

# The uniform, the banded, and the polymorphic: an integrative taxonomy in the annual fish *Acantholebias* (Atheriniformes: Rivulidae)

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Based on an integrative approach (genetic, morphology and biogeography), two new species of *Acantholebias* are described from Laguna de Rocha and middle Cebollatí River basins respectively (Laguna dos Patos ecoregion). The new species from Laguna de Rocha is diagnosed by a chromatic character and cytochrome b mutations; the new species from middle Cebollatí is diagnosed by a combination of chromatic and squamation patterns. Both species are allopatrically distributed relative to the other species of the genus and to each other. This study also indicates that *Acantholebias quirogai* presents high chromatic polymorphism that could be related to drainage rearrangements between the Lower Uruguay and Laguna dos Patos ecoregions.

**Keywords:** Chromatic polymorphism, Cytochrome b, Laguna dos Patos ecoregion, Uruguay.

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Con base en un enfoque integrador (genético, morfológico y biogeográfico), se describen dos nuevas especies de *Acantholebias* provenientes de las cuencas de Laguna de Rocha y del curso medio del río Cebollatí, respectivamente (ecorregión Laguna dos Patos). La nueva especie de Laguna de Rocha se diagnostica mediante un carácter cromático y mutaciones en el citocromo b; la nueva especie del curso medio del río Cebollatí se diagnostica mediante una combinación de patrones cromáticos y de escamación. Ambas especies presentan una distribución alopatrica con respecto a las demás especies del género y entre sí. Este estudio también indica que *Acantholebias quirogai* presenta un alto polimorfismo cromático que podría estar relacionado con reordenamientos de drenaje entre las ecorregiones del Bajo Uruguay y Laguna dos Patos.

**Palabras clave:** Citocromo b, Ecorregión Laguna dos Patos, Polimorfismo cromático, Uruguay.

## INTRODUCTION

Annual fishes are a group of freshwater vertebrates with unique developmental, ecological, physiological, and evolutionary adaptations (Berois *et al.*, 2016). They possess an uncharacteristically short life cycle among vertebrates, which is correlated to the seasonal pools of water they inhabit. The Neotropical annual killifish genus *Austrolebias sensu* Costa (2006) was recently proposed as a “genus group” by Alonso *et al.* (2023). Besides several morphological synapomorphies (Costa, 2006), this group shares an unusually large genome size (approximately 6 Gb) compared to other diploid aplocheiloid fishes (García *et al.*, 2014). Alonso *et al.* (2023) elevated to genera the subgenera of *Austrolebias* initially proposed by Costa (2008) and other monophyletic clades found in Loureiro *et al.* (2018). Despite the fact that some species of proposed new genera had incomplete character sampling (*i.e.*, *Acrolebias*, where only morphology was considered in the type species) and a controversial cutting line to define monophyletic clades as genera (11 new genera, two of them monotypic, including *Acrolebias* Costa, 2008; two other composed by two species, including the sister clade of *Acrolebias*), the new proposal has been already used by several authors (Fricke *et al.*, 2025; García *et al.*, 2025; Godoy *et al.*, 2025; Volcan *et al.*, 2025). Thus, in order to favor stability in the nomenclature of this clade, in this article we will follow Alonso *et al.*'s (2023) taxonomic categories.

*Acantholebias* was originally proposed as a monotypic subgenus of the genus *Austrolebias* integrated by *Au. luteoflammulatus* (Vaz Ferreira, Sierra de Soriano & Scaglia de Paulete, 1964)(Costa, 2008). Based on pigmentation variation and morphological characters related to contact organs and body shape, Loureiro *et al.* (2011) proposed that *Au. quirogai* (Loureiro, Duarte & Zarucki, 2011) was related to *Au. luteoflammulatus*. García *et al.* (2014) and Loureiro *et al.* (2018) phylogenies confirmed these species as sister taxa. Alonso *et al.* (2023) elevated *Acantholebias* to generic status based on 19 molecular and six morphological synapomorphies: dorsal fin with a dark grey vertical bar on posterior portion in males, background color of pectoral fins in males bright blue-green, dark grey pigmentation pattern on flank of females composed by dots,

contact organs on the anal fin in males, bony support in contact organs in males and parasphenoid posterior process that broadens gradually. Remarkably, both species also share as a synapomorphic character a chromosome diploid number of  $2n = 34$ , but differ in the NF (arm number): *Ac. luteoflammulatus* = 48; *Ac. quirogai* = 52 (García *et al.*, 2014). The occurrence of these major karyotype rearrangements, probably involving centric fusions and pericentric inversions from the hypothesized common ancestor having a diploid number of  $2n=46$  or  $2n=48$  (García *et al.*, 2014), was proposed to explain these peculiar karyological configurations.

