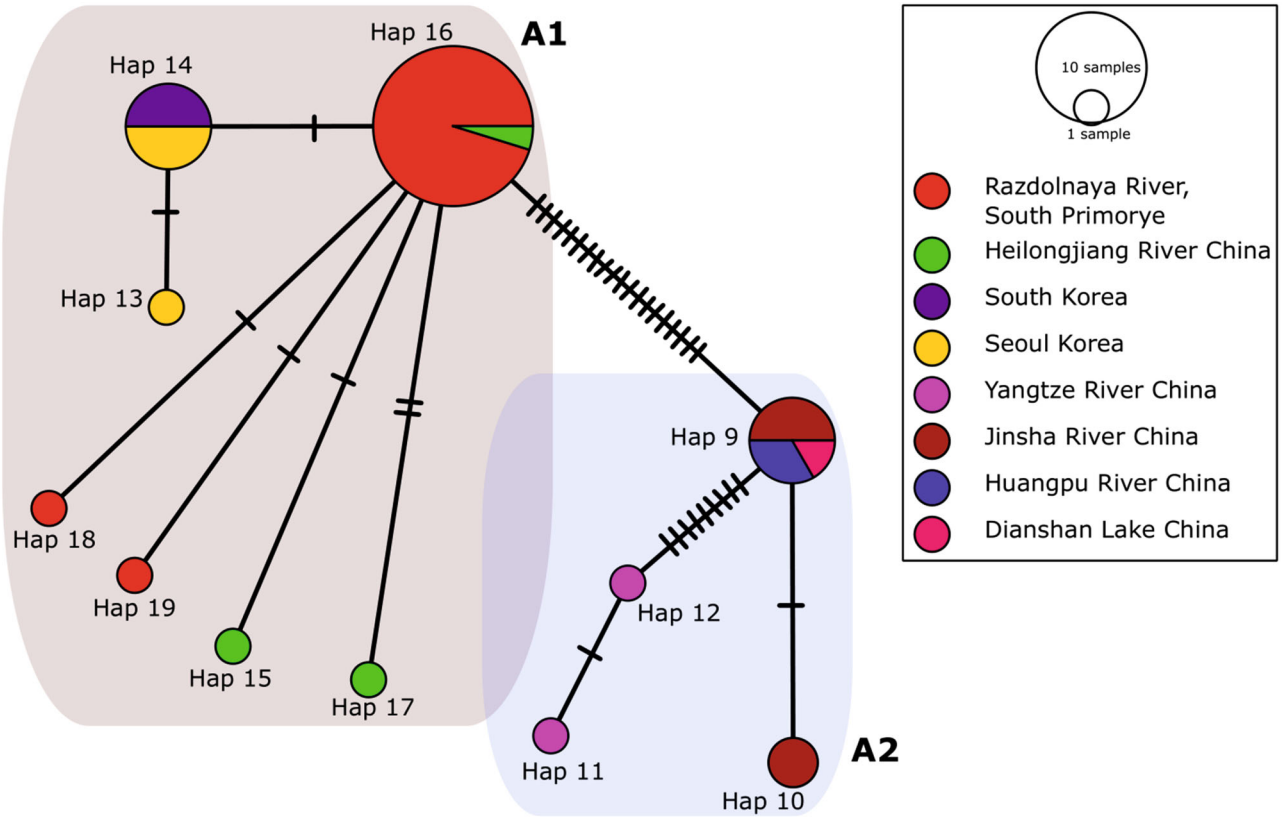

Fig. 4. Minimum spanning network of the Khanka spiny bitterling *Acheilognathus chankaensis* haplotypes based on *cox 1* mtDNA sequences.

the absence of two rows of light spots on the dorsal fin rays, a feature noted for both species by Li et al. (2017, 2020).

We propose dividing the genus *Rhodeus* into two main groups: (1) the core *Rhodeus* s.s., including the type species *R. amarus* and five other species; the *Rhodeus* s.l. group, containing seven species, three of which (*R. lighti*, *R. suigensis*, *R. uyekii*) were historically classified under the synonymized genus *Pseudoperilampus* (originally established by Bleeker (1863)).

Genetic distance analysis reveals that species of the *Rhodeus* s.l. group (*R. ocellatus*, *R. sinensis*, *Rhodeus* sp.) are genetically distinct (5.4–5.6%) from *R. lighti*. The suggestion by Akai and Arai (1998) that *R. sinensis* is a senior synonym of *R. lighti* and *R. uyekii* is not supported by our phylogenetic data. *Rhodeus lighti* clusters within a separate *Rhodeus* s.l. I and II groups, while *R. uyekii* is found in the distant *Rhodeus* s.l. II group (Supplement 4). Given this polyphyly and their historical classification under *Pseudoperilampus*, we recommend reinstating this genus as a broader, s.l. concept (*Pseudoperilampus* s.l.) with two subgroups I and II.

