





# An unexpected discovery of a new species of *Jenynsia* (Cyprinodontiformes: Anablepidae)

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Submitted July 17, 2025  
Accepted November 17, 2025  
Epub April 27, 2026

Associate Editor  Paulo Lucinda  
Section Editor  William Crampton  
Editor-in-chief  José Birindelli

Specimens referred to *Jenynsia* (*Plesiojenynsia*) *unitaenia* from the Tramandaí-Mampituba ecoregion were genetically compared in order to obtain their phylogeographic relationships. Molecular analysis estimated that populations from rio Mampituba and rio Araranguá diverged from rio Maquiné and rio Três Forquilhas populations during the Pleistocene (about 1.2 million years ago). Further, morphological analysis allowed the diagnosis of these two lineages based on the color pattern, as is common among the species of the subgenus *Plesiojenynsia*. Thus they are referred to as separate species: *Jenynsia unitaenia* restricted to the rio Araranguá and rio Mampituba, and a new species from rio Maquiné and rio Três Forquilhas. The pattern of historical relationships of these species of *Jenynsia* among Tramandaí-Mampituba ecoregion drainages differs from those described for other endemic fish taxa.

**Keywords:** *Jenynsia unitaenia*, Paleodrainage, Phylogeography, *Plesiojenynsia*, Tramandaí-Mampituba ecoregion.

Online version ISSN 1982-0224  
Print version ISSN 1679-6225

Neotrop. Ichthyol.  
vol. 24, no. 1, 2026

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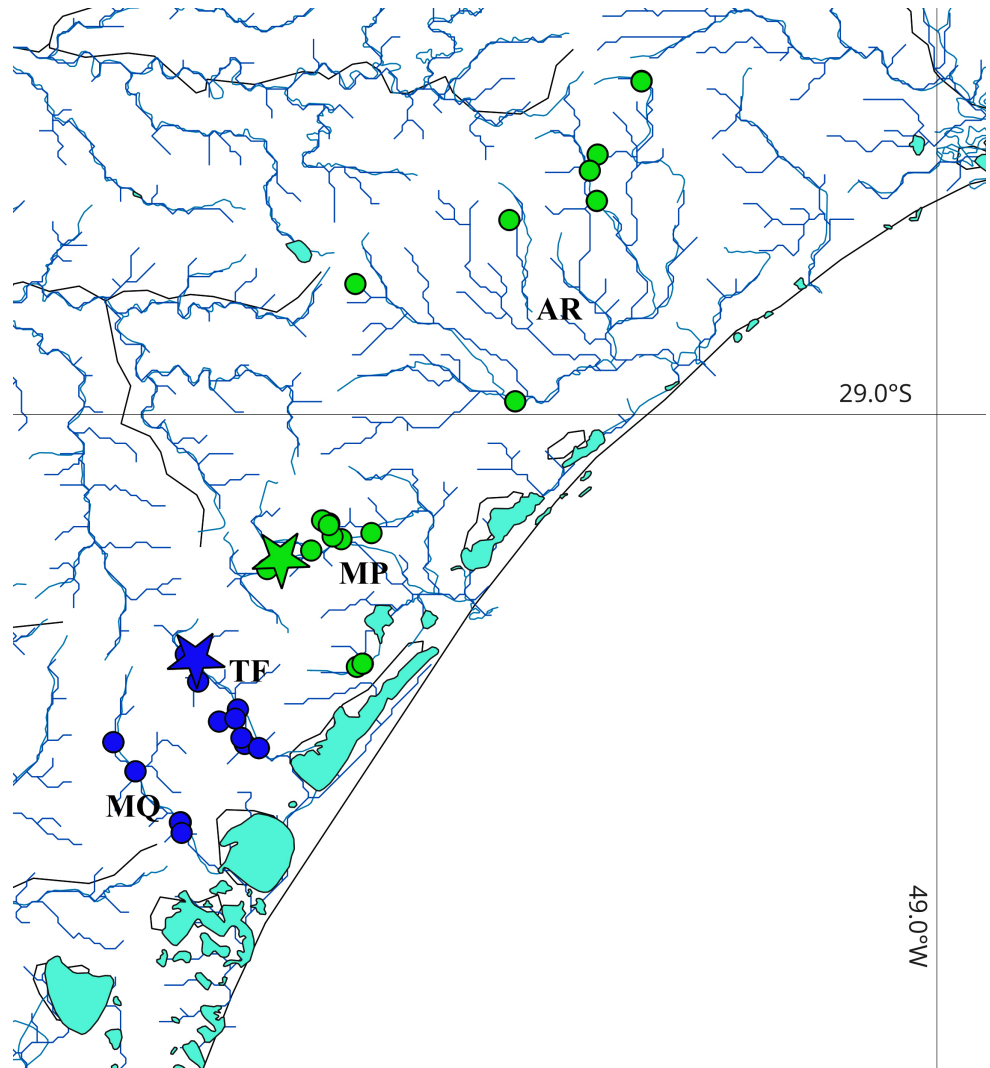
Espécimes referidos como *Jenynsia* (*Plesiojenynsia*) *unitaenia* da ecorregião Tramandaí-Mampituba foram comparados geneticamente com o objetivo de recuperar suas relações filogeográficas. A análise molecular estimou que as populações dos rios Mampituba e Araranguá divergiram das populações dos rios Maquiné e Três Forquilhas durante o Pleistoceno (há cerca de 1,2 milhão de anos). Além disso, a análise morfológica permitiu o diagnóstico dessas duas linhagens com base no padrão de colorido, como usualmente empregado nas espécies do subgênero *Plesiojenynsia*, sendo assim referidas como espécies distintas: *Jenynsia unitaenia* restrita aos rios Araranguá e Mampituba, e uma nova espécie dos rios Maquiné e Três Forquilhas. O padrão de relações históricas destas espécies de *Jenynsia* entre as bacias da ecorregião Tramandaí-Mampituba difere daqueles descritos anteriormente para outras espécies de peixes endêmicas destas bacias.

**Palavras-chave:** Ecorregião Tramandaí-Mampituba, Filogeografia, *Jenynsia unitaenia*, Paleodrenagem, *Plesiojenynsia*.

## INTRODUCTION

The genus *Jenynsia* Günther, 1866 presently contains 16 species assigned to the subgenera *Jenynsia* (11 species) and *Plesiojenynsia* Ghedotti, 1998 (5 species) (Ghedotti, 1998; Aguilera *et al.*, 2013, 2019). Species of the subgenus *Jenynsia* are typically associated with fine-grained sand sediments, often in estuarine areas, while species of the subgenus *Plesiojenynsia* are found in clear-water streams associated with basalt beds located in the southern portion of the Serra Geral formation in Brazil. The subgenus *Plesiojenynsia* includes *Jenynsia unitaenia* Ghedotti & Weitzman, 1995 (type locality in the rio Mampituba drainage), *J. diphyes* Lucinda, Ghedotti & da Graça, 2006 (type locality at rio Iguaçu drainage, Paraná, Brazil), *J. eigenmanni* (Haseman, 1911) (type locality at rio Iguaçu drainage, Brazil), *J. eirmostigma* Ghedotti & Weitzman, 1995 (type locality at rio Uruguay drainage), and *J. weitzmani* Ghedotti, Meisner & Lucinda, 2001 (type locality at rio Tubarão drainage, Santa Catarina State, Brazil).

The Tramandaí-Mampituba ecoregion proposed by Abell *et al.* (2008) has been recognized by a high endemism of freshwater fish species (Malabarba, Isaia, 1992; Reis, Schaefer, 1998; Hirschmann *et al.*, 2015, 2017; Thomaz *et al.*, 2015; Angrizani, Malabarba, 2018) and embraces the rio Araranguá, rio Mampituba, rio Três Forquilhas and rio Maquiné drainages (Fig. 1). This ecoregion is especially interesting with respect to its biogeographic history and speciation, braced on the range of alternative diversification scenarios exhibited by endemic lineages found in this ecoregion (*e.g.*, *Bryconamericus lethostigmus* (Gomes, 1947) by Hirschmann *et al.*, 2017; *Diapoma itaimbe* (Malabarba & Weitzman, 2003) by Hirschmann *et al.*, 2015; *Hollandichthys Eigenmanni*, 1909 by Bertaco, Malabarba, 2013 and Thomaz *et al.*, 2015; *Epactionotus* Reis & Schaefer, 1998 by Delapieve *et al.*, 2020). We herein analyze mitochondrial DNA and morphological data to determine the relationships among four populations of *Jenynsia unitaenia*, *sensu* Ghedotti, Weitzman (1995), endemic to Tramandaí-Mampituba ecoregion.



**FIGURE 1** | Rio Araranguá (AR), rio Mampituba (MP), rio Três Forquilhas (TF) and rio Maquiné (MQ) drainages forming the Tramandai-Mampituba ecoregion, showing the collection locations of analyzed samples of *Jenynsia aurea* (blue) and *J. unitaenia* (green). Stars indicate type-localities.

## MATERIAL AND METHODS

**Taxonomic sampling and DNA sequencing.** Tissue samples for DNA extraction were fixed and maintained in 96% ethanol. Voucher specimens were fixed in 96% ethanol or 10% formalin and transferred to 70% ethanol for permanent storage. Voucher and tissue samples are deposited in the Laboratório de Ictiologia, Departamento de Zoologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil (UFRGS). Additional comparative material belongs to Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil (MCP), and National Museum of Natural History, Smithsonian Institution, Washington, DC, USA (USNM).

