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Original Research Articles

Molecular species delimitation in the genus *Acrossocheilus* based on mitochondrial sequences

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Accurate species delimitation is essential for phylogeny, phylogeography, ecology, conservation, and biogeography. In China, the cyprinid genus *Acrossocheilus* is of economic and ecological importance, but species boundaries remain controversial because several nominal species exhibit overlapping diagnostic characters and uncertain phylogenetic affinities. Here, we analyzed 31 publicly available complete mitochondrial genomes representing 22 cyprinid taxa, including 16 nominal *Acrossocheilus* taxa, three *Onychostoma* taxa, and three *Spinibarbus* outgroups. We extracted and concatenated the 13 mitochondrial protein-coding genes (ATP6, ATP8, COX1, COX2, COX3, CYTB, ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) and reconstructed phylogenetic relationships using Bayesian inference and maximum-likelihood methods. Species boundaries were evaluated using four complementary approaches: Generalized Mixed Yule Coalescent (GMYC), Bayesian Poisson Tree Processes (bPTP), Automatic Barcode Gap Discovery (ABGD), and Bayesian Phylogenetics and Phylogeography (BPP). The combined analyses supported 15 molecular operational taxonomic units (MOTUs), with bPTP and ABGD showing high congruence when ABGD prior intraspecific divergence was set between 0.001 and 0.0144. The results highlighted several taxonomically important patterns, including possible conspecificity among *A. longipinnis*, *A. iridescens*, and *A. barbodon*; divergent placements of two A. paradoxus accessions; and unresolved generic boundaries between *Acrossocheilus* and *Onychostoma*. Because the present inference is based only on mitochondrial loci and uneven taxon sampling, these MOTUs should be interpreted as testable molecular hypotheses for future integrative taxonomy using nuclear markers, broader sampling, and morphology-based validation.

INTRODUCTION

Cyprinidae is divided into 12 subfamilies and includes 379 genera. The cyprinid genus *Acrossocheilus* Oshima, 1919 belongs to the Barbinae, which includes at least 27 genera. The distribution of *Acrossocheilus* is restricted mainly to South China and the northern part of Vietnam and Laos, and the genus is especially diverse in China (with 21 species).[1] *Acrossocheilus* species occur in tropical, subtropical, and temperate regions,[2] and they are commonly found in mountain streams and rivers, including freshwater habitats in the middle and lower reaches of drainage systems in Fujian, Anhui, Zhejiang, and other central and southern Chinese provinces.[3]

Species of *Acrossocheilus* are generally identified by morphological characters. To date, 26 species have been recognized in *Acrossocheilus*[1,4]: *A. iridescens*, *A. microstoma*, *A. longipinnis*, *A. jishouensis*, *A. yalyensis*, *A. aluoiensis*, *A. baolacensis*, *A. lamus*, *A. macrophthalmus*, *A. malacopterus*, *A. monticola*, *A. multistriatus*, *A. parallens*, *A. rendahli*, *A. spinifer*, *A. wenchowensis*, *A. wuyiensis*, *A. xamensis*, *A. yunnanensis*, *A. paradoxus*, *A. hemispinus*, *A. ikedai*, *A. fasciatus*, *A. clivosius*, *A. beijiangensis*, and *A. kreyenbergii*. The genus can be tentatively divided into two species groups: a striped group, characterized by a plain or inconspicuous dark stripe along the lateral line of the flank, and a barred group, characterized by five to eight vertical black bars along the flank.[4] However, ontogenetic change and sexual dimorphism can affect body coloration, lateral stripes or bars, dorsal-fin serrations, mouth and lip morphology, and meristic characters; therefore, the systematics of the two groups remains unresolved. Originally, the barred group

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was referred to as *Acrossocheilus sensu stricto*, whereas the striped group was affiliated with *Poropuntius*.[5]

