



New record of the eightbarbel loach, *Lefua costata* (Kessler 1876) in Zhejiang, China, with comments on *Lefua sayu* (Herre and Lin 1936)

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Abstract

In China, the eightbarbel loach *Lefua costata* (Kessler 1876) is widely distributed across northern provinces, with the Huai River as its southern limit. However, anecdotal reports suggested its presence further south in Zhejiang Province. In this study, we collected 17 putative *L. costata* specimens from four Zhejiang sites, alongside 27 specimens from five northern populations for comparison. Analyses included 23 morphometric measurements, five meristic traits, and mitochondrial COI and cytb genes. Morphological characteristics (separated nostrils, tube-like anterior nostrils, absent epural, lower lip with four distinct median papillae) confirmed the Zhejiang specimens as *L. costata*. Phylogenetics showed they represent a distinct southern lineage, sister to the Huang River population within *L. costata*. This disjunct distribution may suggest southeastward glacial dispersal followed by postglacial isolation of this species. The finding also aids in reevaluating the taxonomy of *Lefua sayu* (Herre and Lin 1936), known from a single specimen collected from Zhejiang.

Keywords *Lefua costata* · Taxonomy · Distribution · Morphology · Phylogeny

Introduction

The genus *Lefua* Herzenstein 1888 is a group of small-sized primary freshwater fish encompassing eight valid species, with the majority of its diversity limited to the Japanese archipelago (Ito 2025). Of the eight recognized species, three are native to China, known as *Lefua costata* (Kessler 1876), *Lefua pleskei* (Herzenstein in Warpachowski and Herzenstein 1887), and *Lefua sayu* (Herre and Lin 1936). Among these, *L. pleskei* only has a marginal distribution in China, restricted to the Tumen River basin of Yanbian, Jilin Province (Wang et al. 2021). In contrast, *L. costata* exhibits broader ecological adaptability and occurs widely

in major drainage systems across northern China, including the Heilongjiang River, the Liao River, the Huang River, the Huai River, and the Hai River basins (Zhang 1959; Wang 1984; Zhu 1989; Cheng and Zhou 1997; Wang et al. 2000; Li 2015; Jiang and Bai 2020) with its southernmost reliably recorded locality situated in Ruzhou City, Henan Province (Nie et al. 2019; Wang et al. 2021). Distinct from the northern distribution of widespread *L. costata*, *L. sayu* was recorded from the Qiantang River in southeastern China. Although originally placed in the genus *Oreonectes* Günther 1868 and later transferred to *Lefua* based on the absence of an epural (Prokofiev 2005), this species is known solely from a single type specimen, leaving its generic position uncertain (Ito 2025).

Since around 2010, loaches morphologically similar to *L. costata* have been sporadically collected from Taizhou and Wenzhou in Zhejiang Province by fish enthusiasts. During the wildlife survey of Wenling City from 2020 to 2023, the survey team collected this fish at two sites and reported it as *L. costata* without detailed description (Taizhou News Network 2022); however, Li (2024) reassigned them as *L. sayu* based on their geographic proximity to the latter's type locality. These records from Zhejiang are particularly noteworthy because they are geographically distant from the established range of *L. costata* in northern China, yet raise

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questions about the rediscovery of the mysterious *L. sayu*. Therefore, this study aims to clarify the taxonomic status of the Zhejiang population by integrating morphological and mitochondrial evidence. Our results may also provide new insights into the generic uncertainty of *L. sayu* and the biogeographical history of the genus *Lefua*.

Materials and methods

Field collection and specimen processing. Specimens were collected from four sites in Zhejiang Province and five northern population sites between September 2023 and August

2025 (Table 1). The collection included 17 individuals from Zhejiang and 27 from northern populations. Live fish were anesthetized with eugenol (50 mg/L), euthanized, and then fixed in 10% formalin for 72 h before long-term storage in 75% ethanol. Prior to fixation, a clip from the right pectoral fin of each specimen was preserved in 95% ethanol for genetic analysis. All voucher specimens were cataloged and deposited in the Fish Collection of Shanghai Ocean University (SHOU). For clarity, each specimen is referred to in the text using a code based on its collection site abbreviation and a serial number. Details of collection localities and voucher numbers are provided in Fig. 1, Table 1 and Electronic Supplementary Material (ESM) Fig. S1. For

Table 1 Collection data for *Lefua costata* specimens examined in this study, including localities, populations, voucher numbers, GenBank accession numbers, dates and collectors