*Jenynsia unitaenia* was described by Ghedotti, Weitzman (1995) based on paratypes from the rio Araranguá (AR), rio Mampituba (MP - also the type locality), and rio

Três Forquilhas (TF), and specimens from all these drainages were included in both molecular and morphological analyses. Additionally, we have added samples from the rio Maquiné (MQ) that forms the Tramandaí–Mampituba ecoregion (Abell *et al.*, 2008), along with previously cited drainages. DNA sequences of other species of the subgenus *Plesiojenynsia* found in adjacent drainages (*Jenynsia eirmostigma* and *J. weitzmani*) and of species of the subgenus *Jenynsia* (*J. onca* Lucinda, Reis & Quevedo, 2002; *J. sanctacatarinae* Ghedotti & Weitzman, 1996; and *J. lineata* (Jenyns, 1842)) were used as outgroups in the molecular analysis (Tab. S1).

Extraction of genomic DNA followed a modified CTAB protocol (Doyle, Doyle, 1987). We used PCR the cocktail primers C\_FishF1t1–CFishR1t1 (Ivanova *et al.*, 2007) to amplify the cytochrome oxidase subunit I (COI) gene. PCR reactions included 20  $\mu$ L reactions containing 10–50 ng DNA, 0.2  $\mu$ L of each primer, 0.2  $\mu$ L of each dNTP, 19 Buffer, 1.5  $\mu$ L MgCl<sub>2</sub> and 1-unit Platinum Taq DNA polymerase (Invitrogen, São Paulo, SP, Brazil). The PCR consisted of 4 min at 95 °C (initial denaturation) followed by 30 cycles of 30 s at 95 °C (denaturation), 30–60 s at 52–54 °C (hybridization) and 30–60 s at 72 °C (nucleotide extension). PCR products were checked by electrophoresis in 1% agarose gel, purified using EXOSAP (Exonuclease I and Shrimp Alkaline Phosphatase GE Healthcare, Piscataway, NJ, USA) and sequenced in both directions by ACTGene (Porto Alegre, Brazil).

**Alignment and phylogenetic analyses.** Forward and reverse chromatogram reads were assembled and visualized using Geneious v. 8.0 (Biomatters Ltd., Auckland, New Zealand), and the consensus sequences were obtained. For species delimitation and phylogenetic analyses, we downloaded 40 COI sequences of *Jenynsia* from GenBank (<https://www.ncbi.nlm.nih.gov>) generated by previous studies (Rosso *et al.*, 2012; Díaz *et al.*, 2016; Amorim, 2018). Sequences comprising the final matrix were aligned with 649 bp, and 75 taxa using Muscle software (Edgar, 2004), within Geneious v. 8.0, under default parameters. Vouchers, locality information and GenBank accession numbers are summarized in the Tab. S1. Basic statistics, such as nucleotide (p) and haplotype diversity (hd) as well as neutrality tests were calculated in the software DNASP v. 5 (Librado, Rozas, 2009).

We also constructed haplotype networks with the median-joining method (MJN) (Bandelt *et al.*, 1999) using the program network v. 4.1.0.8 ([www.fluxus-engineering.com](http://www.fluxus-engineering.com)). Calculation of F-statistics ( $\Phi$ ST) and Analysis of Molecular Variance (AMOVA) were carried out in the program ARLEQUIN 3.5 (Excoffier *et al.*, 2005). For these analyses, individuals sampled in the same river drainage were merged into a single population to quantify the amount of genetic structure amongst them. To test if MQ and TF may represent isolated populations, even though they are connected through freshwater coastal lagoons in the rio Tramandaí drainage, we kept them as two distinct populations. Thus, the analysis considered one group with four populations.

Phylogenetic relationships among populations of *J. unitaenia* and between *J. unitaenia* and outgroups were inferred by Bayesian inference (BI) and species tree analysis. PartitionFinder v. 1.1.0 (Lanfear *et al.*, 2012) was utilized to select the best-fit model of nucleotide evolution to be used in downstream analyses, and was partitioned by codon positions (1° codon – K80+G; 2° codon – HKY, 3° codon – TrN+G). Beast v. 2 (Drummond *et al.*, 2012) was utilized to estimate the ultrametric gene and species tree under the exponential growth coalescent model (Griffiths, Tavare, 1994) and the

lognormal relaxed clock model (Drummond *et al.*, 2006), assuming that the rates of molecular evolution are uncorrelated but log-normally distributed among lineages. In both analyses, Markov chains included a total of 30 million generations, sampling trees every 3,000 generations to obtain a total of 10,001 trees. Tracer v. 1.5 (Rambaut *et al.*, 2014) was used to examine the average standard deviation of split frequencies (<1%) and the convergence of MCMC searches. The first 1 million generations (10%) were discarded as burn-in and the remaining trees were used to summarize the results of the Bayesian analysis, using the maximum clade credibility tree from the posterior distribution in TreeAnnotator BEAST v. 2.

Estimates of Evolutionary Divergence between sequences were conducted using the Kimura 2-parameter model Kimura (1980). This analysis involved 75 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 494 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). Phylogenetic relationships were inferred by Bayesian Inference (BI) using a gene tree and carried out in MrBayes v. 3.2.2 (Ronquist *et al.*, 2012).

**Morphological data.** Measurements were taken as straight lines with digital calipers. All morphometric comparisons among populations were performed separately for males and females, as distinguished by the presence of a gonopodium in males and a ventrolateral dark spot above the genital opening in females. We further coded *Jenynsia aurea*, new species, based on the character list given by Aguilera *et al.* (2019: S1 tab.) and compare to the morphological character matrix given by Amorim (2018) and Aguilera *et al.* (2019: S1 tab.) in order to check for possible additional characters to diagnose *J. aurea* from other species of the subgenus *Plesiojenynsia*.

Meristic data were taken using a stereomicroscope, including nine counts: total number of dorsal fin rays; total number of anal fin rays; total number of pelvic fin rays; total number of pectoral fin rays; number of main rays of the caudal fin, including the branched rays plus two unbranched rays, these corresponding to the upper and lower margins of the caudal fin; number of predorsal scales; number of scales on the upper lateral line (L1); number of scales on the lower lateral line (L2); number of scales around the caudal peduncle. The perforated scales of the lateral line are presented here as two counts (L1 and L2) as they are distributed in two distinct longitudinal series, unlike the description given by Ghedotti, Weitzman (1995), where the two series are presented as a single score.

Nomenclature of sensory canal system follows Gosline (1949). Osteological data, number of vertebrae, gill rakers, and anal-fin rays of males were obtained from cleared and stained specimens (c&s) prepared according to Taylor, Van Dyke (1985). In the descriptions, values marked with an asterisk represent the values of the holotype. The compound caudal centrum was counted as one caudal vertebra; the number of gill rakers in the lower branch includes the raker at angle between the lower and upper branches. Osteological images were done based on photographs taken in a stereomicroscope with a Nikon AZ100M camera attached.

## RESULTS

*Jenynsia aurea*, new species

urn:lsid:zoobank.org:act:440E8BD9-0603-4866-BCB7-3A54465F6B78

(Figs. 2–6; Tabs. 1, 2)

*Jenynsia unitaenia* Ghedotti, Weitzman, 1995:943 (in part; one lot of paratypes from rio Três Forquilhas, USNM 326104, belongs to *Jenynsia aurea*). —Malabarba *et al.*, 2013:103 (identification guide; photo; diagnosis; map of occurrence in the rio Maquiné and rio Três Forquilhas). —Bertaco *et al.*, 2016:423, tab. 1 (listed paratypes USNM 326104 from rio Três Forquilhas as vouchers of the occurrence in the rio Tramandaí drainage).

**Holotype.** UFRGS 29990, 56.7 mm SL, TEC 2867, Brazil, Rio Grande do Sul, Itati, arroio Carvalho, tributary of rio Três Forquilhas, rio Tramandaí system, 29°23'25"S 50°11'02"W, 6 Jun 2012, L. R. Malabarba, J. Ferrer, L. Artioli, P. Neto-Carvalho.

**Paratypes.** All from Brazil, Rio Grande do Sul. UFRGS 16534, 1, 52.5–66.7 mm SL, TEC 2867, collected with the holotype. MCP 10779, 33, 27.4–68.2 mm SL, 2 c&s, 56.5–62.0 mm SL, Torres, creek tributary of rio Três Forquilhas, Chapéu, 29°32'00"S 50°05'00"W, 25 May 1986, C. A. Lucena, L. R. Malabarba & R. E. Reis. MCP 25663, 14, 34.6–63.7 mm SL, Itati, arroio Carvalho, tributary of rio Três Forquilhas, 29°23'00"S 50°12'00"W, 21 Mar 2000, W. Bruschi Jr. & G. Vinciprova. MCP 25292, 41, 20.7–66.9 mm SL, Terra de Areia, rio Três Pinheiros, 29°31'36"S 50°06'21"W, 29 Dez 1999, L. R. Malabarba, J. P. Silva, V. A. Bertaco & T. Borges. MCP 25306, 19, 21.4–66.9 mm SL, Terra de Areia, creek between Terra de Areia and Itati, 29°31'01"S 50°06'40"W, 29 Dec 1999, L. R. Malabarba, J. P. Silva, V. A. Bertaco & T. Borges. UFRGS 2995,



**FIGURE 2** | *Jenynsia aurea*, UFRGS 29990, holotype, male, 56.7 mm SL, Brazil, Rio Grande do Sul, Itati, arroio Carvalho, rio Três Forquilhas drainage.