Similar issues were identified within the genus *Acheilognathus*. *Acheilognathus rhombeus* is polyphyletic, appearing in clades with *Tanakia* and *Acheilognathus* (as a sister to *A. gracilis*). Importantly, *Rhodeus albomarginatus* consistently clusters with *Acheilognathus* barbatus in all our analyses (BI, MP, ML) (Fig. 2), providing robust molecular evidence for its taxonomic transfer to the genus *Acheilognathus*. This contradicts the proposal by Li and Arai (2014) to retain it in *Rhodeus* based solely on morphology. The sole available mitogenome (MW896838) for *R. albomarginatus* further complicates its classification, as it remains morphologically unverified.

Position and Genetic Diversity of Acheilognathus chankaensis

The taxonomic history of *A. chankaensis* is complex. Berg (1907) first applied the combination to *Devario chankaensis*, while Berg (1949) later transferred it to *Acanthorhodeus*. Our analysis clarifies the ongoing confusion. Recent phylogenetic studies (Li et al., 2017; Esmaeili et al., 2020) notably excluded the true *A. chankaensis* s.s. Furthermore, published mitogenomes attributed to this species (Lv et al., 2014; Tan et al., 2019; Xu et al., 2020; Kim and Cho, 2021) actually represent: *A. chankaensis* s.l. (KF695385; MK380726); *A. gracilis* (NC_028416); and *Pseudoperilampus* s.l. I (AP012986; MN683735). The barcoding

data for *A. chankaensis* from Primorsky region was first provided by Maximov et al. (2024).

The true *A. chankaensis* s.s. in our study is represented by two major populations and includes sequences misidentified as *R. sericeus*, *A. gracilis*, *A. macropterus*, and *A. hypselonotus* (Fig. 2, Table 1) (Supplements 2, 3).

Regarding genetic divergence, our *p*-distance values for *A. chankaensis* s.s. haplogroups (0–4.25%) match the intraspecific variability range (0–3.7%) reported for cypriniform fishes by Shen et al. (2016). Notably, distances between *A. chankaensis* s.s. and s.l. (7.52–10.62%) fall within Shen et al. (2016) intrageneric range (*p*-distances 2.42–15.78%, mean 6.60%), while distances within the *A. chankaensis* s.l. group (0–3.27%) remain at intraspecific levels. The inclusion of *A. taenianalis* (PP799048) within this group without morphological verification complicates the taxonomic interpretation. These results indicate that *A. chankaensis* s.l. represents a complex requiring revision and is likely a distinct, new species. However, we refrain from synonymizing *A. taenianalis* and *A. macropterus* due to the latter's unstable phylogenetic position (Fig. 2).

The Issue of a Cryptic Genus

Within the nominal genus *Acheilognathus*, *A. striatus* occupies a phylogenetically ambiguous position, variably forming a basal lineage within the core clade (Fig. 2), a sister relationship to *Pseudoperilampus* s.l. I (Supplement 2), or a trichotomy with *Rhodeus* s.s. and *Pseudoperilampus* s.l. II (Supplement 3).

Genetic *p*-distances further underscore the distinctiveness of *A. striatus*. Its divergence from major groups is substantial: 13.56–15.52% from *Rhodeus* s.s., 13.89–15.85% from *Pseudoperilampus* s.l. I, and 15.52–16.99% from *Acheilognathus* s.s. (Supplement 5). Notably, the maximum distance between *A. striatus* and *Acheilognathus* s.s. (16.9%) exceeds the most pronounced intergeneric distances observed among other taxa (Supplement 6).

This pronounced molecular divergence, coupled with diagnostic morphological traits described by Yang et al. (2010) (barbel-to-eye diameter ratio, body stripe extension, and the presence of black marginal bands on the dorsal and anal fins in males), warrants the establishment of a new genus, *Acheilognathoides* n.g., with *A. striatus* designated as its type and sole species.

Consequently, the family Acheilognathidae now encompasses nine generic-level taxa: six well-supported monophyletic genera (*Acheilognathus* s.s., *Rhodeus* s.s., *Pseudorhodeus*, *Tanakia*, *Paratanakia*, *Sinorhodeus*), the newly proposed *Acheilognathoides*, and two taxa (*Pseudoperilampus* s.l. I and II) pending further systematic revision.