Collecting site	Population	Coordinates	No. of specimens	Voucher number of SHOU*	GenBank accessions	Site code	Collecting date	Collector
Danxi Reservoir, Wenzhou	ZJ	28°12'N, 120°57'E	6	SOU1801020	COI: PX397404–PX397406 cytb: PX431690–PX431692	DX	30 June 2025	Shao Y.K.
Yang'ao Village, Wenzhou	ZJ	28°14'N, 120°56'E	2	SOU1801021	COI: PX397402–PX397403 cytb: PX431704–PX431705	YA	30 June 2025	Shao Y.K.
Yandang Town, Wenzhou	ZJ	28°23'N, 121°08'E	5	SOU1801019	COI: PX399406–PX399407 cytb: PX431706–PX431707	YD	28 December 2024	Shao Y.K.
Taihu Reservoir, Taizhou	ZJ	28°29'N, 121°13'E	4	SOU1801022	COI: PX397397–PX397399 cytb: PX431698–PX431700	TH	12 July 2025	Shao Y.K.
Shibaotou Reservoir, Xinxiang	H-H	35°41'N, 113°58'E	5	SOU1801023	COI: PX397408–PX397409 cytb: PX431693–PX431694	HN	3 September 2023	Tang Y.T.
Likou Mountain, Weihai	H-H	37°27'N, 122°04'E	4	SOU1801024	COI: PX397400–PX397401 cytb: PX431701–PX431703	LK	18 July 2025	Shao Y.K.
Biliu River, Dalian	LD	39°59'N, 122°33'E	6	SOU1801025	COI: PX397394–PX397396 cytb: PX431684–PX431686	BL	25 December 2024	Shao Y.K.
Erdao River, Dandong	LD	40°06'N, 124°19'E	7	SOU1801026	COI: PX397389–PX397391 cytb: PX431687–PX431689	ER	8 August 2025	Shao Y.K.
Songfeng Mountain, Harbin	HLJ	45°19'N, 127°24'E	5	SOU1801027	COI: PX397392–PX397393 cytb: PX431695–PX431697	SF	14 August 2025	Shao Y.K.

*Specimens collected from the same locality are registered under the same voucher number, with suffixes distinguishing individuals (e.g., SOU1801020-1, SOU1801021-2) where applicable

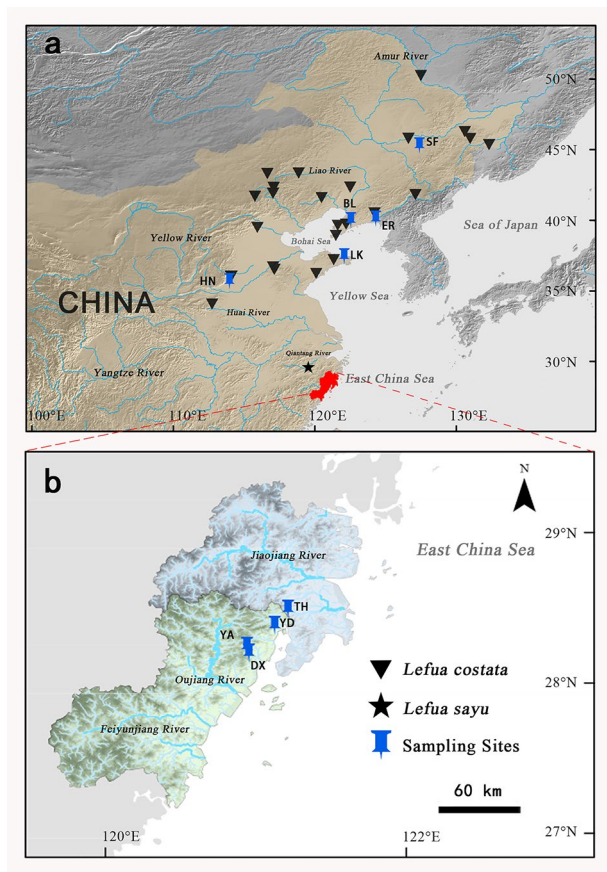
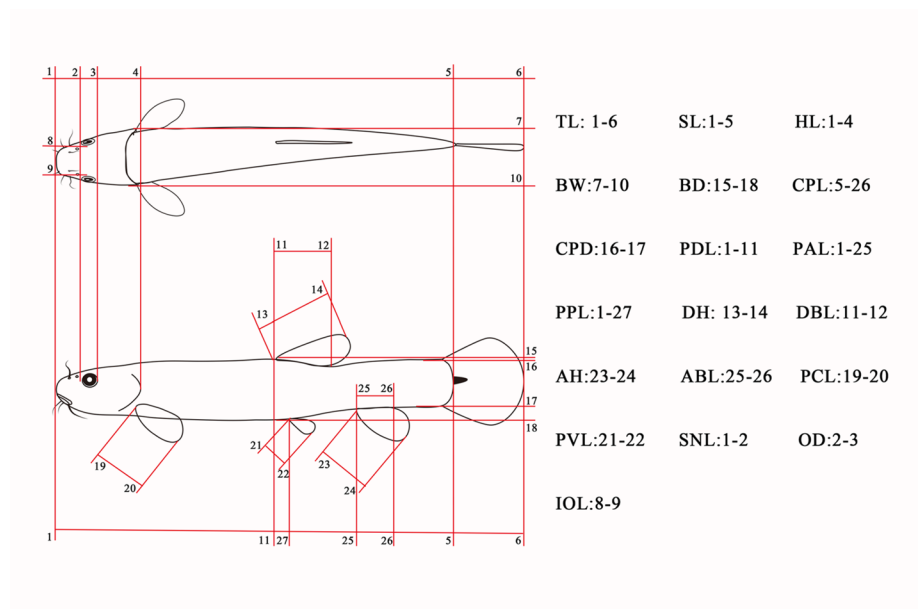