In China, the striped group includes *A. ikedai*, *A. xamensis, A. cf. xamensis, A. yunnanensis*, and *A. malacopterus* (a species formerly misidentified as *A. elongatus*).[2] In contrast, the barred group includes *A. beijiangensis*, *A. clivosius*, *A. fasciatus*; *A. hemispinus*, *A. iridescens*, *A. jishouensis*; *A. kreyenbergii*, *A. lamus*, *A. longipinnis*, *A. microstoma*, *A. monticola*, *A. paradoxus*, *A. parallens*, *A. spinifer*, *A. wenchowensis*, and *A. wuyiensis* and it is mostly distributed in South China. The exception is *A. lamus*, which is endemic to Vietnam.[6] Identification based only on morphology can be difficult, especially when key diagnostic traits such as coloration, bars, stripes, and serrations on the unbranched dorsal-fin ray change during the transition from juvenile to adult stages. Consequently, the distribution of narrowly endemic species may be underestimated, and some GenBank accessions may reflect historical identifications that require voucher-based confirmation.

The taxonomic history of *Acrossocheilus* is marked by many contradictions, disagreements, and revisions. For example, *A. iridescens* was formerly classified as *Cyclocheilichthys* iridescens, *Barbus* barbodon, and *B. paradoxus* quinquefasciatus. *Acrossocheilus* iridescens subsp. yuanjiangensis was later described as *A. microstoma*, and *A. iridescens subsp. zhujiangensis* was later identified as *A. longipinnis*. *Acrossocheilus* stenotaeniatus was once considered synonymous with *A. iridescens* but has been treated as a junior synonym of *A. longipinnis*.[7] In addition, *A. kreyenbergii* was formerly described as *Gymnostomus* kreyenbergii and as *B. hemispinus subsp. cincta*; in China, *A. hemispinus* has been recognized as distinct from *A. cinctus*, with the latter treated as a junior synonym of *A. kreyenbergii*.[8]

Morphological studies have also attempted to clarify relationships among *Acrossocheilus* species and closely related genera. Based on morphological diagnoses, *A. elongatus* was assigned to *Onychostoma*.[9] *Zacco* platypus was treated as a synonym of *A. parallens* based on ontogenetic variation in body characteristics.[10] Overall, uncertainty in *Acrossocheilus* taxonomy can be attributed partly to variation in morphological characters, limited voucher comparison, and the use of historically unstable names. Without definitive integrative diagnoses, synonyms, homonyms, and misidentifications may persist.

Molecular analyses may provide new insights into the systematics of *Acrossocheilus* because the taxonomic status of several nominal species is uncertain based on morphology alone. To resolve the taxonomic status of *A. formosanus*, *A. labiatus*, and *A. fasciatus* in Taiwan, horizontal starch-gel electrophoresis was used, confirming that the three nominal taxa were identical and should be reclassified as *A. paradoxus*.[11] A prior study sequenced the mitochondrial ND4 gene from seven Chinese barred *Acrossocheilus* species to test their validity.[12] That study supported a lineage comprising *A. fasciatus*, *A. hemispinus*, *A. parallens*, *A. beijiangensis*, *A. spinifer*, and *A. hemispinus subsp. cinctus* and proposed that *A. hemispinus* and *A. hemispinus subsp. cinctus* was a separate species.[12] The mitochondrial control region was also used to examine relationships within the barred group; specimens previously identified as *A. fasciatus* and *A. wuyiensis* from Fujian Province were inferred to represent *A. wuyiensis*.[6]

Despite these efforts, most molecular studies have focused primarily on phylogenetic relationships, and explicit species-delimitation analyses within *Acrossocheilus* remain limited.[13] In this study, we used published mitochondrial genomic data to address three specific objectives: (1) reconstruct the mitochondrial phylogeny of sampled *Acrossocheilus* and related *Onychostoma* taxa using the 13 mitochondrial protein-coding genes; (2) test whether the species complexes involving *A. longipinnis-A. iridescens-A. barbodon*, *A. hemispinus-A. parallens*, *A. paradoxus*, and *A. beijiangensis-A. stenotaeniatus-A. spinifer* are congruent with current morphology-based taxonomy; and (3) evaluate whether mitochondrial evidence supports reciprocal monophyly of *Acrossocheilus* and *Onychostoma*. Because only mitochondrial data are available for all included samples, we treat the resulting MOTUs as testable hypotheses for future integrative taxonomy rather than as final species-level decisions.