Haplotype Diversity and Biogeography of Acheilognathus chankaensis

Our study provides the first haplotype diversity analysis for bitterlings using the *cox 1* gene and the Minimum spanning network method (Figs. 3, 4). Unlike previous studies (Saitoh et al., 2016, 2017; Nagata et al., 2018; Miyake et al., 2021; Ito et al., 2024), we included outgroup taxa (*A. typus*, *A. tonkinensis*) to calibrate interspecific divergence.

The haplotype network revealed a deep genetic split between the *A. chankaensis* s.s. and s.l. groups, separated by 46 mutational steps—a distance comparable to that separating other distinct species (45 steps to *A. typus*, 44 to *A. tonkinensis*). This pattern is supported by significant *p*-distances (Table 1) and polyphyly in the phylogeny (Fig. 2), collectively indicating that *A. chankaensis* s.l. represents a separate, undescribed species (*Acheilognathus* sp. 1), distinct from the true *A. chankaensis* s.s.

The analysis revealed clear biogeographical structuring of two *A. chankaensis* s.s. haplogroups (Supplement 1). Haplogroup A1 is associated with the Paleo-Amur and Paleo-Razdolnaya basins (Russian Far East, Northeast China, Korean Peninsula), reflecting historical connections among these river systems during the post-glacial period (Lindberg, 1955, 1957, 1972). Haplogroup A2 is endemic to Southern China (Yangtze River basin), indicating an older vicariance event linked to the Paleo-Huanghe drainage. The s.l. group (*Acheilognathus* sp. 1) shows a complex distribution across China, Korea, and Russia and encompasses records misidentified as *R. sericeus*, *A. taenianalis*, and *A. macropterus*. The modern co-occurrence of *A. chankaensis* s.s. and *Acheilognathus* sp. 1 in South Korea and the Amur River basin confirms the existence of a former unified Amur–Razdolnaya–Huanghe paleo-complex (Goryainov et al., 2014; Burik, 2018), a pattern also supported by data from other fish, such as *Gobiobotia naktongensis* (Gobionidae) (Kim et al., 2022), which links Korean Geum River populations to the Paleo-Huanghe basin.

Based on our findings, we recommend.

(1) Re-evaluating records of *Rhodeus sericeus* from the Artyomovka, Amur, and Razdolnaya river basins (Vainutis and Voronova, 2021; Vainutis et al., 2024a, 2024b; Sayenko and Palatov, 2023; Sayenko, 2025), as these likely represent misidentified *A. chankaensis* or *Acheilognathus* sp. 1.

(2) Prioritizing Khanka Lake as a conservation hotspot for cryptic bitterling diversity.

(3) Implementing targeted monitoring of populations in the Razdolnaya River, which harbors the unique A1 haplogroup of *A. chankaensis* s.s.

CONCLUSIONS

Our phylogenetic analysis of Acheilognathidae reveals substantial taxonomic complexity within the

Acheilognathus chankaensis species complex and confirms the polyphyly of the genera *Acheilognathus* and *Rhodeus*. The key findings are: (1) identification of a cryptic species: The recognition of two distinct genetic lineages—*A. chankaensis* s. s. and *Acheilognathus* sp. 1—supports the existence of a previously overlooked cryptic species; (2) generic-level revision: The resolution of nine generic-level groups within Acheilognathidae, including the newly proposed *Acheilognathoides* for *A. striatus* and two taxa (*Pseudoperilampus* s.l. I and II) requiring further systematic study; (3) persistent taxonomic issues: The identification of persistent polyphyly in several species (e.g., *A. rhombeus*, *R. sericeus*, *A. macropterus*) highlights widespread misidentification.

These findings underscore the critical need for integrative taxonomic approaches combining molecular and morphological data to resolve the systematics of East Asian bitterlings.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S003294522560154X.

AUTHOR CONTRIBUTION

K.S. Vainutis and A.N. Voronova contributed to conceptualization, molecular data, phylogenetic and haplotype analyses, writing and editing. R.A. Maximov contributed to sampling, molecular data. V.V. Bogatov contributed to project administration, sampling, writing and editing.

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DATA AVAILABILITY

All data supporting the findings of this study are available within the paper and its Supplementary Information. Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information with the accession codes PV820667–PV820689.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The research program using mussels *Nodularia douglasiae* and Khanka spiny bitterling *Acheilognathus chankaensis* from floodplain lake of the Razdolnaya River was approved by the Committee for the Control of the Care and Use of Experimental Animals of the Far Eastern State Technical Fisheries University. The committee's establishment was formalized by order no. 491 on June 20, 2024.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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