



Phylogenomics of *Rhinogobius* gobies reveal northern-southern divergence and trait evolution in China

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ABSTRACT

The goby genus *Rhinogobius* represents a prominent radiation of freshwater fishes in East Asia, with China serving as its evolutionary hotspot harboring over 39 endemic species. Despite their ecological dominance in riverine ecosystems, phylogenetic relationships among mainland Chinese *Rhinogobius* species remain poorly resolved due to limited sampling and morphological conservatism, particularly in meristic traits traditionally used for classification. To address these knowledge gaps, we employed a nuclear gene enrichment approach to target 4,434 single-copy nuclear loci across 26 species spanning all major river basins in eastern and southern China. Our phylogenomic analyses based on sequence of 2,137 cleaned-up loci (477,822 bp) recovered a robust topology dividing *Rhinogobius* into three primary lineages: an early-diverging of *R. similis* sister to two major clades, the northern vs. southern clades exhibiting strong geographic structuring. Fossil-calibrated molecular dating placed the crown age of *Rhinogobius* at ~ 9.33 million years ago (Ma) during the late Miocene, with a pivotal north–south divergence at ~ 5.96 Ma coinciding with major paleo-drainage reorganizations in East Asia. Ancestral state reconstruction revealed that the southern clade uniquely evolved unstable first dorsal fin formula and near-universal presence of predorsal scales. By integrating the most extensive geographic and taxonomic sampling to date with phylogenomic data, this study establishes: (1) a revised evolutionary timeline for *Rhinogobius* diversification linked to East Asian geomorphological history, (2) diagnostic morphological characters for major clades, and (3) a robust phylogenetic framework to guide future taxonomic revisions and conservation efforts for this ecologically significant group.

1. Introduction

The genus *Rhinogobius* Gill, 1859 (family Oxudercidae) comprises the most speciose and geographically diversified freshwater gobiid radiation in East and Southeast Asia, occupying river systems from Vietnam to Japan (Yamasaki et al., 2015). China represents the evolutionary center of this genus, containing at least 42 species (39 endemic) distributed across mainland China, Taiwan, and Hainan Island (Wu, 2008). These ecologically versatile fishes demonstrate extraordinary adaptive traits, including specialized egg morphology (Katoh and Nishida, 1994; Yamasaki and Tachihara, 2006) and complex reproductive behaviors

(Kano et al., 2012), making them exceptional models for studying freshwater adaptive radiation. Despite their ecological dominance and economic value, particularly *R. similis* in fisheries and aquaculture and other species for ornamental trade, their phylogenetic architecture remains poorly understood. Current knowledge derives primarily from studies of Japanese populations (Yamasaki et al., 2015), leaving Chinese remarkable species richness genetically unexplored.

Taxonomic confusion plagues *Rhinogobius* due to conserved morphology and rapid speciation. Discrepancies in species counts persist between authoritative sources: 83 valid species in Catalog of Fishes (Fricke et al., 2018) versus 65 accepted from 108 nominal taxa in

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FishBase (Froese and Pauly, 2022). Molecular studies have uncovered extensive synonymy, including *R. similis*-*R. giurinus* (Suzuki et al., 2016) and unrecognized diversity within the *R. brunneus* complex (Akihito et al., 2000, 2002). While species descriptions continue accelerating (Chen et al., 2024, 2022; Chen and Wei, 2024; Xia et al., 2018), most taxa exhibit overlapping meristic ranges (Maeda et al., 2024) and only subtle chromatic differences. This morphological conservatism challenges traditional taxonomy that relies on vertebral counts, scale arrangements, and fin ray formulae, characters increasingly shown to lack diagnostic resolution among sister species.

The phylogenetic relationships within *Rhinogobius* remain controversial, with most studies to date relying exclusively on mitochondrial markers. In the first comparative mitogenomic analysis of five species (*Rhinogobius brunneus*, *R. leavelli*, *R. cliffordpopei*, mainland Chinese *R. similis*, and Taiwanese endemic *R. rubromaculatus*), Zhang and Shen (2019) identified *R. similis* as the basal lineage, a finding subsequently supported by multiple mitochondrial DNA studies (Han et al., 2025; Song et al., 2022; Tuncharoen et al., 2025). However, Tan et al. (Tan et al., 2020) recovered an alternative topology with *R. giurinus* (synonymy of *R. similis*) and *R. leavelli* as sister species. The most

comprehensive mitogenomic study to date (Jia et al., 2025) analyzed 16 species, resolving two primary clades: one containing *R. wuyanlingensis*, *R. filamentosus*, *R. zhoui*, and *R. duospilus*, and the other comprising *R. brunneus*, *R. leavelli*, *R. davidi*, *R. cliffordpopei*, *R. wuyiensis*, *R. lentiginis*, and *R. niger*. These conflicting topologies likely stem from two fundamental limitations of mitochondrial data: their inheritance as a single linkage unit in vertebrates, which conflates gene and species histories, and insufficient taxonomic sampling that fails to capture full diversity of the genus. Consequently, more taxon sampling and additional nuclear DNA data are needed for better resolution in *Rhinogobius*.

Here, we collected 26 *Rhinogobius* species from major river systems across southern and eastern China. Through targeted sequence capture of 4434 nuclear protein-coding loci, we generated phylogenomic datasets for the 26 species (54 specimens). Additionally, a total of 95 specimens were re-examined for 8 meristic characters, supplemented by published morphological data (Wu, 2008). This study addresses three primary objectives: (1) reconstruction of the first species-level nuclear phylogeny for *Rhinogobius* using genome-level data; (2) estimation of divergence times and biogeographic history of mainland Chinese lineages; and (3) evaluation of traditional diagnostic characters within a