MATERIALS AND METHODS

DATA COLLECTION

We downloaded 31 complete mitochondrial genomes representing 22 cyprinid species from the National Center for Biotechnology Information (NCBI) (Table 1). The dataset comprised 25 *Acrossocheilus* mitogenomes assigned to 16 sampled *Acrossocheilus* names in GenBank, three *Onychostoma* taxa, and three *Spinibarbus* outgroup taxa. The *Onychostoma* taxa were *O. barbatulum* (Pellegrin, 1908), *O. meridionale* (Kottelat, 1998), and *O. gerlachi* (Peters, 1881). *Spinibarbus* denticulatus (Oshima, 1926), *S. hollandi* (Oshima, 1919), and *S. sinensis* (Bleeker, 1871) were used as outgroups.

Because 26 species are recognized in *Acrossocheilus* in the Introduction, the sampling gap was made explicit. The recognized species not represented by complete mitogenomes in the present dataset were *A. microstoma*; *A. yalyensis*; *A. aluoiensis*; *A. baolacensis*; *A. lamus*; *A. macrophthalmus*; *A. malacopterus*; *A. multistriatus*; *A. rendahli*; *A. xamensis*; *A. ikedai*; and *A. clivosius*. Their absence may reduce the present dataset's ability to detect additional lineages, test the full monophyly of species groups, and evaluate whether unsampled taxa would alter MOTU membership. Accordingly, all delimitation results are interpreted as hypotheses for the sampled taxa only.

For each complete mitochondrial genome, the 13 protein-coding genes (ATP6, ATP8, COX1, COX2, COX3, CYTB, ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) were extracted according to GenBank annotations. Each gene was checked under the vertebrate mitochondrial genetic code to confirm reading-frame consistency and to screen for obvious annotation errors. Genes were aligned separately in MEGA v6.0[14] using the vertebrate mitochondrial genetic code and default settings unless otherwise specified. PhyloSuite v1.2.2[15] was used for sequence management, concatena-

Table 1. Species, sequence length and GenBank accession number for samples in this study

Species	Sequence length	Accession number
Acrossocheilus barbodon	16596bp	NC_022184.1
Acrossocheilus beijiangensis 1	16600bp	NC_028206.1
Acrossocheilus beijiangensis 2	16596bp	KY131976.1
Acrossocheilus fasciatus	16589bp	NC_023378.1
Acrossocheilus hemispinus	16590bp	NC_022183.1
Acrossocheilus iridescens	16596bp	NC_031551.1
Acrossocheilus jishouensis	16587bp	NC_034917.1
Acrossocheilus kreyenbergii 1	16849bp	NC_024844.1
Acrossocheilus kreyenbergii 2	16596bp	KY094969.1
Acrossocheilus longipinnis	16593bp	NC_047455.1
Acrossocheilus monticola 1	16605bp	KT367805.1
Acrossocheilus monticola 2	16599bp	NC_022145.1
Acrossocheilus paradoxus 1	16595bp	AP009303.1
Acrossocheilus paradoxus 2	16586bp	MG878098.1
Acrossocheilus parallens 1	16590bp	AP011251.1
Acrossocheilus parallens 2	16591bp	KT715479.1
Acrossocheilus parallens 3	16592bp	NC_026973.1
Acrossocheilus parallens 4	16588bp	KP257293.1
Acrossocheilus spinifer	16591bp	NC_034918.1
Acrossocheilus stenotaeniatus	16594bp	NC_024934.1
Acrossocheilus wenchowensis 1	16591bp	NC_020145.1
Acrossocheilus wenchowensis 2	16591bp	KC495074.1
Acrossocheilus wuyiensis	16594bp	NC_034919.1
Acrossocheilus yunnanensis 1	16588bp	NC_028527.1
Acrossocheilus yunnanensis 2	16590bp	MN395748.1
Onychostoma meridionale	16595bp	NC_031603.1
Onychostoma barbatulum	16612bp	AP009311.1
Onychostoma gerlachi	16601bp	NC_026549.1
Spinibarbus hollandi	16521bp	NC_026129.1
Spinibarbus denticulatus	16549bp	NC_021616.1
Spinibarbus sinensis	16591bp	NC_022465.1

tion of the 13 aligned protein-coding genes, format conversion, and preparation of input files for partitioning and phylogenetic analyses.