**FIGURE 3** | Color in life of *Jenynsia aurea*. All figured specimens are paratypes. (A) MCP 25306, male, 50.9 mm SL, (B) MCP 25292, female, 66.9 mm SL, Brazil, Rio Grande do Sul, creek on the road between Terra de Areia and Itati; (C) UFRGS 26517, immature, 35.7 mm SL, (D) UFRGS 26517, female, 54.7 mm SL, Brazil, Rio Grande do Sul, Maquiné, Balneário Municipal do rio Maquiné.

15, 32.4–73.2 mm SL, 2 c&s, 52.3–61.1 mm SL, Três Forquilhas, rio Três Forquilhas, 29°32'00"S 50°05'00"W, 20 Aug 1983, Ichthyology UFRGS team. UFRGS 4994, 2, 41.4–66.6 mm SL, 2 c&s, 1 male, 45.0 mm SL, 1 female, 59.4 mm SL, Maquiné, rio Maquiné, 29°39'07"S 50°12'29"W, 25 Jan 2001, L. R. Malabarba, J. A. Anza, V. A. Bertaco & T. Hasper. UFRGS 6308, 1, 67.7 mm SL, Três Forquilhas, rio Três Forquilhas at Vila Boa União, 29°28'17.58"S 50°07'00.33"W, 26 Aug 2003, J. Anza, P. Colombo & L. Fusinato. UFRGS 12586, 7, 38.5–66.7 mm SL, TEC 1262, Itati, arroio do Padre, 29°29'09.5"S 50°07'15.6"W, 31 Mar 2010, J. Ferrer, T. Hasper, K. Vogel & F. Weiss. UFRGS 16503, 129, 20.9–68.2 mm SL, TEC 2831, 10, 42.0–64.4 mm SL, Itati, mouth of arroio da Barra into arroio Bananeiras, 29°25'37"S 50°10'49"W, 6 Jun 2012, L. R.

Malabarba, J. Ferrer, L. Artioli & P. Neto-Carvalho. UFRGS 17821, 9, 18.0–63.6 mm SL, TEC 3502, Maquiné, Barra do Ouro, 29°34'13.6"S 50°16'49"W, 2 Jun 2013, J. P. Miranda Santos. UFRGS 18439, 1, 24.9 mm SL, TEC 3832, Maquiné, RS-484, tributary of rio Maquiné, 29°34'14.3"S 50°16'49.9"W, 29 Jan 2014, L. R. Malabarba *et al.* UFRGS 18442, 1, 61.0 mm SL, TEC 3835, Maquiné, RS-484, tributary of rio Maquiné, 29°31'26.04"S 50°18'56.6"W, 29 Jan 2014, L. R. Malabarba *et al.* UFRGS 18447, 11, 18.2–69.4 mm SL, TEC 3840, Maquiné, RS-484, tributary of rio Maquiné, 29°31'26.04"S 50°18'56.6"W, 29 Jan 2014, L. R. Malabarba *et al.* UFRGS 18466, 4, 43.7–57.1 mm SL, TEC 3859, Maquiné, RS-484, tributary of rio Maquiné, 29°40'08"S 50°12'24"W, 29 Jan 2014, L. R. Malabarba *et al.* UFRGS 21403, 9, 49.7–80.0 mm SL, TEC 6427, and TEC 6453, Itati, rio do Padre, 29°29'27.4"S 50°08'49"W, 27 Jan 2016, R. Angrizani, J. Wingert, J. Ferrer & F. Kuhn. UFRGS 26517, 9, 35.7–54.7 mm SL, Maquiné, rio Maquiné, 29°39'08"S 50°12'34"W, 27 Oct 2018, UFRGS students.

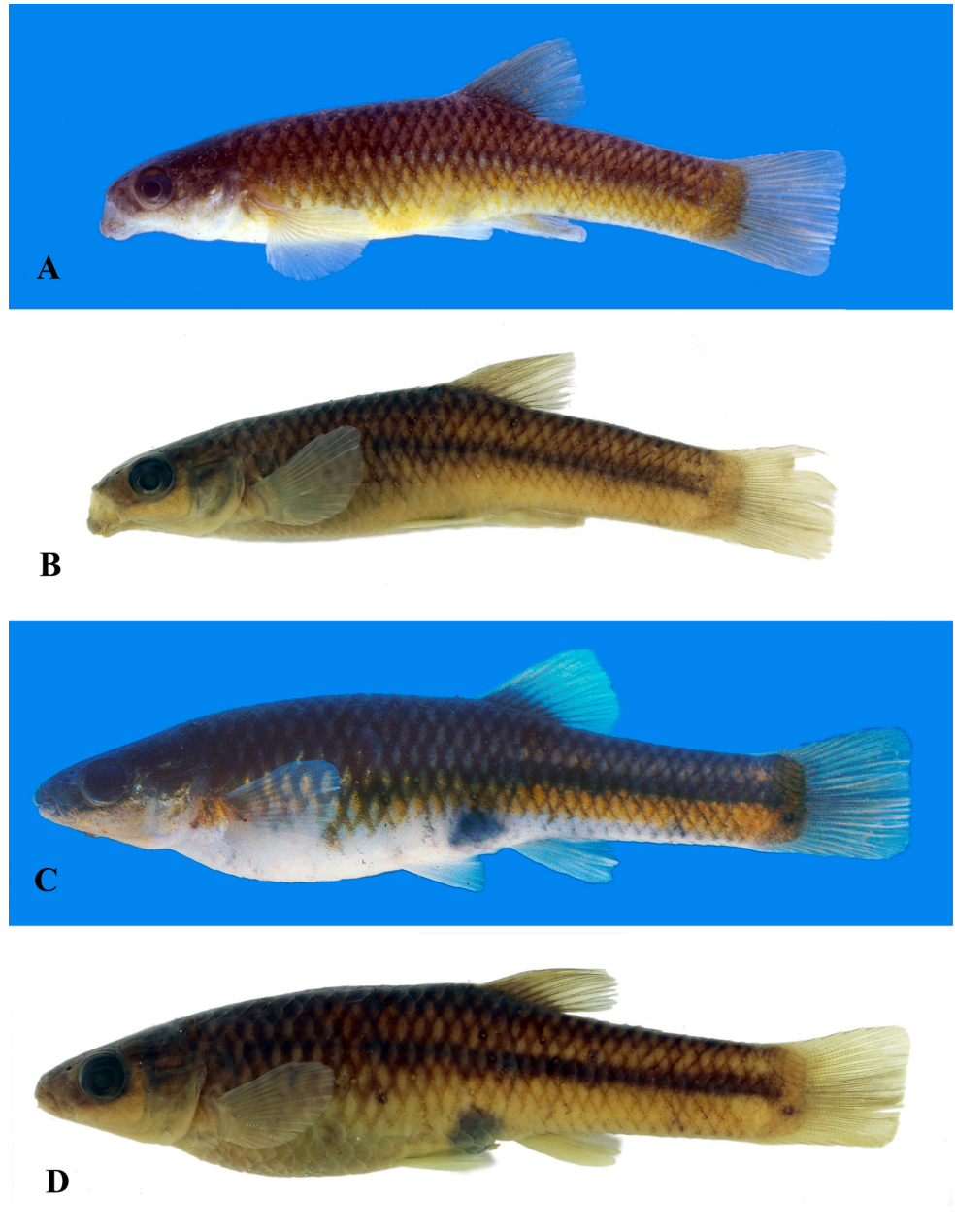
**Diagnosis.** *Jenynsia aurea* belongs to the subgenus *Plesiojenynsia* and is diagnosed from the species of the subgenus *Jenynsia* based on possession of a tubular gonopodium with a short fourth anal-fin ray, less than two-thirds length of ray three. *Jenynsia aurea* is distinguished from *J. diphyes*, *J. eigenmanni*, and *J. eirmostigma* by the possession of a single midlateral stripe in adults (Figs. 3–4; *vs.* discontinuous midlateral and dorsolateral stripes formed by a series of blotches on the body in *J. diphyes*, and three distinct and discontinuous lateral stripes in *J. eigenmanni* and *J. eirmostigma*). *Jenynsia aurea* (except in juveniles) differs from *J. unitaenia* by lacking dorsolateral or ventrolateral chevron marks associated to the midlateral stripe (Figs. 3–4; *vs.* presence of dorsolateral or ventrolateral chevron marks in *J. unitaenia*, Fig. 7). *Jenynsia aurea* is distinguished from *J. eigenmanni*, *J. eirmostigma*, and *J. weitzmani* by the presence of 13 to 15 gill rakers on ventral limb of first gill arch (in 2 c&s specimens) (*vs.* fewer than 13 gill rakers). *Jenynsia aurea* is distinguished from *J. diphyes*, *J. eigenmanni*, *J. eirmostigma*, and *J. weitzmani* by the presence of the first mandibular-canal pore (pore W; *vs.* absence).