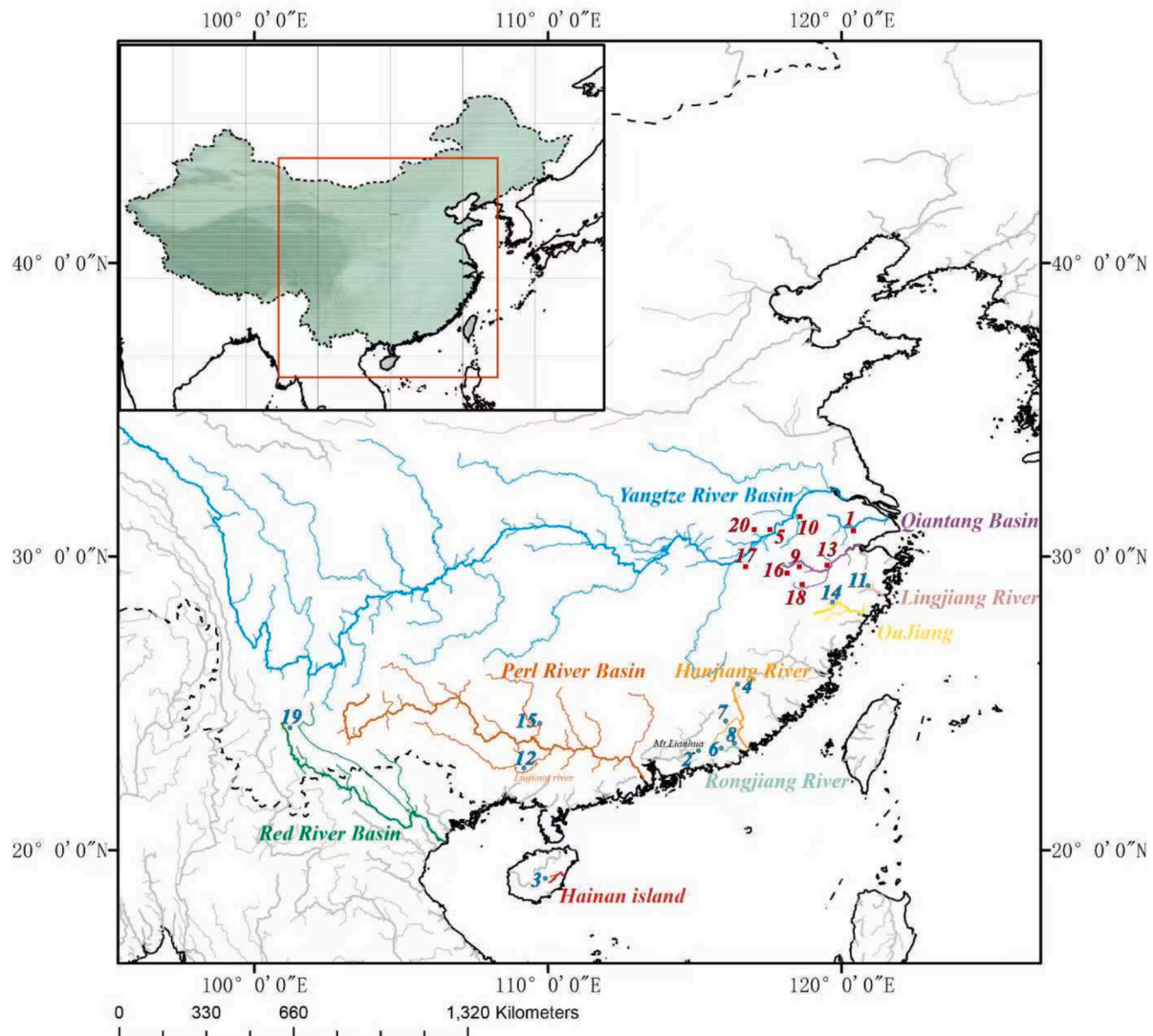


Fig. 1. Sampling locations of *Rhinogobius* across major river basins in China. Collection sites (symbols) are colored according to their phylogenetic clade (see Fig. 2). Rivers with a discharge $> 40 \text{ m}^3/\text{s}$ are shown, representing eight major drainage systems. Sites in the northern basin affiliation (Yangtze and Qiantang Rivers) are marked with red squares. Sites in the southern basin affiliation (Oujiang, Hanjiang, Rongjiang, Red, and Liujiang Rivers, plus the isolated river systems of Hainan Island) are marked with blue circles. The inset shows the regional context within East Asia. The base map was generated using ArcGIS v10.6 (ESRI, 2022) with hydrographic data from HydroSHEDS (Lehner and Grill, 2013).

phylogenetic framework. Our integrated phylogenomic-morphological approach resolves long-standing systematic uncertainties while establishing a foundation for future taxonomic revisions.

2. Materials and methods

2.1. Sampling and DNA extraction

Twenty-six *Rhinogobius* species were collected from major river systems in southern and eastern China, including the Yangtze, Qiantang, Oujiang, Hanjiang, Rongjiang, Red, and Liuijiang river basins, as well as the isolated drainages of Hainan Island (Fig. 1). There are 16 described species from the 26 species and 10 undescribed species (details of these can be found in Supplementary Table S1). Fin clips or muscle tissue samples were collected from fifty-four specimens of the 26 species (Table 1). The tissue samples were preserved in 95% ethanol immediately after collection. Two *Tridentiger* species (*T. barbatus* and *T. bifasciatus*), along with *Pterapogon kauderni* and *Sphaeramia orbicularis* representing the Apogonidae, *Kurtus gulliveri* representing the Kurtidae, *Odontobutis potamophilus* representing the Odontobutidae, *Butis koilomatodon*, *Kribia nana*, and *Oxyeleotris marmoratus* representing the Butidae, as well as *Valenciennae puellaris* representing the Gobiidae, were selected as outgroup taxa based on recent gobioid phylogenies

Table 1

Samples used for molecular analysis. Locality numbers correspond to those in Fig. 1.

Species	specimen ID	River/River system	Locality no.
<i>R. sp. ORAZJ</i>	2228_3, 2229_4	Anji County, Yangtze River Basin	1
<i>R. multimaculatus</i>	2530, 2531	Anji County, Yangtze River Basin	1
<i>R. zhoui</i>	1902_1,2, 1904_1	Haifeng County, Mt. Lianhua	2
<i>R. sangenloensis</i>	2222_2,3	Hainan	3
<i>R. changtiniensis</i>	2495_1	Hanjiang River	4
<i>R. sp. LLVC27</i>	090801, 090803	Huangshan County, Yangtze Basin	5
<i>R. sp. XIAO</i>	1901_1,5,8	Jiexi County, Rongjiang River	6
<i>R. sp. YLGD</i>	1903_2,3,4,6	Jieyang City, Hanjiang River	7
<i>R. duospilus</i>	1908_2,4	Jieyang City, Rongjiang River	8
<i>R. cf. lentiginis</i>	2226_2,3	Kaihua County, Qiantang Basin	9
<i>R. niger</i>	2224_1,2	Lin'an City, Yangtze River Basin	10
<i>R. genanematus</i>	2215_1,4	Lingjiang River	11
<i>R. lentiginis</i>	1328_1,6	Lingjiang River	11
<i>R. cliffordpopei</i>	1147_2,9	Liuijiang River	12
<i>R. leavelli</i>	2461_2,3 201803012	Longyou County, Qiantang Basin	13
<i>R. wuyanlingensis</i>	1850_1,4	Oujiang	14
<i>R. filamentosus</i>	2216_1,2,3	Peri River Basin	15
<i>R. davidi</i>	2227_1,4	Qimen County, Qiantang Basin	16
<i>R. sp. LLAH</i>	2231_4	Qimen County, Yangtze River Basin	17
<i>R. sp. OSVSAH</i>	2232_3,4	Qimen County, Yangtze River Basin	17
<i>R. cf. wuyiensis</i>	2230_2	Qimen County, Yangtze River Basin	17
<i>R. sp. P18L33AH</i>	2233_3	Qimen County, Yangtze River Basin	17
<i>R. maxillivirgatus</i>	2016100002, 0095	Qimen County, Yangtze River Basin	17
<i>R. wuyiensis</i>	1327_1,2	Quzhou City, Qiantang River Basin	18
<i>R. honghensis</i>	2220_3,4,5	Red River Basin (Yunnan)	19
<i>R. sp. CBBSAH</i>	2225_2	Shitai County, Yangtze River Basin	20

(Kuang et al., 2018). All specimens were assigned unique voucher numbers (Supplementary Table S1) and deposited in the Fish Collection of Shanghai Ocean University (SOU), China, (contact person: Dr Ya Zhang, zhangya@shou.edu.cn). Genomic DNA was extracted from ethanol-preserved tissues using the Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China). DNA concentration and quality were assessed using a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Wilmington, DE, USA) and verified by 1% agarose gel electrophoresis. Only samples with A260/A280 ratios between 1.8–2.0 and showing minimal degradation were used for downstream analyses.

2.2. Library preparation and targeted sequencing

Library preparation and target enrichment procedures were conducted on the basis of established protocols (Meyer and Kircher, 2010) with adaptations for divergent species (Li et al., 2013). Initially, 50 µl of purified DNA was fragmented to ~ 250 bp with a SCIENTZ18-A Ultrasonic DNA Interrupter (Scientz, Ningbo, China), employing a protocol consisting of 22 cycles of ultrasonication (20 s on, 20 s off) at 4°C with 300 W processing power. Library construction began with 300 ng of sheared DNA per sample. A set of biotinylated RNA baits (120 bp length, 2 × tiling density) was synthesized by MYcroarray (Ann Arbor, Michigan, USA; Catalog #150901-Li-Goby), designed based on 4434 single-copy nuclear exon markers previously established for phylogenetic studies of gobies (Hu et al., 2023; Jiang et al., 2019; Kuang et al., 2018). Two successive rounds of hybridization capture were performed for each library to optimize target enrichment efficiency. All libraries were uniquely identified with 8 bp dual-index combinations on P5 and P7 adapters to enable multiplexing. Following quality control, enriched libraries were pooled in equimolar concentrations and subjected to 150 bp paired-end sequencing on an Illumina HiSeq X Ten platform (Illumina, San Diego, CA, USA) through Novogene Technology (Beijing, China).