MOLECULAR PHYLOGENETICS

We used Bayesian inference (BI) and maximum likelihood (ML) methods to estimate phylogenetic relationships using the concatenated dataset. The optimal partitioning strategy for the dataset was inferred using PartitionFinder v2[16] in PhyloSuite, under a greedy search algorithm with linked branch lengths based on the corrected Akaike Information Criterion (AICc). The general time-reversible model with gamma-distributed rate variation and a proportion of invariable sites (GTR+G+I) was selected as the most appropriate model for subsequent analyses. BI analyses were performed using MrBayes v3.2.6.[17] 20 million Markov Chain Monte Carlo (MCMC) generations were used, with tree sampling every 1,000 generations to calculate posterior probabilities (PPs). We used four chains and two independent runs, and the first 25% of generations were discarded as burn-in. Convergence was assessed by examining the average standard deviation of split frequencies and the effective sample size (ESS) for all parameters in Tracer v1.7.[18] An ESS > 200 was used as a good indicator of convergence. ML analyses were conducted with the best partitioning scheme selected in PartitionFinder and run in IQ-TREE v1.6.8[19] independently ten times with 1,000 ultrafast bootstrap (BS) replicates to obtain branch support values. A BS value > 70 was considered to indicate strong support for a given clade in the ML analyses.

MOLECULAR SPECIES DELIMITATION

First, we used the Generalized Mixed Yule Coalescent (GMYC) model[20] through the online server (https://species.h-its.org/gmyc/) to infer molecular clades based on the phylogeny. The GMYC analysis was conducted

with the multiple-threshold version. This is a likelihood-based method for delimiting species by fitting within- and between-species branching models to reconstructed gene trees using an ultrametric tree. In this analysis, an ultrametric tree was obtained in BEAST v1.8.2.[21] For the BEAST analysis, the Yule process of speciation and the uncorrelated log-normal relaxed clock model were used as tree priors. MCMC chains were run for 50 million generations with tree sampling every 5,000 generations. The first 10% of generations were discarded as burn-in.

Second, we used the Bayesian Poisson Tree Processes (bPTP) model[22] through the online server (https://species.h-its.org/ptp/). This is an updated version of the original ML Poisson Tree Processes (PTP) model with Bayesian PPs, providing more accurate results. Compared with the GMYC model, the bPTP model is a more robust and simpler method. We used the ML tree as input and ran bPTP analyses for 500,000 MCMC generations, setting the thinning value at 100 and burn-in at 0.1.

Third, we used the Automatic Barcode Gap Discovery (ABGD) method[23] through its online server (https://bioinfo.mnhn.fr/abi/public/abgd/). This is a distance-based method that relies on the gap in the distribution of interspecific and intraspecific genetic distances. The analyses were performed with the following settings: a prior limit to intraspecific diversity (P) from 0.001 to 0.1, a relative gap width (X) of 0.9, 20 steps, and the Jukes-Cantor (JC69) model.

Fourth, we used Bayesian Phylogenetics and Phylogeography (BPP) v4.3.8[24,25] to confirm the delimitation results from the above methods. This is a validation method that applies reversible-jump Markov Chain Monte Carlo (rjMCMC) to estimate the PPs for different hypotheses of species delimitation. The species recovered from the GMYC, bPTP, and ABGD analyses were used as putative species, yielding a total of 15 taxa to test. We used default settings with speciesdelimitation = 1, speciestree = 1, algorithm = 0, finetune = 2, usedata = 1, and cleandata = 0. The reversible-jump MCMC analyses were run for 100,000 generations and sampled every two generations, with 8,000 samples discarded as burn-in.

RESULTS

ALIGNMENT DATASET

The final dataset consisted of 31 complete mitochondrial genomes listed in Table 1. The sequence lengths reported in Table 1 refer to complete mitochondrial genome lengths, not to the concatenated 13-gene alignment. The final alignment length, number of variable sites, number of parsimony-informative sites, and mean pairwise sequence divergence should be calculated directly from the final concatenated 13-PCG alignment before submission. These statistics cannot be derived reliably from the accession table or figure images alone; therefore, no numerical values are fabricated here.