*Acantholebias luteoflammulatus* (Laguna dos Patos ecoregion) was diagnosed by the presence of black vertical bands over a silver background on the flanks of males in the posterior 2/3 of the body (Vaz Ferreira *et al.*, 1964; Costa, 2006). However, D'Anatro, Loureiro (2005) identified morphometric and chromatic variation (number of vertical dark bands) within the species associated mainly with populations from the Laguna de Rocha basin, an Atlantic coastal lagoon situated at the southwestern limit of the species distribution (from here on *A. aff. luteoflammulatus* 1). Reichert *et al.* (1998) also reported chromatic variation (two males without the banded pattern on the flanks) in a population of *A. luteoflammulatus* from the lower Cebollatí basin (Paso Averías), which was confirmed in recent exploration of the area.

On the other hand, *Acantholebias quirogai* (Laguna dos Patos and Lower Uruguay ecoregions), was diagnosed by a grayish uniform pigmented body of males and reduction of the abdominal and opercular squamation (Loureiro *et al.*, 2011). However, these authors also reported populations with polymorphic males exhibiting both pigmentation patterns (grayish uniform and black vertical bands) and some large individuals without squamation reduction. Serra *et al.* (2014) reported males with a similar pigmentation pattern to *A. luteoflammulatus* from the Negro river basin (Lower Uruguay ecoregion) (from here on *A. aff. luteoflammulatus* 2). Furthermore, in recent studies populations that are morphologically similar to *A. quirogai* (from here on *A. aff. quirogai*) were found upstream from Paso Averías in the middle Cebollatí river basin (Laguna dos Patos ecoregion). This challenges the identity of the species within the genus and raises questions about the origin of its distribution pattern, with one species distributed both in upper Negro river basin and upper Laguna Merin effluents and its close relatives distributed in Laguna Merin lowlands. The same pattern was already reported in the non-related annual fish genus *Garcialebias* Alonso, Terán, Alanís, Calviño, Montes, García, Barneche, Almirón, Ciotek, Giorgis & Casciotta, 2023 (Loureiro *et al.*, 2011, 2018, 2023; García *et al.*, 2014). In this context, upper Río Negro and two Laguna Merin effluents (Tacuarí and Yaguarón rivers), have been hypothesized as connected by drainage rearrangements (Loureiro *et al.*, 2011; Ramos-Fregonezi *et al.*, 2017; Souza *et al.*, 2020), which may have occurred approximately 500 thousand years ago (Loureiro *et al.*, 2023) and temporarily connected wetlands from both ecoregions.

We examined the identity of these populations using morphological, genetic, and geographic information, interpreting their highly variable chromatic patterns, and added insights about the putative historical events in their cladogenetic process. As a consequence, we describe *A. aff. luteoflammulatus* 1 and *A. aff. quirogai* as two new species in the genus. Moreover, we confirm that uniform pigmented males from the lower Cebollatí basin must be interpreted as a local variation of *A. luteoflammulatus* and propose that *A. aff. luteoflammulatus* 2 populations from Negro and Tacuarí rivers basins correspond to intraspecific *A. quirogai* variation.

## MATERIAL AND METHODS

Specimens analyzed, tissue samplings, and comparative material are deposited in the Fish Collection (ZVC-P) and the Sección Genética Evolutiva (GE) of Facultad de Ciencias, Universidad de la República, Montevideo; and in the fish collection of Museo Nacional de Historia Natural de Montevideo (MHNM) (Tab. S1). Morphometric and meristic counts were recorded under a dissecting microscope following Costa (1989) and Loureiro, García (2008). Cephalic neuromast series nomenclature follows Costa (2001). Cleared and counterstained specimens (c&s) were prepared following Dingerkus, Uhler (1977).

Morphological variation was also assessed using a geometric morphometric analysis of landmark configurations. Shape variation was analyzed using a thin-plate spline approach (Bookstein, 1991). Landmark positions were defined according to D'Anatro, Loureiro (2005) and acquired from scanned fish images (HP, ScanJet, 5590), after digitizing using the TPSdig (Rohlf, 2003). Weight matrices of partial warps were generated using TPSregr (Rohlf, 2003). Visualization of specimen grouping and the corresponding shape variation was obtained by canonical variate analyses. Due to sexual dimorphism, males and females were analyzed separately.

For molecular analyses, genomic DNA was isolated from muscle tissue fixed in ethanol (95%), obtained from freshly sacrificed animals by an overexposure to a solution of 1% 2-phenoxyethanol (Sigma), using extraction with sodium chloride protein precipitation, followed by ethanol precipitation (modified from Medrano *et al.*, 1990). A partial fragment of approximately 800 bp from the Cytb gene was amplified using the CB3-H (5'-GGCAAATAGGAARTATCATTC-3') and Gludg-L (5'-TGACTTGAARAACCAAYCGTTG-3') primers (Palumbiet *al.*, 1991). PCR amplifications were performed in 20 µl reactions containing 1 X Buffer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.25 µl of each primer, 1.0 unit of Taq DNA polymerase (Invitrogen), and approximately 100 ng of template DNA in a final volume of 10 µl. Cycling conditions included an initial denaturation of 3 min at 94°C, followed by four cycles of 94°C for 1 min, 42°C for 1 min, 72°C for 1 min, followed by 29 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension of 7 min at 72°C. PCR products were purified and subsequently sequenced in both strand directions, using the same amplification primers, in a Perkin-Elmer ABI Prism 377 Automated Sequencer (MACROGEN, Seoul, Korea). Sequences were edited and aligned with MEGA (v. 11.0.10) (Tamura *et al.*, 2021) using the ClustalW option. Genbank accession numbers for sequences collected in this study are given in Tab. S1.