2.3. Data processing and assembly

Raw sequence reads were demultiplexed according to the 8 bp sample-specific indices. Subsequently, Cutadapt v1.2.1 (Martin, 2011) and Trim Galore v0.6.7 (Krueger et al., 2021) were employed to remove adapter sequences and low-quality bases (Q < 20) from demultiplexed reads by default parameters. Sequence assembly followed Yuan et al. (2019) using a custom Perl pipeline to acquire locus-specific contigs from trimmed reads. The pipeline identified paralogous sequences by aligning putative targets to the reference genome, with sequences failing to match target regions being excluded as probable paralogs. Loci with low enrichment efficiency (< 80% taxonomic coverage and < 50% sample coverage) were removed from the final assembly results. The filtered outputs loci were initially aligned in batch using Clustal Omega (Sievers et al., 2011) based on codons, then back-translated to nucleotide alignments while preserving codon structure. The alignment dataset was further refined by excluding loci showing excessive pairwise distances that could not be manually corrected.

2.4. Phylogenetic analyses

Aligned sequence were concatenated and subjected to Maximum likelihood (ML) trees reconstruction using RAxML v8.2.8 (Stamatakis, 2014) under the GTR + Γ model with 1,000 bootstrap replicates (Kalyaanamoorthy et al., 2017). Both codon-position partitioning and an optimized scheme selected by PartitionFinder v2.1.1 (Lanfear et al., 2017) were evaluated. The partitioning optimization, initiated from codon-based data blocks, employed the rcluster algorithm under GTR + Γ and selected the best-fit scheme using Bayesian Information Criterion (BIC).

2.5. Divergence time estimation

Divergence time estimation was conducted basing on clock-like loci selected from aligned loci in [section 2.3](#) but with 100% of taxonomic coverage and containing at least 90% individuals ($n > 58$) for all 36 analyzed species (26 ingroup and 10 outgroup taxa). The most clock-like loci were identified using a Perl script developed by [Yuan et al. \(2019\)](#), which rank loci by comparing log likelihood scores between strict-clock and relaxed-clock models for individual gene trees. After excluding loci rejected for molecular clock ($p < 0.05$), 612 loci were used for divergence time estimation.

Subsequently, these loci were converted into the.nex format and configured in BEAUti v2.7.8 for Bayesian dating analyses in BEAST v2.7.8 ([Bouckaert et al., 2019](#); [Drummond and Rambaut, 2007](#)) with following parameters: codon-partitioned data (positions 1 + 2 + 3) with linked clock models and trees but unlinked site models. The substitution model was GTR + Γ with 4 gamma categories and 1.0 substitution rate for all partitions. Clock model was relaxed clock log normal with 1.0 mean clock rate and tree model was birth–death. To prevent potential convergence failures associated with random starting trees in multiple BEAST runs, the rooted RAxML tree topology obtained from previous concatenated analysis ([Section 2.4](#)) was employed as the foundation and subsequently calibrated with fossil dates (listed in the following paragraph) using the penalized likelihood method implemented in r8s ([Sanderson, 2003](#)). The resulting time-calibrated tree served as the starting topology for all BEAST analyses. In addition, four parameters: Birth Death Subtree Slide, Narrow, Wide and Wilson-Balding were disabled to ensure tree topology is fixed throughout each BEAST runs. Two independent MCMC runs of 60 million generations each were performed, sampling parameters every 1,000 generations. Convergence was assessed in Tracer v1.7.2 ([Rambaut et al., 2018](#)), requiring effective sample sizes > 200 for all parameters. The resulting maximum-clade credibility (MCC) tree with means and 95% highest posterior density of divergence times was generated with TreeAnnotator v2.7.8 after discarding 20% burn-in using LogCombiner v2.7.8 and visualized in FigTree v1.4.4.

Four nodes were constrained using well-documented fossil calibrations within Gobiiformes. The first calibration point established a hard minimum age of 47.8 Ma for the crown group of all gobiiformes species, based on *†Carlomonnus quasigobius* ([Bannikov and Carnevale, 2016](#)), the oldest known gobioid fossil from the Lower Eocene of northern Italy (47.8–56.0 Ma) along with a soft maximum age of 122 Ma (95% CI) derived from mitochondrial-based estimates of the gobiiformes root age ([Chakrabarty et al., 2012](#)). This node was modeled with a lognormal prior (mean = 1.9, SD = 0.5, offset = 47.8). The second fossil calibration established age constraints for the most recent common ancestor of Oxudercidae (*Tridentiger* and *Rhinogobius*), with a hard minimum bound at 17.2 Ma based on the oldest skeleton-based member of the Oxudercidae, *†Simpsonigobius nerimanae* ([Dirnberger et al., 2024](#)) from the Lower Miocene of Turkey with the geological age estimated between 17.2 and 19.5 Ma., and a 95% soft maximum bound at 56 Ma based on maximum geographical age of *†Carlomonnus quasigobius* (Lognormal prior settings: mean = 2.343, SD = 0.8, offset = 17.2). Fossil for setting hard minimum bound is *†Simpsonigobius nerimanae*, which was placed under the Oxudercidae by sharing two phylogenetically informative morphological characters with the family: (1) premaxilla with a weak or absent postmaxillary process and (2) an anal fin with one ray more than in the second dorsal fin ([Dirnberger et al., 2024](#)). For the *Rhinogobius* crown group, a hard minimum of 0.2 Ma was assigned based on Middle Pleistocene fossils from Japan (1.1–0.2 Ma) ([Yabumoto, 1987](#)). Though species-level identification remains uncertain currently, generic assignment to the *Rhinogobius* is supported by following meristic traits: (1) 10 abdominal + 16 caudal vertebrae; (2) first dorsal fin with 6 spines, second dorsal fin with 1 spine + 8 soft rays; (3) anal fin with 1 spine + 8 soft rays. The soft maximum for this node (19.5 Ma) aligns with *†S. nerimanae*'s maximum age (Lognormal prior settings: mean =

1.62, SD = 0.8, offset = 0.2). An additional calibration point was applied to constrain the divergence between *Kribia* and *Butis* (hard minimum: 23.03 Ma, based on *†Lepidocottus aries*; soft maximum: 56 Ma, based on *†C. quasigobius*, lognormal prior settings: mean = 2.18, SD = 0.8, offset = 23.03) to facilitate calibration in r8s.

2.6. Morphological statistics

Morphological statistics were derived from a total of 95 specimens. It should be noted that while the samples for morphological assessment and phylogenetic analysis were sourced from identical collection batches, a direct specimen-for-specimen correspondence was not always feasible; the complete sample mapping is detailed in Supplementary Table S1. The methodology involved microscopic observation and statistical analysis for scale patterns (predorsal, longitudinal, transversal). Fin ray counts (dorsal, pectoral, anal); vertebral counts (including urostyle) were mainly obtained from a 3D rendering scanned by X-ray microtomography (NSI X50, U.S.A.). Owing to preservation limitations, biological replicates for full morphological analysis were unavailable for some individuals. As a result, morphological data for *R. cliffordpopei* are referenced solely from the Fauna Sinica ([Wu, 2008](#)), and data for *R. changtinensis*, *R. sangenloensis* and *R. lentiginis* incorporate both a single examined specimen and the prior descriptions ([Chen and Miller, 2013](#); [Huang and Chen, 2007](#); [Wu, 2008](#)).