Fig. 1 Geographic distribution and sampling sites of *Lefua costata*. **a** Overall distribution range of *L. costata* in East Asia. **b** Detailed sampling localities in Zhejiang Province. Blue pins represent sampling sites in the present study; black triangles denote reliable historical records of *L. costata* obtained from literature; the black star indicates the approximate distribution of *Lefua sayu*

Fig. 2 Standards for the 19 morphometric measurements conducted in this study. Measurements of the dorsal fin height (DH), anal fin height (AH), and pelvic fin length (PVL) were defined as the length of the longest unbranched ray. Measurements of barbels were not illustrated in this graph



comparative analyses, specimens were categorized into four geographic populations based on their river basins: (1) Zhejiang Coastal Rivers Population (ZJ), composed of specimens from streams in Taizhou and Wenzhou; (2) Huang He Population (H-H), including specimens from Xinxiang and Weihai, both located in Huang River (the Yellow River) basin; (3) Liaodong Coastal Rivers Population (LD), consisting of specimens from Dalian and Dandong; and (4) Heilongjiang Population (HLJ), represented by specimens from the Heilongjiang River basins.

Morphological analyses. We analyzed 23 morphometric variables and five meristic traits. Morphological terminology adheres to the standards set in Fauna Sinica: Osteichthyes—Cypriniformes Volume II (Chen 1998). Morphometric data, including total length (TL), standard length (SL), head length (HL), body width (BW), body depth (BD), caudal peduncle length (CPL), caudal peduncle depth (CPD), pre-dorsal fin length (PDL), pre-anal fin length (PAL), pre-pelvic fin length (PPL), dorsal fin height (DH), dorsal fin base length (DBL), anal fin height (AH), anal fin base length (ABL), pectoral fin length (PCL), pelvic fin length (PVL), snout length (SNL), orbital diameter (OD), interorbital length (IOL), and length of four types of barbels were measured with a digital caliper to the nearest 0.01 mm; or, for smaller structures, under a dissecting microscope using the MShot Image Analysis System v1.1.6 (MShot Optoelectronics Technology Co., Ltd., Guangzhou, China). Measurement standards are illustrated in Fig. 2.

Osteological examination of four specimens (SOU1801019-5, SOU1801022-1, SOU1801025-5, SOU1801027-1) was conducted by X-ray microtomography (voltage 60 kv, current 280 μ A, optimum 2.88 microns; NSI X50, USA). Vertebral counts were recorded as the sum of

the first four vertebrae (comprising the Weberian apparatus), abdominal vertebrae, and caudal vertebrae (including the terminal centrum fused to the urostyle). Fin-ray counts were made under a dissecting microscope and subsequently verified against micro-CT images (ESM Fig. S2). The cephalic sensory system was examined from macro-photographs of the head region.

Morphometric data analysis. To eliminate the effect of size on morphological comparisons, 21 morphometric measurements (excluding TL and SL) were standardized as ratios to SL prior to analysis. As no evident sexual dimorphism in meristic or morphometric characters has been reported for this species (Ohgita and Matsunuma 2020), data from all specimens were pooled for analysis. The analysis was conducted on the size-corrected ratio data using the scikit-learn library (Pedregosa et al. 2011) in Python. The first two principal components (PC1 and PC2) were extracted to visualize the variation in a two-dimensional morphospace. To visualize the distribution range of each lineage, 95% confidence ellipses (calculated based on the chi-square distribution with 2 degrees of freedom) were superimposed on the scatter plot.

DNA extraction, amplification, and sequencing. Genomic DNA was extracted using a commercial kit (Tiangen Biotech, Beijing, China). Two mitochondrial gene fragments, the cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (*cytb*), were amplified using primers specifically designed from the complete mitochondrial genome of *Lefua costata* (GenBank: NC_029385.1; ESM Table S1). PCR conditions were optimized for each reaction (ESM Table S2).

PCR products were verified on 1.5% agarose gels, purified, and sequenced bidirectionally using Sanger sequencing (GENEWIZ Biotechnology Co., Ltd., Suzhou, China). High-quality sequences were obtained for 22 individuals. The resulting chromatograms were assembled, and the forward and reverse reads for each gene were aligned using the Clustal W algorithm (Thompson et al. 1994) in Geneious Prime 2024.0.5. Low-quality base calls at the sequence termini were trimmed manually. The final consensus sequences for each gene were submitted to GenBank; the corresponding accession numbers are listed in Table 1.