**Description.** Morphometric data in Tabs. 1 (males) and 2 (females). Body terete and laterally compressed posteriorly. Greatest body depth at pelvic-fin origin or anterior to that point in pregnant females. Head relatively short and pointed. Mouth terminal to slightly subterminal in large specimens, clearly displaced ventrally when protracted (Figs. 4A, B). Dorsal-fin origin at a vertical between pelvic and anal fins. Largest female (73.2 mm SL) and largest male (67.7 mm SL).

Adult males with tubular gonopodium composed of the anal fin with distal tip directed laterally, dextral or sinistral. Intromittent organ supported by eight anal-fin rays, with posterior one or two anal-fin rays not forming the gonopodium. Females with median urogenital opening, lacking flaps or swelling areas.

Head-squamation pattern E type. Supraorbital-pore pattern represented by three disjunct canals (1, 2a; 2b, 3, 4a; 4b, 5, 6, 7). Preopercular canal with seven pores. Mandibular canal with five pores (W, X, Va, Vb, Z). Infraorbital canal with four pores.

Teeth on dentary and premaxilla tricuspid, with distal tip expanded and twice wider than its mid length (Fig. 5). Ascending process of premaxilla in adults present and prominent.



**FIGURE 4** | Color just after fixation in formalin and color after preservation in alcohol of the same specimens of *Jenynsia aurea*. Paratypes. (A, B) UFRGS 4994, male, 63.6 mm SL, (C, D) female, 66.0 mm SL, Brazil, Rio Grande do Sul, Maquiné, rio Maquiné.

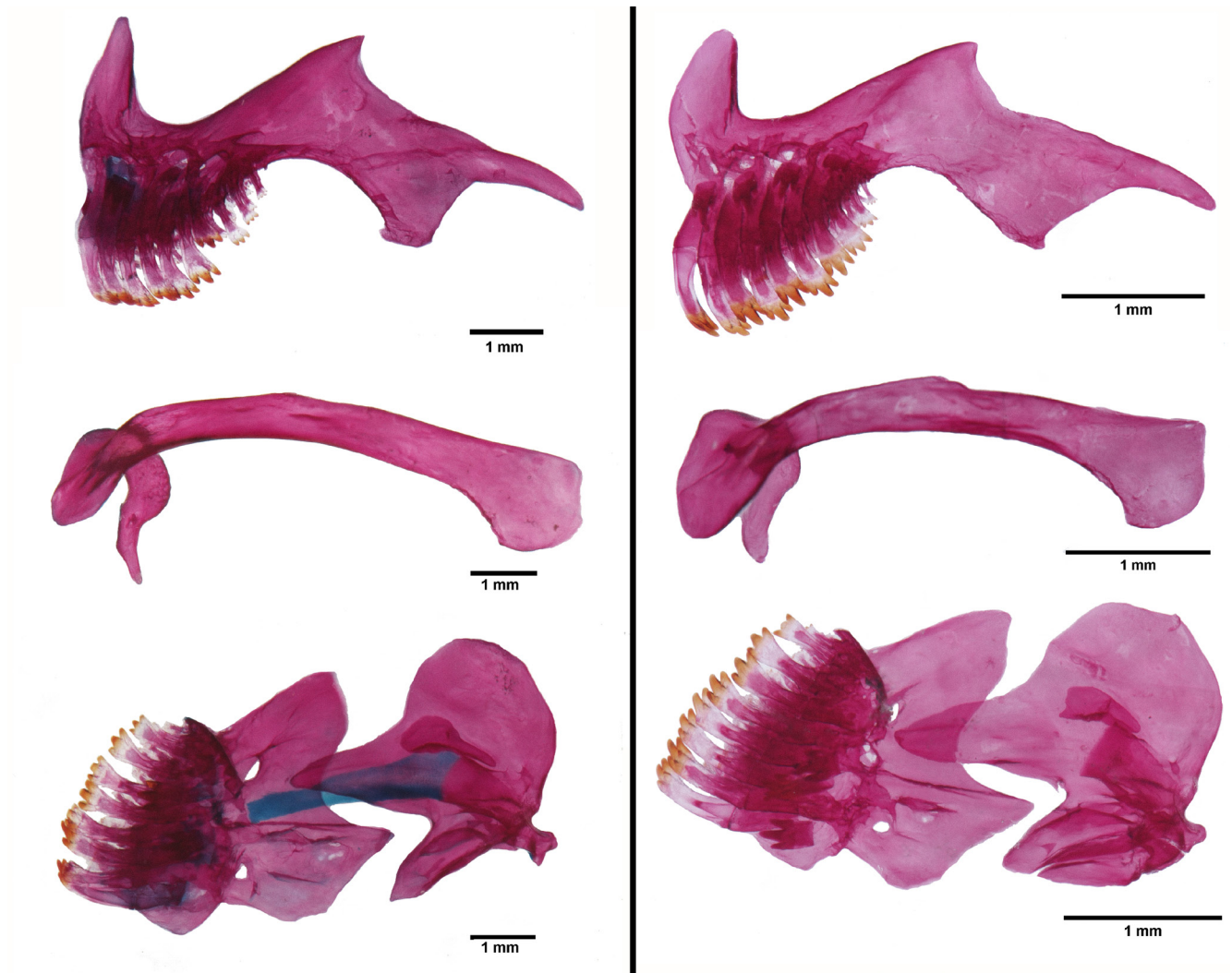
Dorsal-fin rays 9 [1], 10 [22], 11 [19] or 12\* [1]. Pectoral-fin rays 15 [2], 16\* [29], 17 [8], or 18 [1]. Pelvic-fin rays 6\* [34], or 7 [6]. Anal-fin rays (females) 9 [10], 10 [2]; (males) 9 [1], 10 [1]. Principal caudal-fin rays 16 [4], 17 [12], or 18\* [28]. Predorsal scales 14 [8], 15\* [20], or 16 [12]. L1 scales 8 [3], 9 [6], 10\* [4], 11 [8], 12 [7], 13 [2], or 14 [1]. L2 scales 20 [1], 21 [2], 22\* [12], 23 [14], 24 [6], 25 [1]. Scales around caudal peduncle 13 [3], 14\* [18], 15 [9], or 16 [3]. Gill rakers on ventral arm of first gill arch 13 [1], 15 [1]. Total vertebrae 33 [3], 34 [3], abdominal vertebrae 14 [4], 15 [2], caudal vertebrae 18 [1], 19 [3], 20 [2].

**TABLE 1** | Morphometric data of males of *Jenynsia aurea* from rio Maquiné (UFRGS 4994, 18466) and rio Três Forquilhas (UFRGS 6308, 16503, 16534), and males of *J. unitaenia* from rio Mampituba (UFRGS 15386, 15959, 25823) and rio Araranguá (UFRGS 6184, 12553, 19916, 22961). SD = Standard deviation.

	Holotype	<i>Jenynsia aurea</i>				<i>Jenynsia unitaenia</i>			
		Min	Max	Mean	SD	Min	Max	Mean	SD
Standard length (mm)	56.7	52.0	67.70	58.50	-	36.7	67.40	49.67	-
<b>Percentage of standard length</b>									
Body depth	21.3	20.6	23.3	21.9	0.87	21.2	24.3	22.8	0.89
Predorsal length	59.2	56.3	63.3	59.3	2.14	56.6	61.8	59.0	1.38
Preanal length	65.3	63.2	66.8	65.5	1.37	62.3	67.6	64.6	1.31
Prepelvic length	48.9	46.9	49.9	49.1	0.94	48.0	53.0	50.8	1.29
Caudal peduncle length	30.5	28.0	32.3	30.0	1.66	27.8	31.2	29.7	1.08
Caudal peduncle depth	13.2	13.2	15.9	14.0	0.82	13.1	15.2	14.1	0.63
Anal-fin length	18.2	17.9	22.7	20.1	1.80	17.7	23.9	20.8	1.63
Pelvic-fin length	14.6	14.5	16.3	15.0	0.57	13.4	16.7	15.3	0.79
Head length	24.5	24.2	26.7	25.2	0.82	24.3	29.7	25.9	1.31
<b>Percentage of head length</b>									
Horizontal orbit diameter	27.3	23.4	27.3	25.4	1.24	21.5	30.2	26.3	2.38
Head depth	46.8	43.8	48.8	46.6	1.82	45.0	51.3	47.9	2.11
Interorbital width	40.3	38.4	45.7	42.1	2.43	36.7	45.0	41.0	2.32

**TABLE 2** | Morphometric data of females of *Jenynsia aurea* from rio Maquiné (UFRGS 4994) and rio Três Forquilhas (UFRGS 2995), and females of *J. unitaenia* from rio Mampituba (UFRGS 10850, 12564, 16079, 15888, 25823) and rio Araranguá (UFRGS 6184, 6186, 19916). SD = Standard deviation.