2.7. Character mapping and ancestral state reconstruction

Morphological data for eight meristic characters were compiled in a matrix format, as summarized in [Table 2](#). This character matrix was imported into Mesquite version 4.01 ([Maddison and Maddison, 2025](#)). Using the software's character mapping module, each character state was explicitly linked to its corresponding terminal taxon on the phylogeny, thereby overlaying the morphological dataset onto the tree topology for subsequent analysis.

Ancestral state reconstructions were then performed for three key meristic traits: predorsal scale condition, first dorsal fin spine stability, and vertebral count. Analyses were conducted on the majority-rule consensus tree derived from the input maximum likelihood phylogeny produced by RAxML v8.2.8 (see [Section 2.4](#)). Each character was coded as a discrete trait with two states for maximum likelihood (ML) reconstruction under the Markov k-state 1 parameter model (Mk) model: Predorsal scales: coded as 'absent/incomplete' (count = 0, or variable within a range including 0) versus 'complete' (consistently present with a count > 0). First dorsal fin spine count: coded as 'unstable' (variable count) versus 'stable' (fixed at VI). Vertebral count: coded as 'low' (< 27) versus 'high' (≥ 27).

For comparative purposes, an additional reconstruction was performed for these traits using the parsimony criterion, with characters specified as 'ordered'. All analyses utilized the default settings in Mesquite. The results of both the ML and parsimony reconstructions were visualized and compared directly on the consensus tree topology.

Fisher's exact test was applied to determine if the distribution of meristic traits differed significantly between two phylogenetic clades (Clade S and Clade N). The test assesses whether a statistically significant association exists between two categorical variables. The data consisted of binary outcomes (as coded in ancestral state reconstruction) for each sample. All calculations were performed using GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts, USA; www.graphpad.com).

3. Results

3.1. Phylogenetic relationships and divergence patterns

Final analyses included 2137 loci (477,822 bp) after those with low enrichment efficiency ($< 80\%$ taxonomic coverage and $< 50\%$ sample

Table 2Meristic characters of 26 species of *Rhinogobius* species.

species	VC	Pre-D	LR	TR	D1	D2	A	P
<i>R. sp. XIAO</i>	26	9 ~ 14	28 ~ 32	8	VI	I-6~8	I-6~7	18~19
<i>R. zhoui</i>	26	8~9	30	8~9	VI	I-8~9	I-7~8	16~18
<i>R. filamentosus</i>	27	5~11	34~35	8~10	V~VI	I-8~9	I-8	15~17
<i>R. wuyanlingensis</i>	27	5	29	9~10	V~VI	I-8~9	I-8	17~18
<i>R. genanematus</i>	25	0~5	27~29	8~9	V~VI	I-8~9	I-7	15
<i>R. maxillivirgatus</i>	27	5~9	28~30	6~7	V~VI	I-8~9	I-7~8	13~15
<i>R. changtingensis</i>	27	0~2	28~30	7~8	VI	I-8	I-7~8	16~17
<i>R. duospilus</i>	27	10	28	7	VI	I-8	I-7~8	16~17
<i>R. honghensis</i>	27~28	3	28~30	8	VI	I-8~9	I-7~8	16~17
<i>R. sp. YLGD</i>	26	5~8	29~32	8~9	V~VI	I-7~8	I-7~8	16~17
<i>R. sangenloensis</i>	26	9~10	29~30	9~10	V~VII	I-8~9	I-7~8	16~17
<i>R. lentiginis</i>	27	0	29~32	10~11	VI	I-8	I-7~8	14~15
<i>R. cf. lentiginis</i>	27	0	31	7~8	VI	I-8	I-7	15
<i>R. sp. CBBSAH</i>	27	0	NA	NA	VI	I-8	I-7	16
<i>R. sp. LLAH</i>	25~26	0~4	31	8	VI	I-8	I-7	16
<i>R. sp. OSVSAH</i>	27	0~1	32	8	VI	I-8	I-7	15
<i>R. wuyiensis</i>	26	3~6	30~31	9~11	VI	I-8~9	I-7~9	15~18
<i>R. cf. wuyiensis</i>	26	0~7	31~32	8	VI	I-8~9	I-7~9	15~18
<i>R. sp. LLVC27</i>	27	3~4	33~35	7~8	VI	I-8	I-7~8	16~17
<i>R. niger</i>	27	0	35~36	9~10	V~VI	I-8~9	I-7~8	16~18
<i>R. sp. P18L33AH</i>	26	0	33~34	9	VI	I-8	I-7	18
<i>R. leavelli</i>	26	5~9	29~31	7	VI	I-8~9	I-8~9	14~15
<i>R. cliffordpopei</i>	26	0	31	9~10	VI	I-8~9	I-7~9	17~21
<i>R. davidi</i>	27~28	0	30~31	7~9	VI	I-9~10	I-6~8	14~15
<i>R. sp. ORAZJ</i>	29	0	35~37	8~11	VI	I-9~10	I-8	16~17
<i>R. multimaculatus</i>	29	0	34~35	9	VI	I-9~11	I-7~8	14~16

coverage) were removed. The concatenated nuclear phylogenetic analysis strongly supports the monophyly of *Rhinogobius* (bootstrap = 100%, Fig. 2, Supplementary Fig. 1), with *R. similis* branched first and the other sampled species forming two well-supported clades exhibiting distinct biogeographic patterns. Clade S (bootstrap = 100%) comprises eleven southern Chinese species: *Rhinogobius* sp. XIAO, *R. zhoui*, *R. filamentosus*, *R. wuyanlingensis*, *R. genanematus*, *R. maxillivirgatus*, *R. changtingensis*, *R. duospilus*, *R. honghensis*, *Rhinogobius* sp. YLGD, and *R. sangenloensis*. Clade N (bootstrap = 100%) contains fifteen northern Chinese taxa including *R. lentiginis*, *R. cf. lentiginis*, *Rhinogobius* sp. CBBSAH, *Rhinogobius* sp. LLAH, *Rhinogobius* sp. OSVSAH, *R. wuyiensis*, *R. cf. wuyiensis*, *Rhinogobius* sp. LLVC27, *R. niger*, *Rhinogobius* sp. P18L33AH, *R. leavelli*, *R. cliffordpopei*, *R. davidi*, *Rhinogobius* sp. ORAZJ, and *R. multimaculatus*.

While the major clade structure aligns with Jia et al. (2025), we recovered distinct interspecific relationships within these clades. Notably, *R. zhoui* shows stronger phylogenetic affinity to *R. filamentosus* than to *R. duospilus* (bootstrap = 93%), contrasting with previous mitochondrial-based topologies. The *R. similis* forms a distinct early-diverging lineage sister to all other *Rhinogobius* species (bootstrap = 100%), consistent with most mitogenomic studies (Han et al., 2025; Song et al., 2022; Zhang and Shen, 2019). Generally, our phylogenomic analyses suggests an early diversification of *R. similis* and the ancestral populations of other Chinese *Rhinogobius* species, followed by the diversification of S and N clade from their common ancestor.

3.2. Divergence time, lifestyle and biogeographic pattern

BEAST analysis estimates crown age of *Rhinogobius* in the late Miocene at 9.33 Ma (95% HPD: 6.37 Ma–12.70 Ma), followed by a major divergence during Early Pliocene at 5.96 Ma (95% HPD: 4.22 Ma–7.92 Ma) that split the genus into the two primary clades identified in the phylogenetic analyses (Fig. 3A & C). All species within Clade S are fluvial or landlocked. In contrast, Clade N is predominantly composed of fluvial/landlocked species but includes two amphidromous members: *R. cliffordpopei* and *R. leavelli*. Notably, the early-diverging species *R. similis* is also amphidromous (Fig. 3A).