Phylogenetic analysis. Sequences of the COI (1,094 bp) and *cytb* (910 bp) genes were concatenated into a single 2,004 bp alignment using Geneious Prime 2024.0.5. We supplemented our data with sequences from a Korean *L. costata* individual (GenBank accessions: EU670769, EU670788) and the complete mitochondrial genomes of *L. costata* (GenBank accessions: NC_029385) and *Lefua nikkonis* (Jordan and Fowler 1903) (GenBank accessions: NC_027662), the latter of which was used as an outgroup for rooting the phylogenetic tree. All reference sequences were aligned with our newly generated data. The final concatenated dataset had a length of 2,004 bp and included 25 sequences. Phylogenetic reconstruction was performed using IQ-TREE v2.0

(Minh et al. 2020) under the ModelFinder option to determine the best-fit substitution model (HKY+G). The resulting maximum-likelihood tree was visualized and annotated using iTOL (Letunic and Bork 2021) and Adobe Illustrator. Genetic distances were calculated based on the Kimura two-parameter (K2P) model (Kimura 1980) in MEGA11 (Tamura et al. 2021).

Haplotype network analysis. Unique haplotypes were identified using DnaSP v6.12.03 (Rozas et al. 2017). A haplotype network was then reconstructed from the concatenated sequences using the TCS algorithm (Clement et al. 2000) implemented in PopART (Leigh and Bryant 2015). The input file was manually annotated to assign each sequence to its respective geographic population defined above (ZJ, H-H, LD, HLJ).

Results

Morphological identification and variation. The Zhejiang specimens exhibited diagnostic morphological characters consistent with *Lefua costata*, including separated anterior and posterior nostrils (Fig. 3g, h), a tube-like extension on the anterior nostril (Fig. 3g, h), the absence of an epural bone (ESM Fig. S2), a lower lip with four distinct median papillae (ESM Fig. S3c), and a blackish longitudinal stripe in males (Fig. 3e; ESM Fig. S4). Meristic counts (e.g., fin rays and vertebrae) also fell within the ranges observed in northern populations (Table 2). Principal component analysis (PCA) based on size-corrected morphometric ratios extracted two main components accounting for 50.72% of the total variance (PC1: 31.25%; PC2: 19.47%; ESM Table S3). The scatter plot revealed a morphological continuum along the first principal component (Fig. 4). The Zhejiang (ZJ) population exhibited a broad range of morphological variation, spanning the morphospace between the Huang He (H-H) and Liaodong (LD) populations. Its 95% confidence ellipse overlapped extensively with those of the H-H population (located mainly along the negative side of PC1) and the LD population (located mainly along the positive side of PC1). Furthermore, the 95% confidence ellipses of H-H and LD populations showed marginal overlap in the intermediate region, supporting the presence of a continuous morphological transition rather than a strict separation. In contrast, the Heilongjiang (HLJ) population formed a relatively distinct cluster differentiated primarily along the second principal component (PC2). It occupied the lower section of the morphospace, showing no overlap with the H-H and ZJ populations and only slight marginal overlap with the LD population, suggesting a clearer morphological divergence compared to the southern and central populations.

It is worth noting that an individual from the Taihu Reservoir (SOU1801022-4) fell outside the 95% confidence



Fig. 3 Live specimens of *Lefua costata* from the Danxi Reservoir, Wenzhou (non-types). **a–c** Dorsal, lateral, and ventral views of a female; **d–f** dorsal, lateral, and ventral views of a male; **g–h** close-

up views of snout region of two individuals; *thin arrows* indicate the positions of anterior and posterior nostrils. *an* Anterior nostril, *pn* posterior nostril

ellipse of the ZJ population. This deviation is likely attributed to allometric effects, as this specimen is relatively small (20.2 mm SL) in individuals examined in this study.

Molecular phylogeny. The maximum-likelihood phylogeny, based on concatenated COI and *cytb* sequences, is presented in Fig. 5. All Zhejiang specimens formed a well-supported subclade (bootstrap = 87%) within the larger *L. costata* clade. This Zhejiang subclade was recovered as the sister lineage to the Huang-He population and one haplotype (Hap_9) from the Heilongjiang population, and together they constituted a highly supported clade (bootstrap = 100%) distinct from other northern lineages. Genetic distances, calculated using K2P model, were as follows: 0.000–0.025 within the major *L. costata* clade, and 0.077–0.084 between *L. costata* and *L. nikkonis*.