PHYLOGENETIC RELATIONSHIPS

Phylogenetic analyses of the mitochondrial dataset using ML and BI approaches produced identical topologies with high BS support and PPs. *Acrossocheilus* was divided into two major clades, defined here as C1 and C2, that were supported with strong PPs and BS scores (100%) (Fig. 1).

The C1 clade was split into several well-supported subclades. *A. longipinnis* and *A. iridescens* grouped together and then clustered with *A. barbodon* into one clade with 1.00 PP and 100% BS support. *A. hemispinus* nested within a clade of *A. parallens*, and together these taxa were sister to a clade including one individual of *A. paradoxus* and *A. jishouensis* with 100% BS support and 0.995 PP. *A. wenchowensis* formed a paraphyletic group with the inclusion of *A. fasciatus*, and together these taxa were sister to a monophyletic group of *A. kreyenbergii* with 1.00 PP and 100% BS support. A monophyletic group of *A. beijiangensis* formed a clade with *A. stenotaeniatus* and *A. spinifer*, which was sister to a clade comprising *A. wuyiensis* and another individual of *A. paradoxus* with 100% BS support and 1.00 PP.

The C2 clade included only two species, each comprising two individuals: *A. monticola* and *A. yunnanensis*. Both species were monophyletic with 100% BS support and 1.00 PP. Notably, the genus *Onychostoma* formed a well-supported clade (100% BS and 1.00 PP), defined here as C3, which was nested between the two *Acrossocheilus* clades (C1 and C2) (Fig. 1). The clades C1 and C3 were more closely related and together were sister to C2.

SPECIES DELIMITATION

Across sampled *Acrossocheilus* and *Onychostoma* lineages, the number of inferred molecular operational taxonomic units (MOTUs) varied from six to 15 depending on the method and parameter settings (Fig. 1). The multiple-threshold GMYC analysis estimated 13 putative species, with a confidence interval of four to 19 and a branching-pattern transition at -0.8592776. The bPTP analysis estimated 15 putative species; the highest Bayesian-supported solution was congruent with the ML solution, with an acceptance rate of 0.29049, 50,103 merge events, and 49,897 split events.

The ABGD results were sensitive to the prior limit of intraspecific divergence (P). Fifteen putative species were recovered at P = 0.0010, 0.0013, 0.0016, 0.0021, 0.0026, 0.0034, 0.0043, 0.0055, 0.0070, 0.0089, 0.0113, and 0.0144, matching the bPTP result. Six putative species were recovered at P = 0.0183, 0.0234, and 0.0298 (Fig. 2). Thus, the six-species ABGD result is reported together with its P-value interval rather than as an isolated conclusion.

The BPP analysis of the 15 candidate MOTUs showed that 11 MOTUs had posterior probabilities greater than 0.95. The four remaining MOTUs, corresponding to MOTUs 4, 5, 7, and 8 in Fig. 3, received lower support, ranging from approximately 0.66 to 0.88. These moderately supported units should therefore be regarded as provisional mitochondrial hypotheses that require validation with nuclear markers and morphology.