Phylogeographic analysis revealed a clear but imperfect association between clade membership and basin affiliation. The sampled specimens encompass major river basins of eastern and southern China

(Fig. 1), which we classified into northern (Yangtze and Qiantang) and southern basins (Lingjiang, Hanjiang, Oujiang, Rongjiang, Pearl, and Liujiang Rivers plus Hainan Island's drainages) based on geographic position and drainage patterns. Clade S predominantly occurs in southern basins, while Clade N is primarily found in northern basins. Notable exceptions include *R. maxillivirgatus* from the northern Yangtze basin, which clusters within Clade S, and *R. lentiginis* (Lingjiang) and *R. leavelli* from southern basins, which are both placed in Clade N (Fig. 3A & B).

3.3. Meristic character mapping and systematic significance of morphological traits

The traditional morphology-based taxonomy of *Rhinogobius* relies heavily on meristic characters, such as vertebral counts, scale patterns (predorsal, longitudinal, and transversal), and fin ray numbers. However, the phylogenetic signal of these characters within the genus *Rhinogobius* remains unclear. To evaluate their phylogenetic distribution, we mapped eight key meristic traits (Table 2) onto the phylogeny using character mapping module in Mesquite 4.01. This analysis revealed distinct phylogenetic clustering patterns (Fig. 4A & B).

Most species within Clade S consistently possess predorsal scales. This group includes *R. sp. XIAO*, *R. zhoui*, *R. filamentosus*, *R. wuyanlingensis*, *R. maxillivirgatus*, *R. duospilus*, *R. honghensis*, *R. sp. YLGD*, and *R. sangenloensis*. The exceptions are *R. genanematus* and *R. changtingensis*, in which this trait is variably absent in some individuals. Clade N is characterized by a marked reduction or complete loss of predorsal scales. The absence of this feature is fixed in *R. lentiginis*, *R. cf. lentiginis*, *R. sp. CBBSAH*, *R. cf. wuyiensis*, *R. niger*, *R. sp. P18L33AH*, *R. cliffordpopei*, *R. davidi*, *R. sp. ORAZJ*, and *R. multimaculatus*. In contrast, the trait is a bit variable (rare or absent) in *R. sp. LLAH*, *R. sp. OSVSAH*, *R. wuyiensis*, *R. sp. LLVC27*, and *R. leavelli*. The significant inter-clade divergence in predorsal scale absence (F-test, $p < 0.01$) indicates that its loss is an ancestral state in Clade N, a hypothesis directly supported by the ancestral state reconstruction results in Mesquite 4.01 (Supplementary Fig. 2).

A significant difference was also observed in the stability of first dorsal fin spine counts between clades ($p < 0.01$). Variability was common in Clade S, as seen in *R. filamentosus*, *R. wuyanlingensis*,

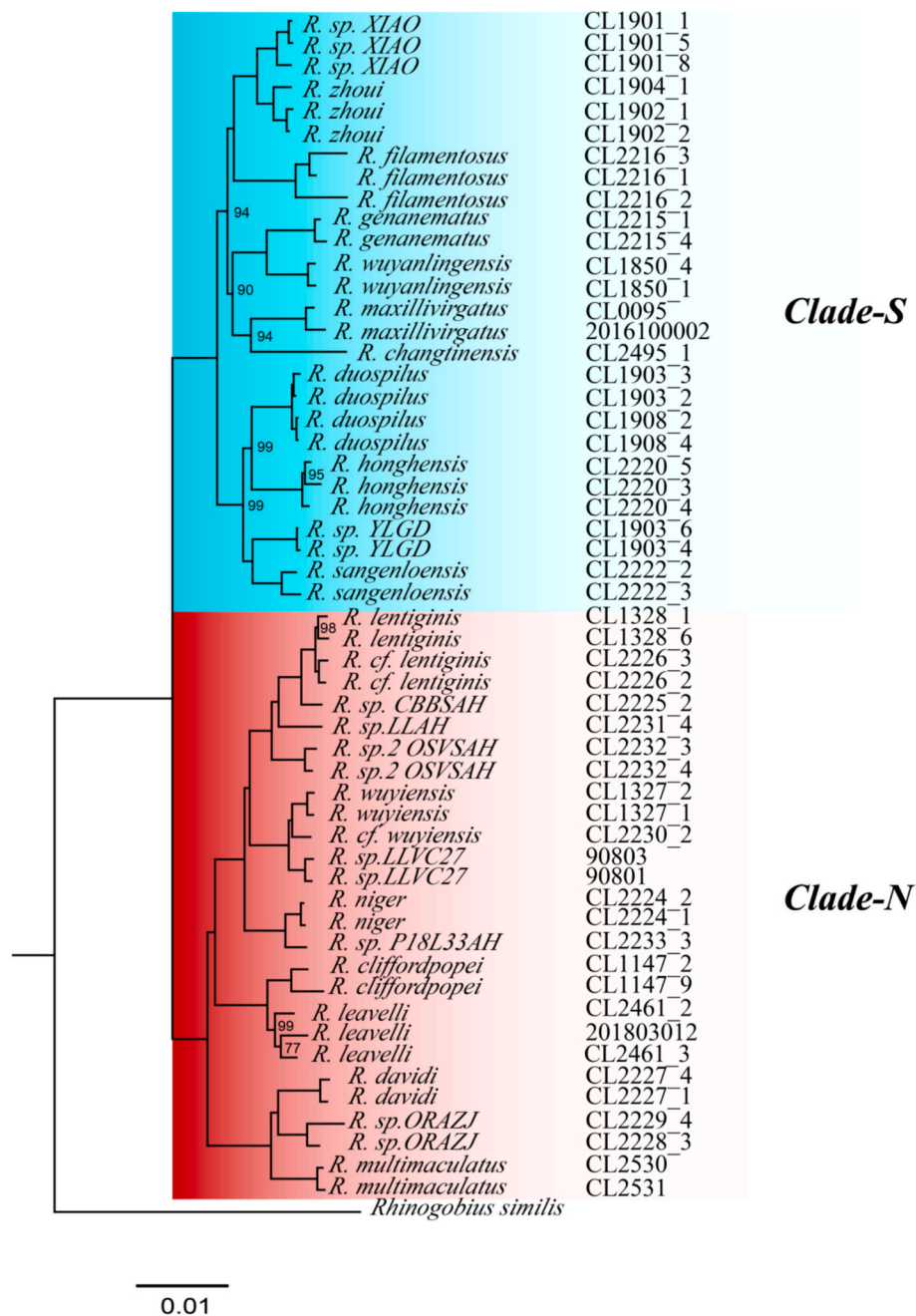


Fig. 2. Maximum likelihood phylogeny of *Rhinogobius* based on concatenated analysis of 2,137 nuclear protein-coding loci, reconstructed using RAxML under the GTR + Γ model. Nodes with bootstrap support values < 100% are labeled (100% support omitted for clarity). Color gradients at branch tips reflect sampling localities as shown in Fig. 1.

R. genanematus, *R. maxillivirgatus*, *R. sp. YLGD* (all V–VI), and *R. sangenloensis* (V–VII). Clade N, however, was characterized by a conserved count of VI, with the exception of *R. niger* (V–VI). However, ancestral state reconstruction did not identify variable spine counts as an ancestral state for Clade S (Supplementary Fig. 2).