Haplotype network. The TCS network, based on 22 concatenated sequences, revealed 16 distinct haplotypes with no shared haplotypes among the four geographic populations (Fig. 6). The network displayed a multi-branched, star-like topology, clearly separating the Zhejiang (ZJ) population from the northern populations. Within the ZJ lineage, two subgroups were evident: one containing specimens from various Wenzhou sites (Group 1), and another comprised exclusively of individuals from Taizhou (Group 2). Among the northern populations, haplotypes from the Liaodong (LD) and Heilongjiang (HLJ) populations formed a group (Group 3), within which the Dalian (LD population) haplotypes clustered with those from HLJ population, while the Dandong (LD population) haplotypes were distinct. A separate group (Group 4) grouped a single HLJ haplotype with haplotypes from Weihai and Xinxiang (the Huang He population). This topology indicates restricted gene flow and suggests long-term geographic isolation between the southern (Zhejiang) and northern populations.

Discussions

Taxonomic status and phylogenetic position of the Zhejiang population. Based on integrated morphological and phylogenetic evidence, we propose that the newly discovered Zhejiang population represents a distinct southern population of *Lefua costata*. Morphologically, we identify these specimens as belonging to the genus *Lefua* based on key characteristics: (1) separated anterior and posterior nostrils (Fig. 3g, h); (2) a posteriorly extended, tube-like anterior nostril (Fig. 3g, h); and (3) the absence of epural bone (ESM Fig. S2). At the species level, the Zhejiang specimens possess four distinct median papillae on the lower lip (ESM Fig. S3c), a stable diagnostic character that clearly distinguishes them from *L. pleskei*, which is characterized by a thinner lower lip with inconspicuous median papillae (Wang et al. 2021). Additionally, no clear differences in meristic characters were observed between the Zhejiang population and Northern populations of *L. costata*. This morphological consistency is further supported by the PCA result (Fig. 4), which reveal that the two groups cannot be completely distinguished due to broad overlap, reinforcing its classification as *L. costata*.

Phylogenetically, the Zhejiang specimens formed a well-supported subclade within the larger *L. costata* clade. Critically, the genetic divergence within *L. costata* (K2P distance: 0.000–0.025) was substantially lower than the interspecific divergence between *L. costata* and *L. nikkonis* (0.077–0.084). This supports the classification of the Zhejiang population as a distinct geographic lineage of *L. costata* rather than a separate species. This conclusion is reinforced by the haplotype network, which showed strong phylogeographic structure with no haplotypes shared among regions, consistent with the limited dispersal capability previously reported for *L. costata* (Yang et al. 2021).

Table 2 Morphometric measurements and meristic counts for 44 specimens of *Lefua costata*, locality abbreviations correspond to those in Table 1