	<i>Jenynsia aurea</i>				<i>Jenynsia unitaenia</i>			
	Min	Max	Mean	SD	Min	Max	Mean	SD
Standard length (mm)	47.8	73.2	63.68	-	49.9	76.7	66.38	-
<b>Percentage of standard length</b>								
Body depth	20.5	23.0	21.8	1.06	20.0	24.4	22.6	1.15
Predorsal length	59.0	65.0	61.6	2.81	57.6	62.5	60.0	1.42
Preanal length	64.4	67.5	65.6	1.39	63.1	69.4	65.8	1.52
Prepelvic length	49.3	51.9	50.5	1.17	46.0	52.2	49.5	1.81
Caudal peduncle length	26.8	30.8	29.2	1.81	26.7	33.0	28.9	1.82
Caudal peduncle depth	12.6	13.5	13.2	0.45	12.4	14.3	13.6	0.55
Anal-fin length	15.0	17.4	15.9	1.02	13.6	16.9	15.6	0.99
Pelvic-fin length	12.8	15.4	13.5	1.31	12.1	14.7	13.2	0.73
Head length	24.3	26.3	25.0	0.90	23.4	26.8	24.5	0.95
<b>Percentage of head length</b>								
Horizontal orbit diameter	21.5	29.3	24.8	3.30	21.4	29.3	25.8	1.61
Head depth	42.5	50.0	46.8	3.11	47.8	55.0	51.1	1.89
Interorbital width	41.4	45.7	43.5	1.92	40.3	48.1	44.2	1.92



**FIGURE 5** | Premaxilla, maxilla and dentary of specimens cleared and stained for bone and cartilage, showing the teeth of *Jenynsia aurea* from rio Maquiné (left, UFRGS 4994) and rio Três Forquilhas populations (right, MCP 10779). The premaxillae and dentaries are in medial view and the maxillae are in lateral view.

**Coloration in alcohol.** Body background color grading from brown dorsally to pale yellow on ventral surface (Figs. 4B, D, 6). Black chromatophores distribution differs between small and mature specimens. Specimens from 20 mm SL up to about 40 mm SL show irregular reticulated black pattern bordering body scales, with lighter, non-reticulate areas more numerous in smaller than in larger specimens, and distributed mostly on caudal peduncle and lower half of body (Fig. 6). A black stripe with irregular borders and sometimes interrupted along its length is present from the posterior edge of the operculum to caudal fin (Fig. 6). Large males and females (nearly 45 mm SL and larger) show a more regular reticulate pattern that is progressively lighter ventrally, with dark chromatophores on scale margin, lacking lighter non-reticulate areas. A continuous black stripe is also present, without interruptions and with smooth dorsal and ventral margins (Figs. 4B, D). Some specimens show the black stripe bending ventrally near the caudal fin (Figs. 3A, D). Specimens with intense black pigmentation



**FIGURE 6** | Color after fixation in formalin and preservation in alcohol of juveniles of *Jenynsia aurea*, UFRGS 16503, paratypes, (A) 25.9 mm SL, (B) 29.3 mm SL, and (C) 33.9 mm SL, Itati, mouth of arroio da Barra into arroio Bananeiras.

on scales may turn black above the belly region (Figs. 3A, B). Mature females with a genital spot. Head brown dorsally grading to pale yellow ventrally. Fins unpigmented except for a few dark chromatophores bordering the rays in dorsal fin, caudal fin and dorsal part of pectoral fin.

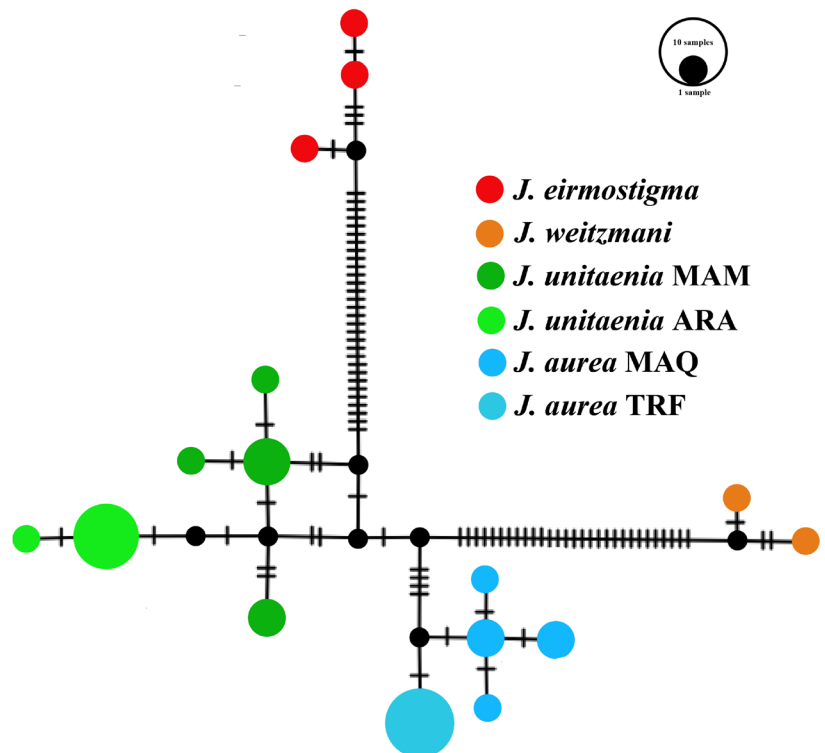
**Coloration in life.** Color patterns vary according to body size and sex, with intense golden reflections and less discernible black pigments in mature males (usually more than 55 mm SL; Figs. 2, 4A) than in females or small specimens (Figs. 3C, D, 4C, 6). Mature males are golden brown on the back and upper half of the body and head; golden yellow on ventral half of body and on the pectoral, dorsal, anal and caudal fins; hyaline on pelvic fins; usually white on ventral surface of head and body (Figs. 3A, 4A), but the holotype is golden yellow also on ventral portions of head and body (Fig. 2). Females are dark brown on the back and upper half of the body and head; golden yellow on ventral half of head and body; all fins hyaline; white on ventral surface of head and body (Figs. 3B, 4D). Specimens smaller than 50 mm SL have the overall body color light brown on upper half of head and body, light brown on ventral half of body and white on ventral surface of head and belly (Fig. 3C). All specimens show a continuous lateral stripe, hardly discernible in fully colored males whose scale row on the lateral stripe show light reflections over a dark brown stripe (Figs. 2, 4A). Black marks as described in alcohol preserved specimens.

**Geographical distribution.** The species is found in the rio Maquiné, rio Três Forquilhas, rio Tramandaí system, and their tributaries in the valleys of the Serra Geral Formation (Fig. 1).

**Etymology.** The name “aurea” comes from Latin “*aurum*” meaning golden, in a reference to the color in life, especially of mature males. An adjective.

**Conservation status.** The new species is widely distributed in the rio Maquiné and rio Três Forquilhas and no specific threat can be assigned to this species. According to the International Union for Conservation of Nature (IUCN, 2024) categories and criteria, *Jenynsia aurea* would be classified as Least Concern (LC).

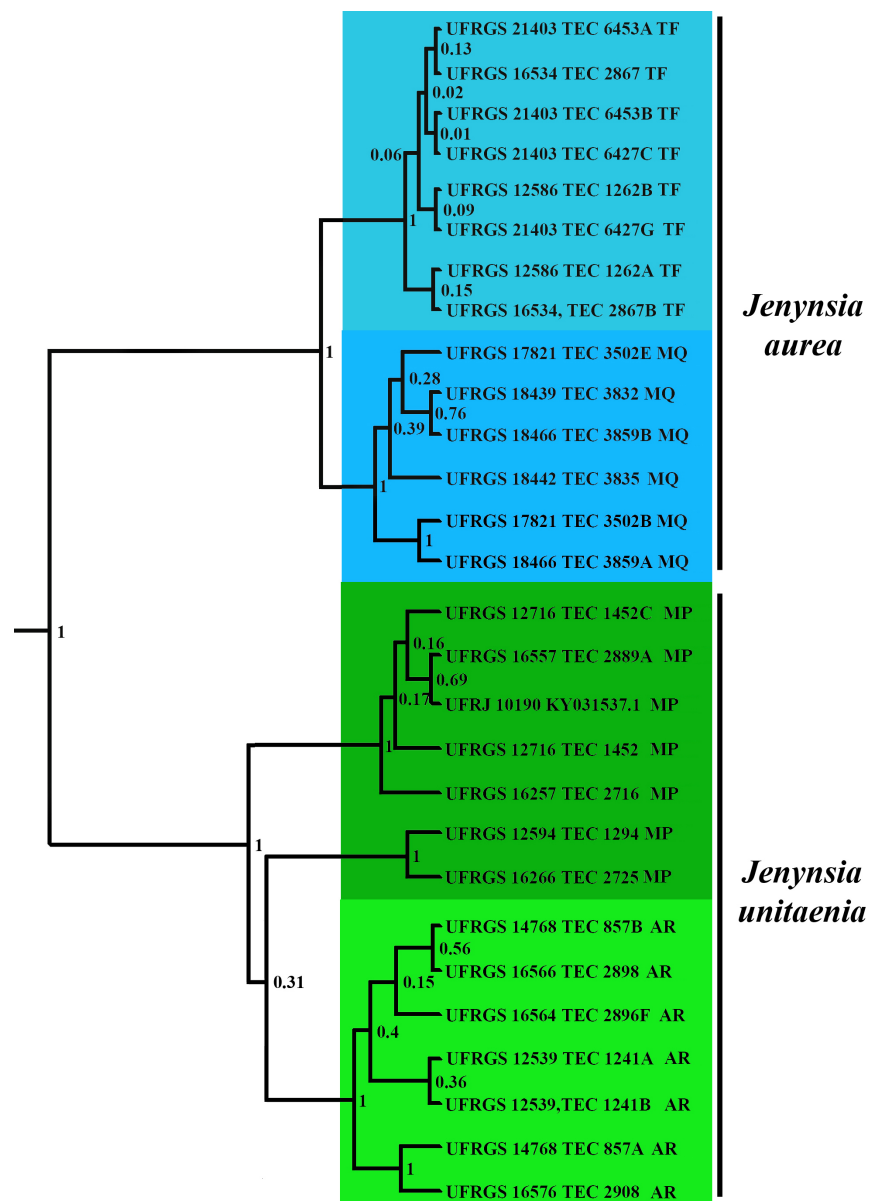
**Molecular analysis.** A total of 664 base pairs were aligned for the mtDNA sequences of 27 samples for the COI gene. A total of 26 polymorphic sites were found, resulting in 16 haplotypes and a haplotypic diversity  $hd = 0.92$ . The haplotype network (Fig. 7) of the species available of the subgenus *Plesiojenynsia* recovered four genetically distinct groups. Two correspond to *Jenynsia eirmostigma* and *J. weitzmani*. The MP-AR group corresponds to *J. unitaenia* and the MQ-TF group corresponds to *J. aurea*. The AMOVA test showed a high genetic differentiation between the analyzed populations ( $F_{st} = 0.85$ ), demonstrating that 85% of genetic differentiation is between populations and only 15% is within populations. This value was very high when we analyzed the variance between the northern (MP-AR) and southern (MQ-TF) groups ( $F_{st} = 0.88$ ), corroborating the results of the haplotype network.