4. Discussion

4.1. Life history evolution of *Rhinogobius*

The genus *Rhinogobius* exhibits a distinct life history polymorphism. Some species display amphidromous traits, while others spend whole life cycle in freshwater environment, display landlocked or fluvial life-style. It is widely hypothesized that the freshwater form of *Rhinogobius*

evolved from an amphidromous ancestor (Katoh and Nishida, 1994; Masuda et al., 1989; Mizuno, 1960). This evolutionary trajectory was further examined by mathematical modeling, which confirmed its feasibility (Omori et al., 2012), and supported by the phylogenetic studies of Japanese *Rhinogobius* (Yamasaki et al., 2015). In our research, the early-diverging *R. similis* is typically amphidromous, but most species from China in the later-diverging S and N clade are landlocked/fluvial (Fig. 3A). This phylogenetic pattern suggests that the radiation of inland freshwater *Rhinogobius* species in China likely originated from an amphidromous ancestor, supporting a marine-to-freshwater transition in the evolutionary history of this group.

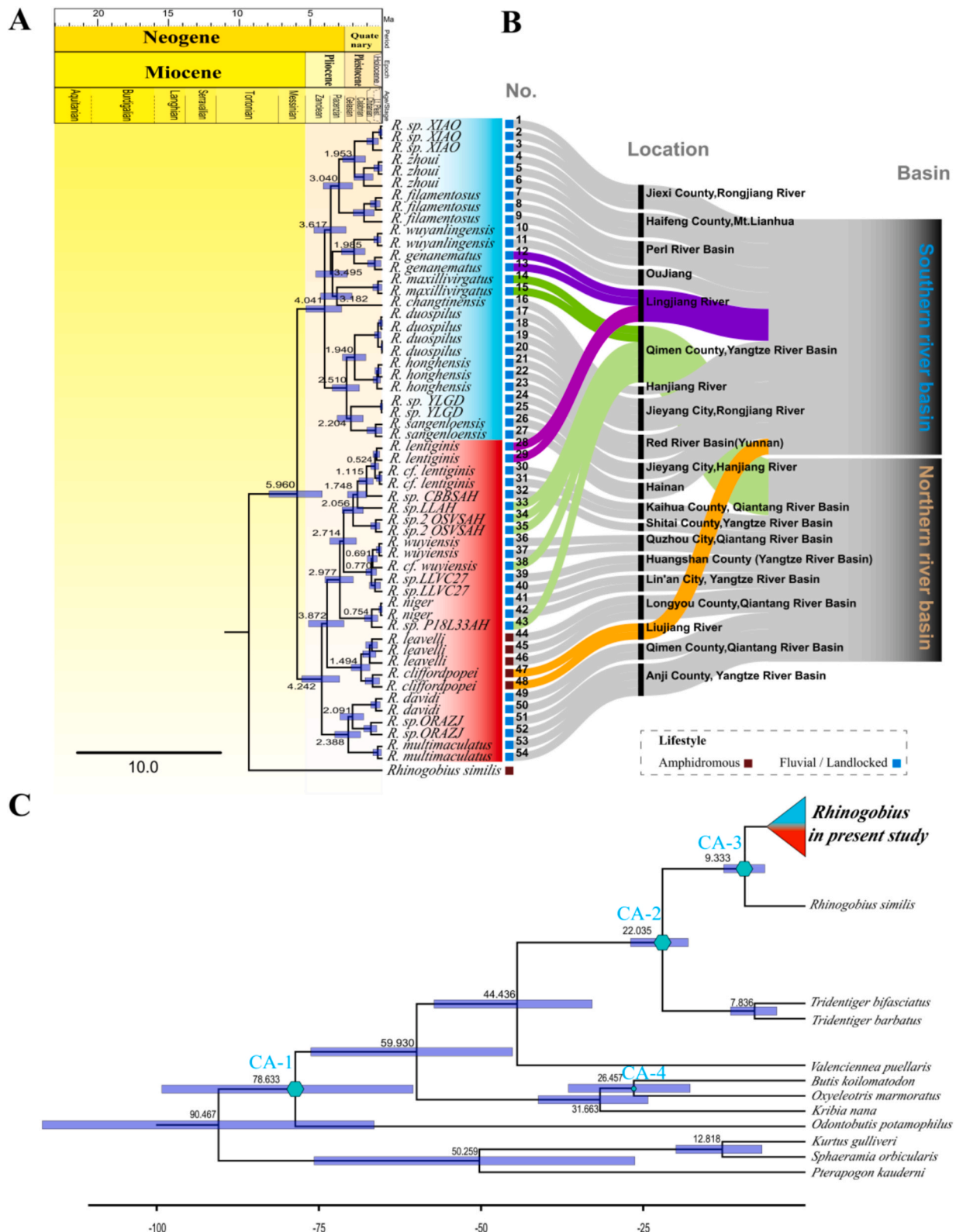


Fig. 3. Divergence history, life history and geographical distribution of *Rhinogobius* lineages. (A) Time-calibrated species tree of *Rhinogobius* reconstructed using BEAST v2.7.8 (calibration points and outgroups see Fig. 3C). The life history of each species is indicated as colored box, (Amphidromous [dark red] vs. Fluvial/Landlocked [light blue]). Node ages are shown with 95% highest posterior density intervals (blue bars). (B) Alluvial plot illustrating specimen-clade-geography relationships, showing concordant (gray) and discordant (colored) patterns between phylogenetic clades (Clade S [blue] vs. Clade N [red]) and basin affiliations (Southern vs. Northern). (C) Time-calibrated tree showing outgroup of *Rhinogobius*. Calibration point indicated by blue hexagon: CA-1, 47.8–56.0 Ma, *Carlomonnius quasigobius* fossil; CA-2, 17.2–19.5 Ma, *Simpsonigobius nerimanae* fossil; CA-3, 0.2–0.8 Ma *Rhinogobius* sp. fossil; CA-4, 23.03–56 Ma, *Lepidocottus aries* fossil.