Population	Zhejiang (<i>n</i> = 17)					Northern (<i>n</i> = 27)				
	YD	DX	YA	TH		HN	LK	ER	BL	SF
No. of specimens	5	6	2	4	Modes	5	4	7	6	5
Dorsal fin rays	iii + 6 or 7	iii + 6 or 7	iii + 6	iii + 6	iii + 6	iii + 6 or 7	iii + 6 or 7	iii + 6	iii + 6	iii + 6
Pectoral fin rays	i + 9 or 10	i + 9 or 10	i + 9	i + 9	i + 9	i + 9 or 10	i + 9 or 10	i + 9	i + 9 or 10	i + 10
Anal fin rays	iii + 5	iii + 5	iii + 5	iii + 5	iii + 5	iii + 5	iii + 5	iii + 5	iii + 5	iii + 5
Pelvic fin rays	i + 5	i + 5	i + 5	i + 5	i + 5	i + 5	i + 5	i + 5 or 6	i + 5	i + 5
Total vertebrates*	4 + 18 + 17	–	–	4 + 17 + 17	–	–	–	–	4 + 20 + 16	4 + 18 + 16
Total length (mm)	41.7–54.4	31.3–35.7	28.1–30.3	25.1–32.5	Means	44.1–50.9	54.6–63.1	40.0–54.5	43.3–56.0	50.6–69.0
Standard length	34.7–46.6	26.4–30.3	23.1–25.3	20.2–27.9	30.8	36.5–43.2	45.5–52.1	33.2–43.1	34.2–46.8	42.6–55.8
Head length (% of SL)	18.4–20.9	17.3–20.1	17.6–18.3	20.6–23.3	19.8	20.9–22.3	20.4–23.7	20.4–22.5	21.7–25.1	19.3–23.4
Snout length	4.5–7.2	4.7–7.8	5.1–5.2	6.1–9.2	6.3	6.4–7.7	6.8–7.3	6.6–7.4	7.2–8.8	7.2–8.2
Body width	11.9–12.3	10.0–12.3	11.0–12.0	12.2–13.4	11.9	10.6–11.8	11.3–14.5	9.9–12.1	11.8–12.6	6.8–9.4
Body depth	13.8–16.8	11.2–14.2	11.9–12.7	13.1–14.7	14.1	11.2–11.8	12.7–15.3	9.0–14.0	13.1–14.5	8.5–10.5
Caudal peduncle length	13.2–15.8	14.6–16.5	14.7–15.7	11.5–15.2	14.8	13.8–16.7	14.5–16.0	13.0–17.5	12.2–15.2	13.9–17.8
Caudal peduncle depth	11.7–12.9	10.4–12.4	12.0–12.7	12.6–14.3	12.3	10.3–11.3	11.5–13.5	9.1–11.9	9.6–12.0	6.2–9.4
Pre-dorsal-fin length	59.9–62.7	60.1–65.2	59.4–67.6	60.6–64.5	61.9	55.6–58.0	59.2–63.6	56.1–62.9	57.2–64.1	58.1–62.3
Pre-anal-fin length	76.5–80.1	73.6–82.9	79.7–83.8	72.2–86.4	78.3	72.4–77.2	74.0–78.2	76.5–83.8	74.7–81.7	73.9–78.7
Pre-pelvic-fin length	53.9–57.6	52.6–57.6	58.7–58.7	56.6–63.5	56.0	49.1–55.3	54.7–58.4	55.8–61.9	56.4–60.3	53.8–59.3
Height of dorsal fin	9.9–11.1	11.8–13.3	12.7–14.7	11.6–13.2	12.0	9.8–11.6	12.0–13.2	12.2–15.8	12.0–14.5	10.0–12.7
Length of dorsal fin base	8.1–9.0	7.8–9.0	8.9–11.5	8.9–9.3	8.9	8.2–9.0	9.0–9.5	8.6–11.0	9.2–11.2	8.0–11.0
Height of anal fin	7.5–10.5	9.3–12.0	9.2–14.2	10.4–10.5	10.5	8.5–9.1	10.0–11.2	10.3–13.0	10.6–11.1	12.2–14.4
Length of anal fin base	6.1–7.1	6.5–7.8	7.2–8.0	5.7–7.0	7.0	6.6–7.1	6.9–7.5	6.1–7.8	7.0–9.2	7.1–8.5
Pectoral fin length	11.8–13.6	14.9–16.2	14.0–15.7	16.0–16.7	14.6	11.5–15.6	13.8–15.0	12.5–17.5	15.3–16.5	13.3–14.7
Pelvic fin length	8.7–9.8	9.6–12.7	9.6–10.4	10.0–10.7	10.2	8.3–9.8	10.0–11.4	10.0–12.7	10.0–13.2	10.8–13.0
Orbit diameter	4.0–5.0	4.1–4.8	4.3–5.6	4.4–4.6	4.6	3.2–3.6	3.2–4.2	4.4–5.3	4.9–5.4	3.6–4.0
Interorbital length	7.6–8.2	8.0–9.7	9.4–12.8	9.7–10	9.1	7.9–9.4	9.4–10.6	8.8–10.1	7.5–9.4	8.1–9.5
Nasal barbel length	5.0–6.0	6.7–7.6	6.5–6.9	6.5–8.3	6.6	5.6–6.6	5.4–6.4	5.4–6.4	5.0–6.1	4.0–5.8
Inner rostral barbel length	3.9–4.7	6.0–6.8	5.8–6.4	5.5–5.7	5.6	5.6–6.3	5.3–6.9	4.4–6.1	4.3–5.2	4.1–5.3
Outer rostral barbel length	5.1–5.5	5.6–6.4	6.4–7.0	5.7–6.4	5.9	6.1–6.6	5.5–7.2	4.3–6.1	5.4–7.1	4.0–5.8
Mandibular barbel length	6.3–6.9	7.5–9.3	7.2–7.3	8.0–8.4	7.7	6.7–7.4	5.4–8.7	5.6–8.0	6.8–7.9	4.6–7.2

*Only for the 4 specimens with a micro-CT scanning model

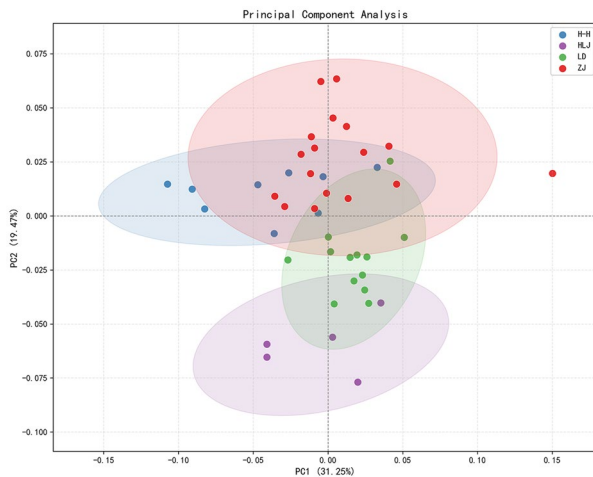


Fig. 4 Principal component analysis (PCA) of *Lefua costata* populations based on 21 standardized morphometric measurements. Colors indicate geographic populations: Zhejiang (ZJ, red), Huang He (H-H, blue), Liaodong (LD, green), and Heilongjiang (HLJ, purple). Shaded ellipses represent 95% confidence ellipses

An expansion of the distribution records of *L. costata* in China. This study confirmed eastern Zhejiang Province as a newly recognized distribution area of *L. costata* in China. The discovery of the *L. costata* population in Zhejiang is significant, as it lies at least 500 km south of the species' known distribution range. We also note a reported occurrence of *L. costata* in Sichuan Province (Guo 2021). However, species of *Lefua* had never been recorded in Sichuan during previous surveys (Ding 1994; Zhang et al. 2019) and we were unable to obtain specimens, so its validity deserves further investigation.