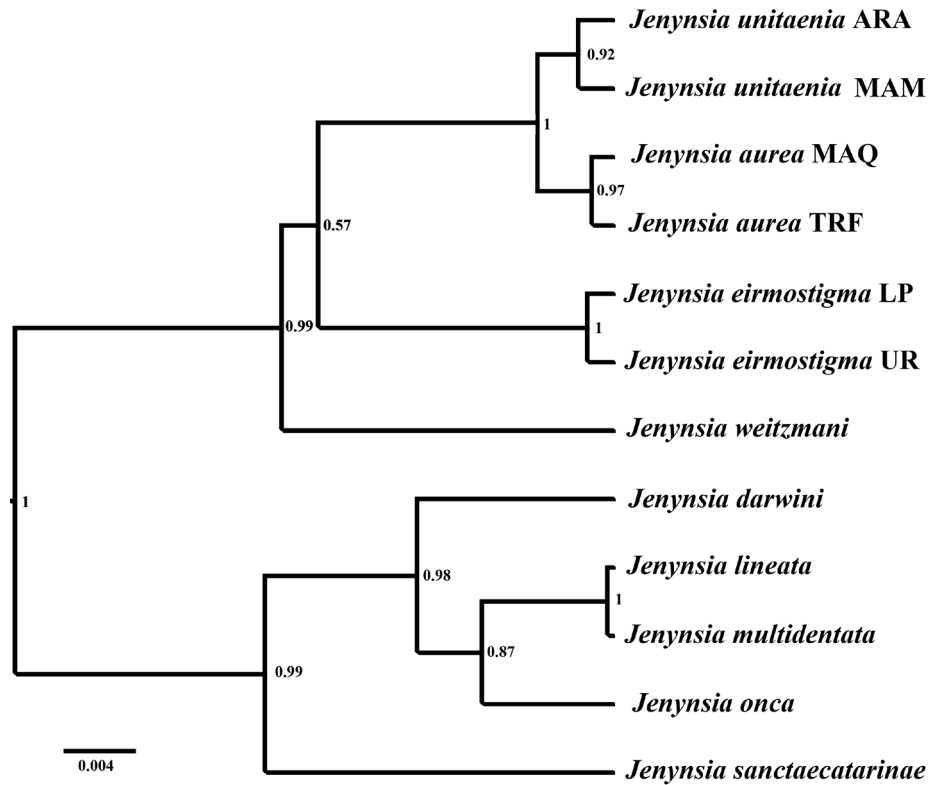
**FIGURE 7** | Haplotype network using Median-Joining for the species of the subgenus *Plesiojenynsia* for the COI gene. Each circle represents a single haplotype and its size is proportional to its frequency. Populations from rio Mampituba (MAM), rio Araranguá (ARA), rio Maquiné (MAQ), rio Três Forquilhas (TRF).

Bayesian inference analyses recovered similar results. The COI gene tree recovered a topology similar to the groups recovered in the COI haplotype network. Both MP-AR and MQ-TF clades proved to be monophyletic and sister groups, with high support values (PP = 1) (Fig. 8). MQ and TF also form separate clades with high support values (PP = 1) between the two populations assigned herein to *J. aurea*.

The species tree generated presented the same topology as the previous results. Strict molecular clock analysis showed that the clade *Jenynsia unitaenia* + *J. aurea* dates back to at least 1.2 Ma, when the two species diverged. Within *J. unitaenia*, MP and AR populations diverged 0.4 Ma ago. Within *J. aurea* MQ and TF populations diverged 0.2 Ma ago (Fig. 9).



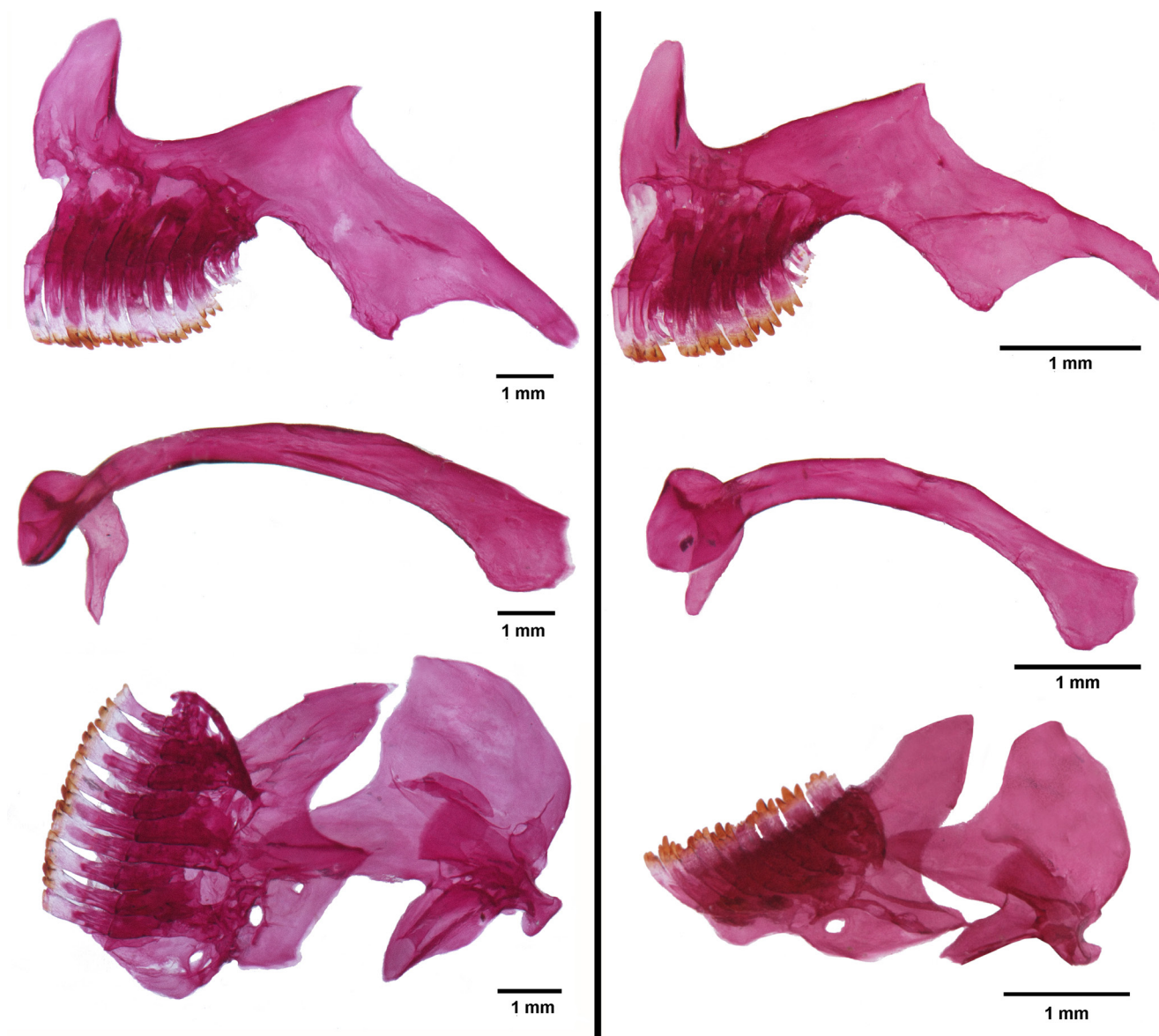
**FIGURE 8** | Gene tree for the COI gene and for the CytB gene generated by Bayesian inference in Beast. *Jenynsia aurea* is represented by the blue clade: populations from rio Três Forquilhas (TF) and rio Maquiné (MQ). *Jenynsia unitaenia* is represented by the green clade: populations from rio Mampituba (MP) and rio Araranguá (AR). The numbers at the nodes are the posterior probability.



**FIGURE 9 |** Species tree. Numbers at the nodes represents the posterior probability. Populations from rio Mampituba (MAM), rio Araranguá (ARA), rio Maquiné (MAQ), rio Três Forquilhas (TRF), Laguna dos Patos (LP), rio Uruguai (UR).