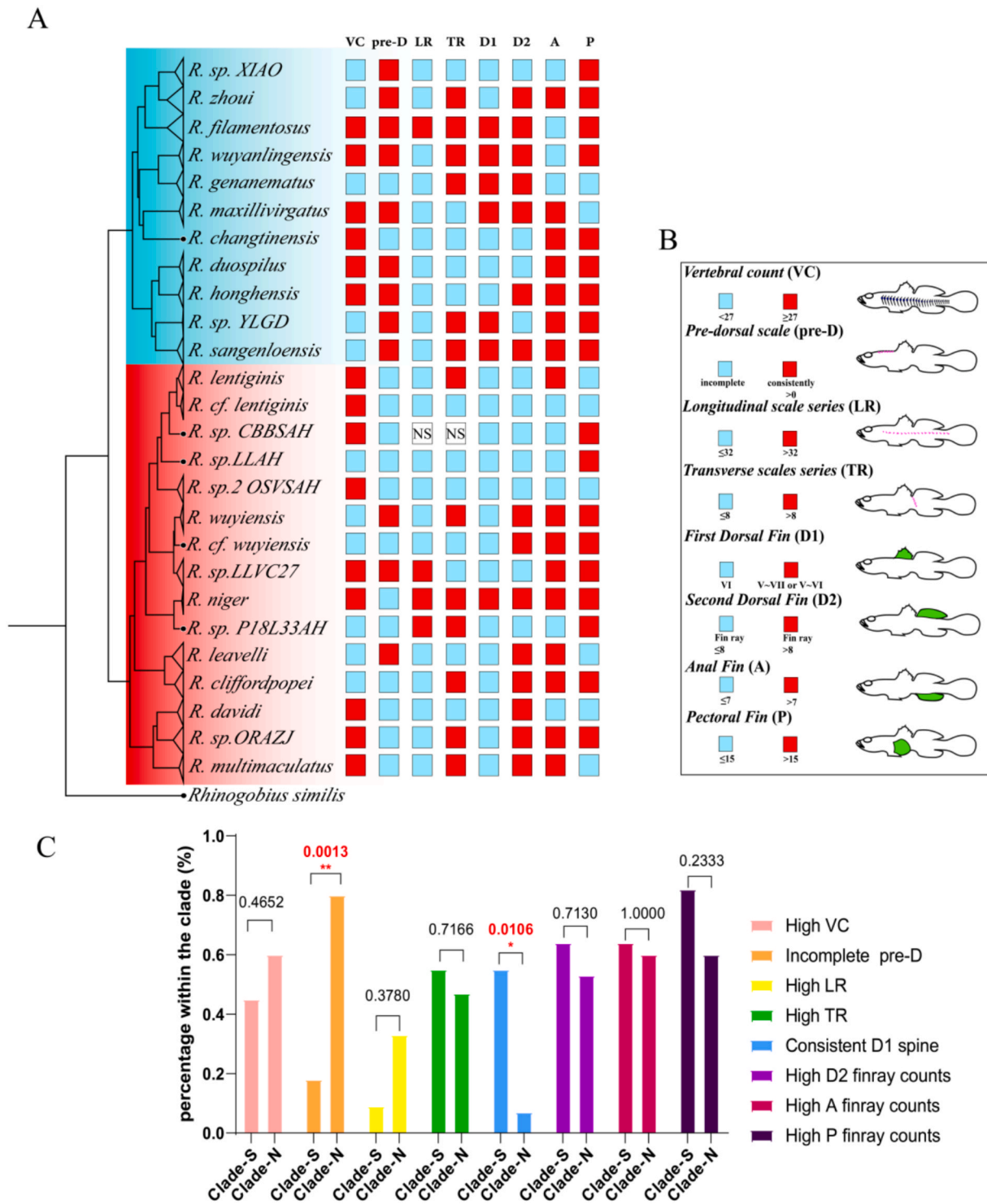


Fig. 4. Morphological trait evolution in *Rhinogobius*. (A) Phylogenetic distribution of meristic characters mapped onto the BEAST-derived species tree (Clade S = blue, Clade N = orange). (B) Schematic representation of eight diagnostic meristic traits (coded as binary states: red = higher values, blue = lower values). (C) Results of Fisher's exact tests comparing meristic character states between N and S clade, with p-values indicating significance of inter-clade differences.

4.2. Paleoclimate fluctuations shaped *Rhinogobius* distribution pattern

Amphidromous *Rhinogobius* typically exhibit strong dispersal capacity in inland freshwater systems, while showing minimal genetic differentiation among populations across the China continent (Ju et al., 2016). In this research, the *R. cliffordpopei*, also is found in the Liujiang Basin outside our main sampling area, reinforces the high dispersal potential of such species. For freshwater *Rhinogobius*, gene flow can be

significantly restricted even between adjacent streams less than 5 km apart (Wu et al., 2016). In this research however, two freshwater species (*R. maxillivirgatus* from the Yangtze, northern basins and *R. lentiginis* from the Lingjiang, southern basins) exhibit discordant placements in the phylogenetic clade. The discordant phylogenetic placements of the freshwater *R. maxillivirgatus* and *R. lentiginis* imply that their current distributions and relationships may not reflect recent gene flow restrictions alone.

Dispersal of freshwater *Rhinogobius* is limited, and is primarily reliant on rearrangements of drainage networks (Wu et al., 2016). Similar drainage network reorganization in ancient past could be the possible reason that results in current geographical and phylogenetic discordant of *R. maxillivirgatus* and *R. lentiginis*. Uplift of the Tibetan Plateau and subsidence in eastern China, following the cessation of Cretaceous arc magmatism within the Cathaysia block during the Cenozoic (Zhou et al., 2008) have acted to reverse an earlier Cretaceous southeast-to-northwest regional topographic gradient and allow the Yangtze to flow eastward into the East China Sea (Zheng et al., 2013). This extensive drainage reorganization facilitated the long-range dispersal of cyprinid fishes from Southeast Asia into the Yangtze River Basin (Chen et al., 2023). Regional Drainage reorganization within the Yangtze River Basin was pervasive between the Miocene and Pliocene. Successive river capture events progressively shaped the distribution of its tributaries (Wu et al., 2024; Zheng et al., 2013). Such geological event may have provided limited historical connectivity between adjacent tributaries—for instance, among the geographically proximate Yangtze (*R. maxillivirgatus* habitats) and Lingjiang rivers (*R. lentiginis* habitats) (Supplementary Fig. 3).

Island speciation presents another compelling biogeographic scenario. Historical sea-level fluctuations, by periodically creating land bridges, facilitated gene flow between populations in different continent (Shen et al., 2011). This geological process is likely a key driver behind the formation of several island-endemic species within *Rhinogobius* (Yamasaki et al., 2015). The divergence between *R. sangenloensis* and *R. sp. YLGD* (approximately 2.204 Ma) coincides with the pronounced global sea-level decline of the Pleistocene, when glacial maxima lowered sea levels by 120–150 m (Clark et al., 2025). This drop would have exposed a broad land connection between Hainan Island and the Chinese mainland, simultaneously altering coastal drainage configurations and creating potential corridors for dispersal.

4.3. Meristic variability challenges morphology-based diagnosis in *Rhinogobius*

Predorsal scales are counted or described (e.g., large, keeled, smooth, in rows) as specific characteristics in phylogenetic analyses, such as in *Homaloptera*, *Opsariichthys* or *Betta* species (Ding et al., 2025; Randall and Page, 2015; Silva-Oliveira et al., 2021). Our results show predorsal scales and first dorsal fin spines are significantly different in S and N clade, suggesting they are key diagnostic meristic traits for identification. However, some meristic characters are usually variable among the populations within same species. Our sample set included topotypic specimens of *R. maxillivirgatus* (holotype and paratypes), *R. zhoui*, *R. genanematus*, and *R. multimaculatus* collected from type localities. During morphological examination, we frequently observed discrepancies between morphometric data of these specimens (Table 2) and literature records, implying intraspecific variation. For example, (1) *R. genanematus*: specimens from the Lingjiang River exhibited fewer vertebrae (25) than documented (27; Chen and Fang, 2006) despite matching other characters (Zhong and Tzeng, 1998); (2) *R. niger*: TR counts (9–10) were lower than the reported range (10–12; Huang et al., 2016); (3) *R. davidi*: Some specimens displayed fewer vertebrae (27–28 vs 28) and TR rows (7–9 vs 11–12; (Chen et al., 1998); (4) *R. wuyanlingensis* also showed deviations: lateral scale rows (LR) (29 vs 30–32) (Huang et al., 2016). Historical literature corroborates meristic instability, causing inconsistent species descriptions. For example, in *R. duospilus*, LR counts vary (29–31 vs 30–32).

Collectively, these findings question the primacy of meristic traits in *Rhinogobius* taxonomy, their variability challenges their sufficiency as standalone diagnostic characters. This concern is amplified by recent species descriptions relying solely on morphological data for affinity assessments (Chen et al., 2024; Chen and Wei, 2024).

4.4. *Rhinogobius* as a potential model for study morphological evolution in freshwater fishes

Variation in the number of scales and fin spines is a common feature of adaptive evolution in fishes (Lemopoulos and Montoya-Burgos, 2021; Price et al., 2015). Throughout the evolutionary history of ray-finned fishes, scales have been lost on multiple occasions, but once lost, they are rarely regained, suggesting evolutionary irreversibility (Lemopoulos and Montoya-Burgos, 2021). Strikingly, our ancestral state reconstruction of predorsal scales uncovers an exception: this trait was lost in the common ancestor of the northern *Rhinogobius* clade but subsequently re-evolved in *R. wuyiensis*, *R. sp. LLVC27*, and *R. leavelli*. (Supplementary Fig. 2). The development of meristic traits in fishes, such as vertebral counts and the number of postocular scales, is known to be influenced by temperature, with higher temperatures often promoting an increase in scale counts (Osgood, 1978; Sfakianakis et al., 2011). Since species in Clade S inhabit lower latitudes with generally warmer environments than those in the northerly-distributed Clade N, this temperature differential may explain the higher average number of predorsal scales observed in the southern clade.