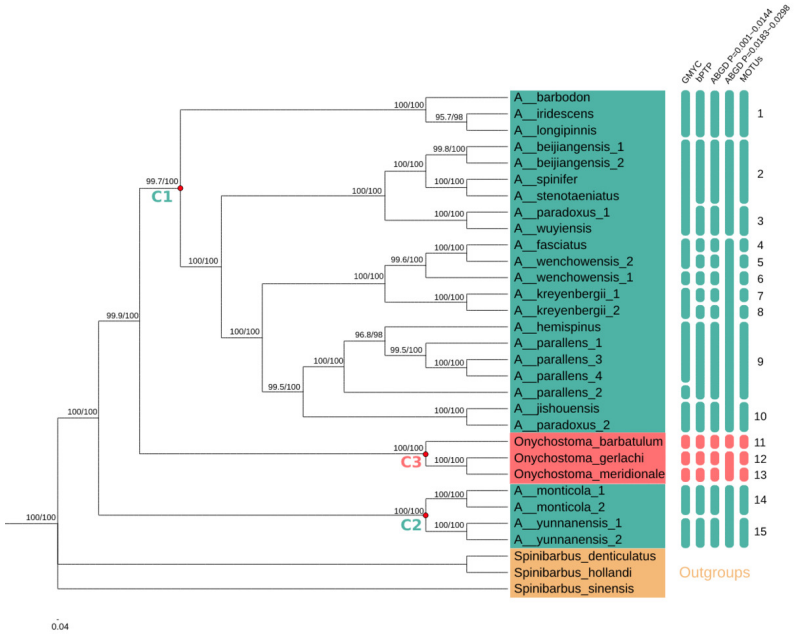

Figure 1. Molecular phylogenetic relationships and species boundaries in Acrossocheilus.

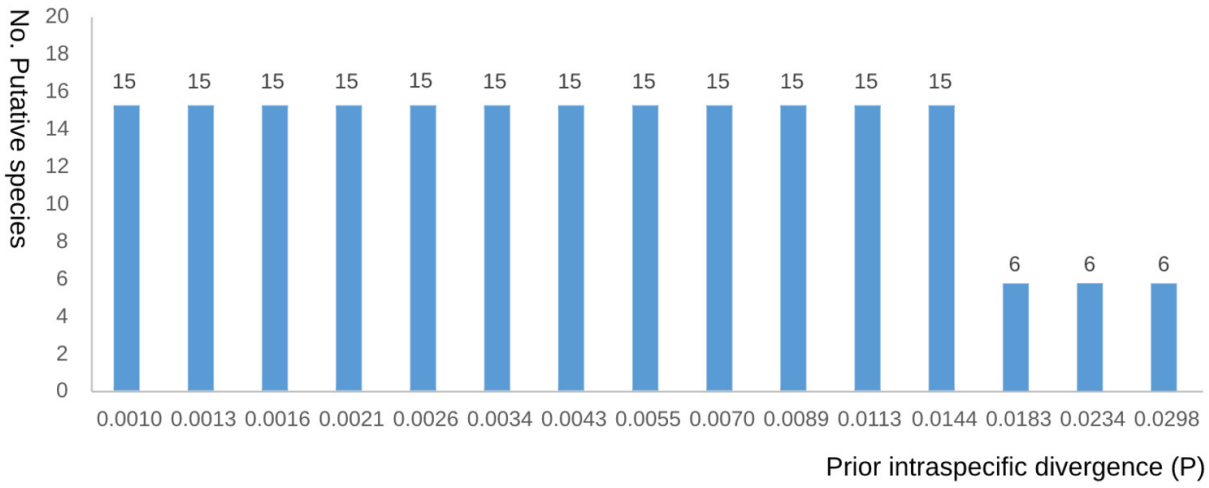

Figure 2. The number of putative species estimated from Automatic Barcode Gap Discovery depending on prior intraspecific divergence.

DISCUSSION

Overall, the four molecular species-delimitation methods yielded broadly congruent results but were not identical. bPTP and ABGD were highly consistent when ABGD P values ranged from 0.0010 to 0.0144, both of which supported 15 candidate MOTUs. GMYC recovered 13 putative species and therefore provided partial support for the same general pattern, although GMYC can overestimate species numbers when single-locus branching patterns are uneven.[26] ABGD yielded only six putative species under higher P values (0.0183-0.0298), emphasizing that distance-based delimitation is sensitive to prior assumptions about intraspecific divergence. We therefore interpret the 15-MOTU scheme not as a final taxonomy, but as the most informative working hypothesis for the present mitochondrial dataset.

The proposed MOTUs highlight several groups requiring taxonomic reassessment. *A. longipinnis, A. iridescens*, and *A. barbodon* formed a strongly supported mitochondrial clade and were assigned to the same MOTU under the most