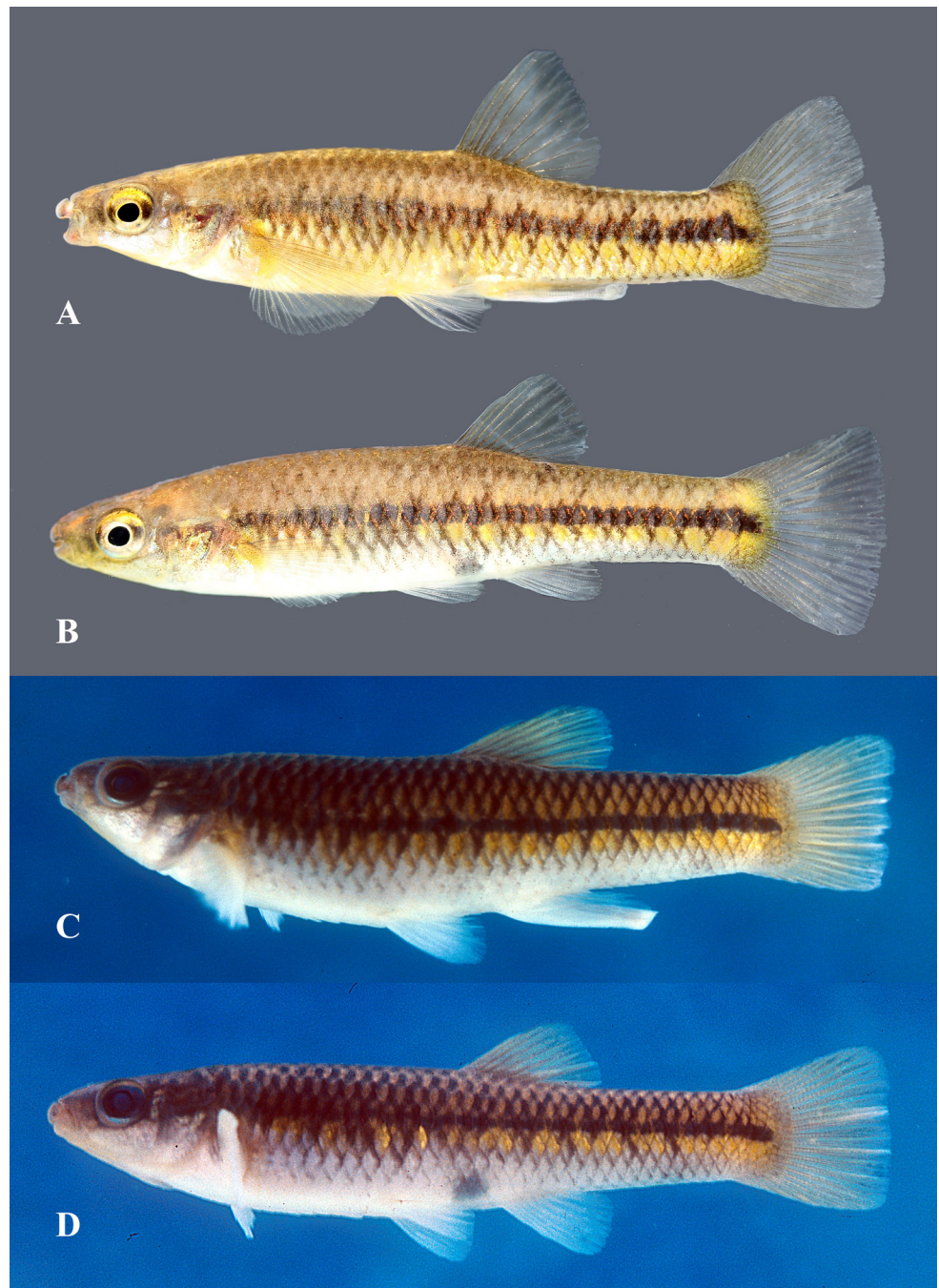
**Morphological comparison between *J. aurea* and *J. unitaenia*.** The analysis of morphometric and meristic characters does not allow a diagnosis between *J. aurea* (MQ and TF) and *J. unitaenia* (MP and AR). A morphometric difference between the two species was observed in head depth of females (Tab. 2) that ranges from 42.5 to 50.0 % of head length (mean = 46.8) in *J. aurea* and 47.8–55.0 (mean = 51.1) in *J. unitaenia*, with a large overlap. Males of *J. aurea* also show a more depressed head (head depth 43.8–48.8 % of head length, mean = 46.6%) than *J. unitaenia* (45.0–51.3%, mean = 47.9%), but the difference in the mean is smaller than in females (Tab. 1).

Populations from MQ and TF, corresponding to *J. aurea*, show jaw teeth slender, spaced and more constricted at their mid length (Fig. 5), while those from MP and AR, corresponding to *J. unitaenia*, show teeth more robust and less spaced (Fig. 10). Populations from MQ and TF, corresponding to *J. aurea* (Figs. 2–4), differ from those of MP and AR, corresponding to *J. unitaenia* (Fig. 11), in the color pattern. Both populations of *J. unitaenia* show a series of irregular chevron marks along the dorsal and ventrolateral margins of the midlateral stripe (Fig. 11), while populations of *J. aurea* from MQ and TF lack such pattern (Figs. 2–4). The chevron marks along the dorsal and ventrolateral margins of the midlateral stripe are observed in juveniles of both species (Fig. 6).



**FIGURE 10** | Premaxilla, maxilla and dentary of specimens cleared and stained for bone and cartilage, showing the teeth of *Jenynsia unitaenia* from rio Mampituba (left, UFRGS 15888) and rio Araranguá populations (right, UFRGS 12553). The premaxillae are in medial view and the maxillae are in lateral view. Dentary of UFRGS 15888 is in medial view and dentary of UFRGS 12553 is in lateral view.

There is one lot of *Jenynsia unitaenia* listed by Ghedotti, Weitzman (1995) from a locality not belonging to MP and AR drainages: the paratypes of USNM 326104 from Rio Grande do Sul state, Terra de Areia municipality, rio Três Pinheiros, belonging to TF drainage. All specimens we have from TF drainage belong to *J. aurea*, including one lot designated herein as paratypes of *J. aurea* (MCP 25292), also collected in the rio Três Pinheiros. Once the paratypes of USNM 326104 are juveniles (3 specimens, 24.4–28.3 mm SL), and juveniles of *J. aurea* (Fig. 6) show irregular chevron marks along the dorsal and ventrolateral margins of the midlateral stripe similar to that found in *J. unitaenia*, we consider this lot of *J. unitaenia* paratypes as belonging to *J. aurea*.



**FIGURE 11** | Color in life of *Jenynsia unitaenia*. UFRGS 22072, (A) male, 54.7 mm SL (B) female, 58.6 mm SL, Brazil, Santa Catarina, Praia Grande, Canyon Malacara, Vila Rosa, rio Mampituba drainage. MCP 25439, (C) male, 56.4 mm SL, (D) female, 56.2 mm SL, Brazil, Santa Catarina, Ermo, rio Itoupava, rio Araranguá drainage.

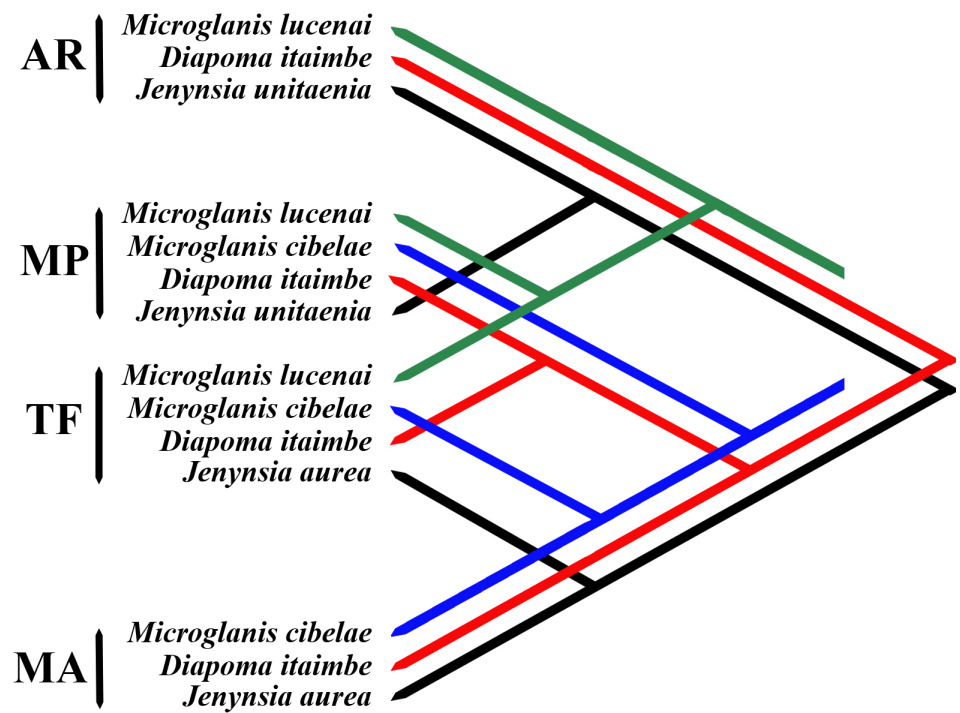
Character coding of *Jenynsia unitaenia* given by Ghedotti, Weitzman (1995) does not seem to include data from specimens of *J. aurea* misidentified as *J. unitaenia*, once paratypes of USNM 326104 do not include cleared and stained specimens for osteological analyses. We further checked characters coded to *J. unitaenia* by Amorim