The discovery of *L. costata* in Zhejiang provides critical new material for reconstructing the biogeographic history of *L. costata*. While previous studies have outlined the colonization of *L. costata* in northern China via the Liaodong

Peninsula (Yang et al. 2021), our phylogenetic results reveal a novel, highly supported clade uniting the Huang-He and Zhejiang populations. We hypothesize that this topological pattern might reflect a southward dispersal event, potentially facilitated by coastal plains exposed during glacial sea-level lowstands. However, as divergence time estimation was not conducted in the present study, we cannot definitively link this lineage splitting to specific glacial cycles. Nevertheless, this lineage may have been subsequently isolated in southern refugia. Therefore, the Zhejiang population represents an excellent model for future studies incorporating molecular dating to investigate glacial paleogeography and calibrate the evolutionary history of *Lefua*.

A comment on *Lefua sayu*. This study confirms that the new record of species of *Lefua* in Zhejiang represents *L. costata* rather than the rediscovery of *L. sayu*, while the generic position of *L. sayu* still needs reevaluation. Our mitochondrial data confirm the Zhejiang population as a distinct, naturally occurring lineage of *L. costata*, which refutes the possibility of its identity as a rediscovery of *L. sayu*. Although the precise phylogenetic position of *L. sayu* remains unresolved molecularly due to the risks of destructive sampling on the unique holotype and the technical challenges associated with uncertain DNA preservation, distinct morphological disparities across morphometric and phenotypic dimensions distinguish it from our specimens.

In terms of morphometric characters, the Zhejiang specimens differ from the holotype of *L. sayu* in several body proportions (Table 3). The most prominent difference lies in orbit diameter, where the Zhejiang specimens possess a markedly larger eye compared to the holotype of *L. sayu* (18.4–26.6% HL vs. 13.9%). Additionally, distinct differences are observed in fin lengths; our specimens are characterized by shorter paired fins (pectoral fin: 11.8–16.7% SL vs. 19.0%; pelvic fin: 8.7–12.7% SL vs. 14.6%) and a lower anal fin height (7.5–14.2% SL vs. 17.5%). These quantitative disparities may suggest a divergence in body shape, although

Fig. 5 Maximum likelihood phylogeny of *Lefua costata* based on a 2,004 bp concatenated alignment of the mitochondrial COI and cytb genes. Numbers at nodes indicate bootstrap support values (%). The sequence labeled "KO01" was obtained from a Korean individual (GenBank accessions: EU670769, EU670788)

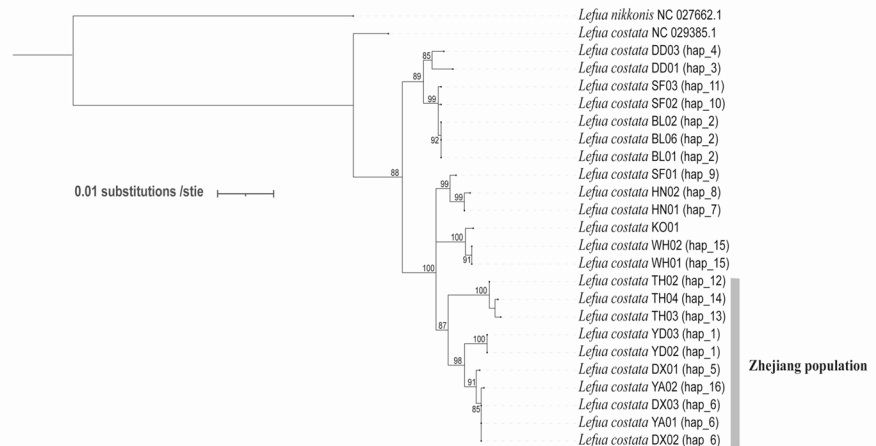


Fig. 6 Haplotype network of *Lefua costata* based on concatenated mitochondrial COI and *cytb* sequences (2,004 bp). Each circle represents a unique haplotype, with its size proportional to its frequency. Colors correspond to geographic populations: Zhejiang (ZJ, red), Huang He (H-H, blue), Liaodong (LD, green), and Heilongjiang (HLJ, purple). Tick marks on the branches denote mutational steps between haplotypes