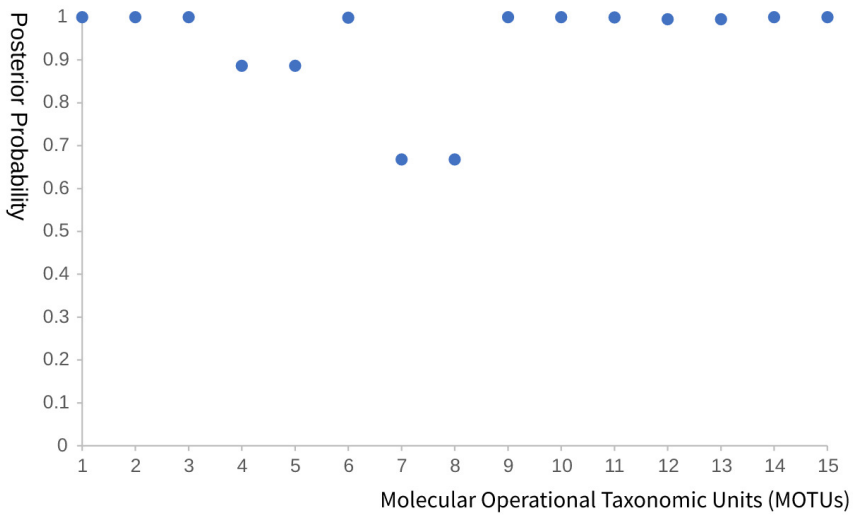

Figure 3. Posterior probabilities from Bayesian Phylogenetics and Phylogeography for 15 putative species.

congruent delimitation settings. Previous morphology-based work treated *A. longipinnis* as a senior synonym of *A. stenotaeniatus*.[7] Rather than concluding that *A. longipinnis* is invalid, the present results indicate that *A. longipinnis* may be closely related to, recently diverged from, introgressed with, or misidentified relative to *A. iridescens* and *A. barbodon*. Additional nuclear loci, topotypic material, and direct examination of diagnostic characters are required before any synonymy is formally proposed. Earlier catalogue-level synonymy treatments should therefore be regarded as taxonomic hypotheses for integrative testing rather than definitive evidence.[27]

The *A. beijiangensis-A. stenotaeniatus-A. spinifer* assemblage also deserves attention. In Figure 1, *A. stenotaeniatus* and *A. spinifer* are placed within a strongly supported subclade associated with *A. beijiangensis*, as indicated by high support values on the tree. This result does not by itself revise the taxonomic status of *A. stenotaeniatus*, but it indicates that the relationship between *A. longipinnis, A. stenotaeniatus, A. spinifer*, and *A. beijiangensis* should be reexamined using both morphology and nuclear evidence.

The two *A. paradoxus* accessions were not recovered as a monophyletic group. *A. paradoxus*_1 was placed with *A. wuyiensis*, whereas *A. paradoxus*_2 was placed near *A. jishouensis*. This pattern could indicate cryptic diversity within *A. paradoxus*, historical introgression, mitochondrial capture, or misidentification of one or both accessions. The taxonomic complexity of *A. paradoxus* is not unexpected because *A. formosanus, A. fasciatus,* and *A. labiatus* have previously been treated as synonyms of *A. paradoxus* in Taiwan.[11] Future work should include voucher-based morphological reexamination of the two *A. paradoxus* samples, broader geographic sampling, and nuclear-marker validation.

The placement of *A. hemispinus* within *A. parallens* is consistent with earlier molecular concerns regarding the taxonomic status of *A. hemispinus*.[12] Nevertheless, this result should be interpreted cautiously because *Zacco* platypus, which has been treated as a synonym of *A. parallens* in morphological comparisons,[10] was not included in the present dataset. The monophyly of *A. monticola* and *A. yunnanensis* was recovered with strong support, suggesting that the sampled mitogenomes for these species are internally consistent.

This work also compares the molecular units with morphology-based species concepts. Some MOTUs correspond closely to currently recognized species, such as *A. monticola* and *A. yunnanensis*, as well as to the sampled *Onychostoma* taxa. Other MOTUs combine more than one nominal species, such as the *A. longipinnis-A. iridescens-A. barbodon* and *A. hemispinus-A. parallens* units, suggesting possible synonymy, recent divergence, or mitochondrial introgression. Still other patterns, especially the two separate placements of *A. paradoxus* accessions, suggest potential cryptic diversity or misidentification. This explicit comparison strengthens the link between molecular delimitation and traditional taxonomy.