(2018) and they actually correspond to specimens of *J. unitaenia* examined herein. *Jenynsia aurea* differs from *J. unitaenia* in one character that further diagnosis the two species (numbered according to Amorim, 2018; Aguilera *et al.*, 2019: S1 tab.). The anterior cleft in anguloarticular is large in *J. unitaenia* (Fig. 10) extending posteriorly beyond posterior border of Meckel's cartilage (ch. 19, state 0), and small in *J. aurea* (Fig. 5), not extending posteriorly beyond posterior border of Meckel's cartilage (ch. 19, state 1). Differences in coding two additional characters are related to polymorphism observed in each species and are not diagnostic. The ossified process on ventrolateral margin of basioccipital at attachment of Baudelot's ligament in adults is polymorphic in *J. unitaenia* (ch. 11, state 0 – absent and state 1 – present) and present in *J. aurea* (ch. 11, state 1). The number of anal-fin rays is 10 in *J. unitaenia* (ch. 40, state 2), but polymorphic in *J. aurea*, ranging from 9 to 10 (ch. 40, states 2 and 3).

## DISCUSSION

Molecular analysis allowed the recognition of four distinct populations of *Jenynsia* in the Tramandaí-Mampituba ecoregion. The results estimated *Jenynsia aurea* (populations from MP and AR) diverged from *J. unitaenia* (populations from MQ and TF) during the Pleistocene (about 1.2 million years ago; COI genetic distance = 1.64–2.48%). During this period, the coast of South America suffered marine transgressions due to climate change and sea level fluctuations (Suguio *et al.*, 1985), causing fish populations to become connected or isolated. The AR population diverged from the MP population at 0.4 Ma (COI genetic distance = 0.61–1.02%) and the populations from MQ and TF were separated by 0.2 Ma (COI genetic distance = 0.41–0.82%). The MQ and TF populations are diagnosed from MP and AR populations by the color pattern, one of the phenotypic morphological characters usually applied as a diagnostic character among species of the subgenus *Plesiojenynsia* (see Ghedotti, Weitzman, 1995; Ghedotti *et al.*, 2001; Lucinda *et al.*, 2006) and are thus referred to as separate species.

Fish in rivers that share a paleodrainage are expected to be genetically more similar to each other compared to fish from rivers with different paleodrainages (Thomaz *et al.*, 2015). In fact, *J. unitaenia* and *J. aurea* form a clade endemic to a single paleodrainage embracing the MQ, TF, MP and AR rivers, that reinforces this pattern. The pattern of diversification or the relationships among fish populations inhabiting the currently isolated river drainages that form this paleodrainage, however, vary according to fish taxa in phylogeographic or phylogenetic studies (Fig. 12). The pattern of area relationships found in *Jenynsia unitaenia* and *J. aurea* [(MA+TF)(MP+AR)] contrasts with those observed in other fish taxa endemic to the Tramandaí-Mampituba ecoregion, such as *Diapoma itaimbe* {[MQ(TF+MP)AR]}; *Microglanis lucenai* Lehmann A., Bartzén & Malabarba, 2024 [(TF+MP) AR] and *Microglanis cibela* Malabarba & Mahler, 1998 [(MQ+ TF) MP] (Hirschmann *et al.*, 2015; Lehmann A. *et al.*, 2024). Such a variation in the patterns of diversification of the fish fauna from a single paleodrainage that constitutes a recognized area of endemism seems to corroborate the hypothesis that besides the studied rivers have been affected by the same cyclical sea-level changes over the Pleistocene, ecological and paleo-landscape sieves have affected differently each taxon and influenced past dispersal of the components of Atlantic coastal river communities (Thomaz, Knowles, 2020).



**FIGURE 12** | Area cladograms of the rio Araranguá (AR), rio Mampituba (MP), rio Três Forquilhas (TF) and rio Maquiné (MQ) drainages, that form the Tramandaí-Mampituba ecoregion *sensu* Abell *et al.* (2008), based on the phylogenetic relationships of endemic fish taxa: *Jenynsia aurea* and *J. unitaenia* (this study), *Diapoma itaimbe* (Hirschmann *et al.*, 2015); *Microglanis lucenai* and *M. cibela* (Lehmann A. *et al.*, 2024).

Although the formation of the studied rivers took place from the Serra Geral and the fish depend on connections between river basins for their dispersal, currently only the rio Maquiné and rio Três Forquilhas are connected by bodies of freshwater. The rio Maquiné flows into the lagoa dos Quadros and the rio Três Forquilhas into the lagoa Itapeva. Both lagoons are connected by a channel and flow into the rio Tramandaí estuary (Ferreira *et al.*, 2006). The fact that the populations of *J. aurea* from the rio Maquiné and rio Três Forquilhas do not share haplotypes, despite being interconnected by coastal lagoons, indicates the absence of gene flow and that the lagoons act as a barrier between populations typical of rapids environments found in rivers in the Serra Geral Formation, as noted by Hirschmann *et al.* (2015) in *Diapoma itaimbe*. Although lowlands and floodplains most often represent dispersal corridors for freshwater fish (Albert, Reis, 2011), such seems not to be the case for *J. aurea* and *D. itaimbe*.

**Comparative material examined.** Brazil, Santa Catarina. *Jenynsia unitaenia*. Paratypes. Rio Mampituba drainage: MCP 17419, 10, 24.9–58.8 mm SL; MCP 17420, 17, 26.1–62.7 mm SL; MCP 17421, 17, 24.2–49.0 mm SL; MCP 17425, 10, 32.5–58.6 mm SL (all collected with the holotype). Rio Araranguá drainage: MCP 17423, 32, 22.1–45.1 mm SL, MCP 17424, 26, 18.1–48.4 mm SL. Non-types. Rio Mampituba drainage: UFRGS 10850, 1, 70.8 mm SL. UFRGS 12564, 11, 28.5–73.1 mm SL. UFRGS 12594, 5, 24.6–46.9 mm SL, TEC 1294. UFRGS 12716, 5, 28.4–45.9 mm SL, TEC 1452. UFRGS 15888, 3 of 5, 62.2–64.6 mm SL, 2 c&s.

UFRGS 15959, 108, 22.8–56.7 mm SL. UFRGS 16079, 47, 21.3–76.7 mm SL. UFRGS 16257, 1, 48.6 mm SL, TEC 2716. UFRGS 16266, 1, 38.5 mm SL, TEC 2725. UFRGS 16555, 4, 47.3–50.9 mm SL, TEC 2887. UFRGS 16557, 13, 21.2–36.3 mm SL, TEC 2889. UFRGS 22072, 9, 53.7–65.9 mm SL, TEC 6973. UFRGS 25823, 10, 27.8–70.1 mm SL, TEC 8940. USNM 326070, 5 (photos), 27.7–43.8 mm SL. Rio Ararangua drainage: UFRGS 6184, 5 of 50, 36.7–69.5 mm SL. UFRGS 6186, 2, 49.9–59.4 mm SL. UFRGS 12539, 2 of 5, 27.2–28.4 mm SL, TEC 1241. UFRGS 12553, 2 of 36, 47.9–55.7 mm SL, 2 c&s. UFRGS 14768, 2, 35.7–37.8 mm SL, TEC 857. UFRGS 15386, 153, 27.1–55.5 mm SL. UFRGS 16564, 6, 20.3–27.3 mm SL, TEC 2896. UFRGS 16566, 3, 32.0–54.5 mm SL, TEC 2898. UFRGS 16576, 1, 27.6 mm SL, TEC 2908. UFRGS 19916, 18, 42.7–71.3 mm SL, TEC 9092. UFRGS 22961, 21, 40.8–42.3 mm SL. MCP 25439, 5, 25.4–56.8 mm SL.

## ACKNOWLEDGMENTS

We thank Carlos A. S. Lucena (MCP) for the loan of specimens and Abigail Reft (SI-NMNH) for sending photos of paratypes of *Jenynsia unitaenia*.

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**Caroline Hartmann:** Formal analysis, Investigation, Methodology, Writing-original draft, Writing-review and editing.

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**Luiz R. Malabarba:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing-original draft, Writing-review and editing.

### FUNDING INFORMATION

LRM is partially funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq grant number 308026/2021-7).

### ETHICAL STATEMENT

Not applicable.

### DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

### AI STATEMENT

The authors did not use any AI-assisted technologies in the creation of this manuscript or its figures.

### COMPETING INTERESTS

The authors declare no competing interests.

### SUPPLEMENTARY MATERIAL

Supplementary material S1

### HOW TO CITE THIS ARTICLE

- **Hartmann C, Wingert JM, Angrizani RC, Malabarba LR.** An unexpected discovery of a new species of *Jenynsia* (Cyprinodontiformes: Anablepidae). *Neotrop Ichthyol.* 2026; 24(1):e250128. <https://doi.org/10.1590/1982-0224-2025-0128>

Neotropical Ichthyology

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Official Journal of the Sociedade Brasileira de Ictiologia