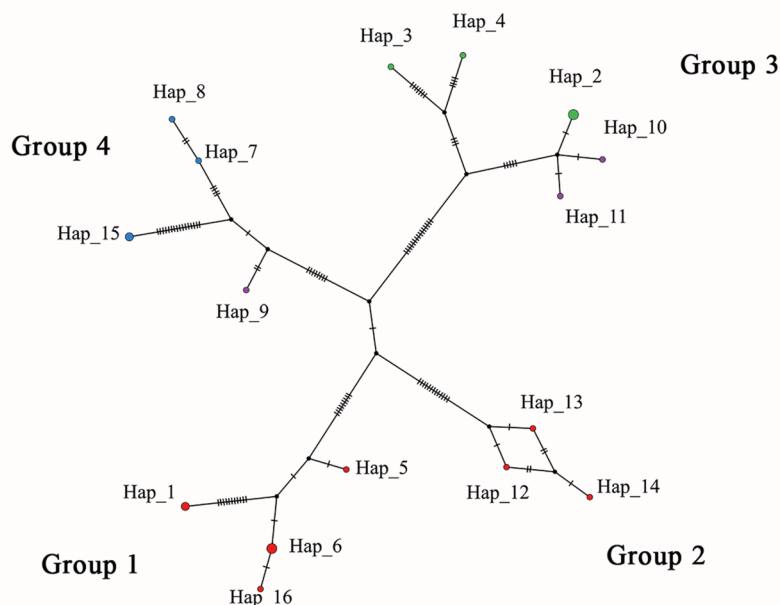


Table 3 Morphometric comparisons for Zhejiang population of *Lefua costata* and holotype of *Lefua sayu*

Species	<i>Lefua costata</i> (n = 17)	<i>Lefua sayu</i> (n = 1)
In % of SL		
Head length	17.3–23.3	20.0
Body width	10.0–13.4	11.2
Body depth	11.2–16.8	15.8
Caudal peduncle length	11.5–16.5	15.3
Caudal peduncle depth	10.4–14.3	11.9
Pre-dorsal-fin length	59.4–64.5	61.6
Pre-anal-fin length	72.2–86.4	70.6
Pre-pelvic-fin length	52.6–63.5	53.1
Height of dorsal fin	9.9–14.7	13.6
Length of dorsal fin base	7.8–11.5	10.6
Height of anal fin	7.5–14.2	17.5
Length of anal fin base	5.7–8.0	8.2
Pectoral fin length	11.8–16.7	19.0
Pelvic fin length	8.7–12.7	14.6
In % of head length		
Orbit diameter	18.4–26.6	13.9
Snout length	21.5–40.0	38.4
Interorbital length	36.5–56.9	41.2
Inner rostral barbel length	19.3–36.1	17.6
Outer rostral barbel length	24.5–39.8	41.7
Mandibular barbel length	32.5–49.2	34.3

Data for *L. sayu* are taken from Ito (2025)

the extent of intraspecific variation in *L. sayu* (known only from the holotype) remains to be assessed.

Specifically regarding anatomical structures, *L. sayu* is described as possessing cephalic lateral line pores (Ito 2025), whereas our specimens completely lack cephalic lateral line pores but with numerous neuromasts (ESM Fig. S3). Furthermore, the orofacial morphology differs markedly; *L. sayu* has a smooth lower lip with a distinct median notch, in contrast to the four distinct median papillae and absence of a notch observed in our specimens (ESM Fig. S3c). Another key difference is the structure of the anterior nostril: in *L. sayu*, the opening is positioned approximately midway along the nasal barbel tube (Ito 2025), whereas in our specimens, it is located at the base of the nasal barbel. These consistent morphological distinctions strongly indicate that the Zhejiang population is not conspecific with *L. sayu*.

The placement of *L. sayu* within *Lefua* relies primarily on the absence of an epural bone (Prokofiev 2005; Ito 2025). However, we caution that relying solely on this single character for generic diagnosis may not be robust, as the loss of the epural bone is known to occur sporadically and independently within Nemacheilidae (Prokofiev 2010). Furthermore, the taxonomy of *Oreonectes* has advanced significantly in recent years, characterized by the description of new and cryptic species and substantial generic revisions (Du et al. 2023; Luo et al. 2024; Liao et al. 2025). Consequently, diagnostic criteria distinguishing these genera in older literature may face limitations, and a broader morphological comparison between species of *Lefua* and *Oreonectes* is warranted to clarify their boundaries. Ultimately, definitive resolution of

the taxonomic status of *L. sayu* is hindered by the persistent lack of additional specimens (Zhu 1989; Chen et al. 1990; Chu 2022). As the absence of recent records may reflect the species' cryptic habits rather than extinction, future research must prioritize targeted collection efforts near the type locality to secure fresh material for both detailed morphological re-evaluation and molecular verification.

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Declarations

Conflicts of interest The author declares no conflict of interest.

Ethics approval All experimental procedures, including specimen handling, followed the Protocol of the Ethics Committee for the Use of Animal Subjects of Shanghai Ocean University (2020), China.

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