Dorsal-fin spines, another adaptive trait linked to predator defense (Price et al., 2015), exhibit marked divergence between the northern and southern *Rhinogobius* clades, similar to landlocked sticklebacks (*Gasterosteus aculeatus*), which rapidly evolve spine number variations post-isolation (Bell and Foster, 1994). Our study reveals spine count variability in recently diverged *Rhinogobius* lineages (Supplementary Fig. 2). Evidence suggests that the evolution of dorsal-fin spine number in sticklebacks resulted from the evolution of *cis*-regulatory sequences in Hox genes (Wucherpfennig et al., 2022). We suggest the difference in dorsal-fin spine number between the northern and southern lineages of gobies is likely driven by similar underlying genetic mechanisms, and selective factors such as convergent evolution under predation pressure change, since freshwater fish diversity in China exhibits a pronounced north-south divide, with southern regions harboring significantly higher species richness and greater topographic complexity than northern regions (He et al., 2020; Kang et al., 2014; Xing et al., 2015). This heterogeneous environment likely supports a wider array of ecological niches and their associated predators for *Rhinogobius* species in Clade S. Collectively, these findings highlight *Rhinogobius* as a promising model for studying evolutionary reversals, adaptive trait diversification, and the genetic basis of morphological evolution in freshwater fishes.

4.5. Species diversity revealed by phylogenetic, morphological, and geographical evidence

Our study reveals significant cryptic diversity within *Rhinogobius*, some lineages identified here likely represent undescribed species. Firstly, some candidate species are with their close phylogenetic relatives but are readily distinguishable based on morphological traits. Specifically in clade S, *R. sp. XIAO* and *R. zhoui* formed a sister lineage. *Rhinogobius sp. XIAO* processes relatively less second dorsal rays (I-6~8 vs. I-8~9 in *R. zhoui*), more pectoral rays (18~19 vs. 16~18), and larger predorsal scale variability (9 ~ 14 vs. 10 ~ 12). Notably, these two species are distributed closely (*R. sp. XIAO* in Jiexi county vs *R. zhoui* in Haifeng county, both in eastern Guangdong province), but their coloration is notably different. *Rhinogobius sp. XIAO* have no organized patch on body side but their upper snout always have an orange stripe whereas *R. zhoui* have 6 ~ 8 big organized orange patch on lateral side and their snout colored white.

In another case, candidate new species are phylogenetically distant from their morphologically similar counterparts, yet they are found in geographic proximity and share similar coloration patterns. Specifically, *Rhinogobius duospilus* (typically 27 vertebrae, predorsal scales = 10, first dorsal fin = VI) and *R. sp. YLGD* (26 vertebrae, predorsal scales 5 ~ 8, first dorsal fin = V occasionally) are phylogenetically disparate, but they

distributed in two closely located rivers (*R. duospilus* in Rongjiang river vs *R. sp.* YLGD in Hanjiang river of Jieyang City). Conversely, *Rhinogobius duospilus* is clustered with *R. honghensis* (red river basin), and *R. sp.* YLGD is clustered with *R. sangelensis* (Hainan island), both pairs are distant geographically. Although these clusters consistent with meristic character differences, *R. sp.* YLGD shares most coloration similarity with *R. duospilus*.

Furthermore, we found that the species composition within certain clades is notably complex. The recognition of the *R. lentiginis* complex is substantiated by a cohesive monophyletic grouping within Clade N, where *R. lentiginis*, *R. cf. lentiginis*, *R. sp.* CBBSAH and *R. sp.* OSVSAH form a well-supported clade. Despite divergent transversal scale counts between sister taxa *R. lentiginis* (10 ~ 11) and *R. cf. lentiginis* (7 ~ 8), this assemblage shares common traits including near-identical coloration and overlapping meristic ranges. However, *Rhinogobius sp.* LLAH phylogenetically nested in the *R. lentiginis* complex shares less similarities with other species in this group.

In summary, our study unveils a substantial yet under-appreciated diversity within *Rhinogobius*. It is recognized that most fish species occupy restricted local habitats instead of whole river basins (He et al., 2020). Their persistence is increasingly compromised by human-induced habitat fragmentation, dam building, and biological invasions, which collectively degrade freshwater biodiversity (reviewed in Olden et al., 2010). Given their highly restricted distributions and weak dispersal ability, many of *Rhinogobius* lineages constitute distinct Evolutionarily Significant Units (ESUs) (Moritz, 1994) and should be prioritized for conservation attention.

5. Conclusion

This study presents the first comprehensive nuclear phylogenomic framework for *Rhinogobius* in mainland China, resolving two deeply divergent clades that originated during the late Miocene (~9.33 Ma) and subsequent major diversification in the Pliocene. By integrating 2,137 nuclear loci with morphological and geographic distribution data, we demonstrate possible ancient climate fluctuations and paleo-drainage reorganizations influenced current distribution patterns, while revealing both the value and constraints of traditional meristic characters for species delineation. Geographic divergences of predorsal scales and the first dorsal spines consisted with deep phylogeographic divergence indicates both morphological characters have good phylogenetic significance, and can be used as key diagnostic features for the classification of *Rhinogobius*. Our robust molecular phylogeny provides a critical foundation for future taxonomic revisions, particularly for morphologically cryptic lineages within *Rhinogobius*. The pronounced north-south divergence patterns and dynamic trait evolution uncovered here highlight the need for further genomic studies to elucidate the mechanisms of freshwater adaptation. We recommend expanding sampling to underrepresented regions and employing population genomic approaches to test hypotheses about dispersal routes and speciation drivers. Beyond systematics, this framework offers essential insights for conserving China's endemic freshwater biodiversity amid growing anthropogenic threats. Ultimately, our findings underscore the importance of combining molecular and morphological data to decipher complex evolutionary histories in rapidly radiating taxa.

6. Limitation of this study

This study has several limitations. First, taxonomic sampling remains incomplete. More comprehensive surveys with broader geographic coverage and denser intraspecific sampling are needed to fully capture the diversity and clarify the phylogenetic relationships within the genus. Second, the proposed evolutionary scenarios, especially those involving paleo-drainage changes rely on correlative evidence from phylogeny and current species distributions. Direct tests of these biogeographic hypotheses will require integration with paleogeological data and

independent evidence from population genomics or paleoclimate modeling. Finally, species delimitation, particularly within complex clades, would benefit from additional evidence such as detailed morphometrics, ecological data, and genomic-scale markers to confirm species boundaries and resolve recent divergences. Addressing these limitations in future studies will be essential for a resolved and mechanistic understanding of diversification in this group.

Ethical statement

All experimental procedures involving animal specimens were conducted in strict compliance with the Ethical Guidelines for Animal Research established by Shanghai Ocean University (2020). The study protocol was reviewed and approved by the Shanghai Ocean University Animal Ethics Committee (Protocol No. SHOU-DW-2025-019).

CRediT authorship contribution statement

Suhan Liu: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Qinwen Xue:** Writing – review & editing, Methodology, Investigation. **Yun Hu:** Software, Resources, Investigation. **Jiantao Hu:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Yiling Pan:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Yun Bu:** Software, Resources, Investigation. **Jiliang Wang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Jianhong Xia 夏建宏:** Writing – review & editing, Validation, Funding acquisition, Conceptualization. **Chenhong Li 李晨虹:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

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Declaration of Competing 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2026.108577>.

Data availability

Sequence alignment can be found at Mendeley Data, V2, <https://doi.org/10.17632/y7dkpb8cs7.2>. Raw sequence reads are available from the NCBI Sequence Read Archive (SRA), under PRJNA1311246. All other types of the data that support the findings of this study are available in the main text and supplementary files.

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