Morphological taxonomy should not be dismissed; rather, it should be integrated with molecular evidence. Controversies in *Acrossocheilus* morphology arise because diagnostic characters can be plastic or ontogenetically variable. In particular, body coloration, lateral stripes, vertical bars, dorsal-fin serrations, mouth and lip structures, and scale-count characters may vary with age, sex, preservation, or geographic population. These factors can generate synonyms, homonyms, and misidentifications when small samples or non-voucher sequences are used. The 15 MOTUs should therefore be treated as candidates for integrative

testing against voucher morphology, morphometrics, meristics, geography, ecology, and nuclear genetic data.

The relationship between *Acrossocheilus* and *Onychostoma* remains unresolved. In the present mitochondrial tree, the *Onychostoma* clade (C3) was nested between two *Acrossocheilus clades*, and C1 was more closely related to C3 than to C2. A similar lack of reciprocal monophyly has been suggested in broader cyprinine phylogenies.[28] However, only three *Onychostoma* taxa were included here, and the conclusion is based on mitochondrial protein-coding genes only. Therefore, the present study should not be used alone to redefine generic limits. Broader sampling of *Onychostoma* and related genera, preferably with nuclear genomic data, is necessary to test generic monophyly robustly.

A major limitation of this study is its reliance on mitochondrial loci. Mitochondrial genomes are maternally inherited as a single linked locus and can be affected by incomplete lineage sorting, introgression, hybridization, selection, sex-biased dispersal, and mitochondrial capture. These processes can cause mitochondrial trees to differ from species trees and can mislead species delimitation. In addition, several recognized *Acrossocheilus* species and many populations are missing from the available mitogenomic dataset, and the three-species sampling of *Onychostoma* is insufficient for strong conclusions about generic boundaries. For these reasons, the proposed MOTUs are best interpreted as mitochondrial hypotheses that require validation with unlinked nuclear markers, genome-wide SNPs, denser geographic sampling, and voucher-based morphology.

Taken together, the phylogenetic, delimitation, and taxonomic results provide a coherent framework for future revision of *Acrossocheilus*. The mitochondrial data identify several stable units, reveal candidate cases of synonymy or misidentification, and highlight lineages where current taxonomy may not match matrilineal history. The strongest practical contribution of this study is therefore not a definitive taxonomic rearrangement but a prioritized list of taxa and species complexes that should be examined using integrative taxonomy.

CONCLUSION

Using 31 complete mitochondrial genomes and the concatenated 13 mitochondrial protein-coding genes, we reconstructed phylogenetic relationships and assessed species boundaries in sampled *Acrossocheilus* and related taxa. The analyses supported 15 candidate MOTUs under the most congruent bPTP and ABGD settings, revealed several taxonomically important complexes, and showed that reciprocal monophyly between *Acrossocheilus* and *Onychostoma* was not recovered in this mitochondrial dataset. These conclusions are deliberately moderated because mitochondrial evidence alone cannot definitively resolve species limits or generic boundaries. Future studies should add nuclear genes or genome-wide markers, include the missing *Acrossocheilus* species and additional *Onychostoma* taxa, examine voucher specimens and diagnostic morphology, and integrate geographic and ecological information. Such integrative work will be essential for a stable taxonomy of *Acrossocheilus* and for conservation and management of these freshwater fishes.

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AUTHOR CONTRIBUTIONS

Conceptualization: YuHe Yang (Equal), TingTing Lin (Equal). Data curation: YuHe Yang (Lead). Formal Analysis: YuHe Yang (Equal), TingTing Lin (Equal). Writing – original draft: YuHe Yang (Lead). Supervision: TingTing Lin (Lead). Writing – review & editing: TingTing Lin (Lead).

CONFLICTS OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY

All sequences analyzed in this study are publicly available from the National Center for Biotechnology Information (NCBI), and the corresponding accession numbers are provided in Table 1. The final concatenated alignment and analysis input files should be deposited as supplementary materials or in an appropriate public repository upon acceptance.

ETHICS STATEMENT

This study was based exclusively on publicly available mitochondrial genome sequences downloaded from NCBI. No live animals were collected, handled, or sacrificed, and no experiments involving humans or animals were conducted. The use of publicly available sequence data complied with relevant ethical and database-use guidelines; therefore, additional ethical approval was not required.

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