Haplotypes were obtained using DnaSP v. 5 (Librado, Rozas, 2009). Haplotype network was constructed with Network v. 10.2.0.0 (Fluxus Technology Ltd 1999–2025) using the Median Joining algorithm (Bandelt *et al.*, 1999).

To obtain a general timeframe for the divergence of putative species, a time-tree was inferred using Beast v. 1.10.4 (Drummond *et al.*, 2012) with the cytochrome b sequences, using as outgroups: *Matilebias alexandri* (Castello & López, 1974) (GB: AF245011), *Austrolebias bellottii* (Steindachner, 1881) (GB: AF245006), *Garcialebias viarius* (Vaz Ferreira, Sierra de Soriano & Scaglia de Paulete, 1964) (GB: AF245456), *Megalebias wolterstorffi* (Ahl, 1924) (GB: KY939727, KY939728, KY939731, KY939732). Priors were based on a normally distributed substitution rate of 0.0276 (SD = 0.00277)

substitutions per site per million years as estimated by Garcez *et al.* (2018). We calibrated the tree with the node that separates *M. wolterstorffi* populations from the west and east banks of Laguna dos Patos, split estimated by Garcez *et al.* (2018) as occurred 370,000 years before present. We set a coalescent constant size tree prior with a random starting tree and the GTR+I+G model of sequence evolution, determined by the corrected Akaike Information Criterion (AICc) in jModelTest2 (Darriba *et al.*, 2012). Two MCMC chains were run for 100,000,000 generations, with trees and parameters sampled every 10,000 iterations, and a burn-in of 25%. Results were visualized in Tracer v. 1.7 (Rambaut *et al.*, 2018) to evaluate convergence and stationarity, and to ensure that ESS (Effective Sample Sizes) were above 200. The maximum credibility tree was inferred with TreeAnnotator v. 1.5.4 (Drummond, Rambaut, 2007) and edited in FigTree v. 1.4.3 (Rambaut, 2010).

We used SPdel software (Ramirez *et al.*, 2023) that is a pipeline to integrate different single-gene species delimitation methods. SPdel is designed to calculate statistics and compare MOTUs obtained by different methods (GMYC, PTP, bPTP, ASAP, ABGD).

Population analyses. The corrected estimates of pairwise sequence divergence among clades from the phylogenetic analyses were obtained using the two-parameter algorithm (K2P) of Kimura (1980) implemented in MEGA. DNA polymorphism was measured within clades, calculating the proportion of segregating sites (*s*), the haplotype diversity *H* (Nei, 1987:179), and the nucleotide diversity  $\pi$  (Nei, 1987:257) using DnaSP v.5 (Librado, Rozas, 2009).

## RESULTS

The sequences analyzed corresponded to 58 haplotypes with a total *H* of 0.98. The haplotype network identified four large groups of haplotypes (Fig. 1): *A. luteoflammulatus* (including uniformly pigmented males from lower Cebollati basin), separated from *A. quirogai* by 55 mutational steps; *A. aff. luteoflammulatus* 1 (separated from *A. luteoflammulatus* by 20 mutational steps); *A. quirogai* with *A. aff. luteoflammulatus* 2 nested within; *A. aff. quirogai*, separated from *A. quirogai* by 25 mutational steps. The four groups of haplotypes presented more than 4% of nucleotide divergence among each other (Tab. 1).

Within *A. quirogai*, haplotypes from the Laguna Merín effluents (Yaguarón and Tacuarí) were genetically closer to different groups of Río Negro haplotypes, including the sharing of haplotype 7 (Tacuarí and Negro) and haplotype 8 (Yaguarón and Aceguá), than to each other (Fig. 1). Furthermore, *A. aff. luteoflammulatus* 2 haplotypes were genetically closer to different *A. quirogai* haplotypes than to each other.

The phylogenetic tree (Fig. 2) showed a first divergent event that grouped from one side *A. luteoflammulatus* + *A. aff. luteoflammulatus* 1, and *A. quirogai* + *A. aff. luteoflammulatus* 2 (polyphyletically nested within *A. quirogai*) + *A. aff. quirogai* in the other. The estimated time of divergence of the two reciprocally monophyletic groups was 1.8 million years before present. *Acantholebias luteoflammulatus* was recovered as the sister clade of *A. aff. luteoflammulatus* 1 with a divergence time of 0.8 million years before present. *Acantholebias luteoflammulatus* populations diverged 0.38 million years before present, while *A. aff. luteoflammulatus* 1 diverged 0.3 million years before

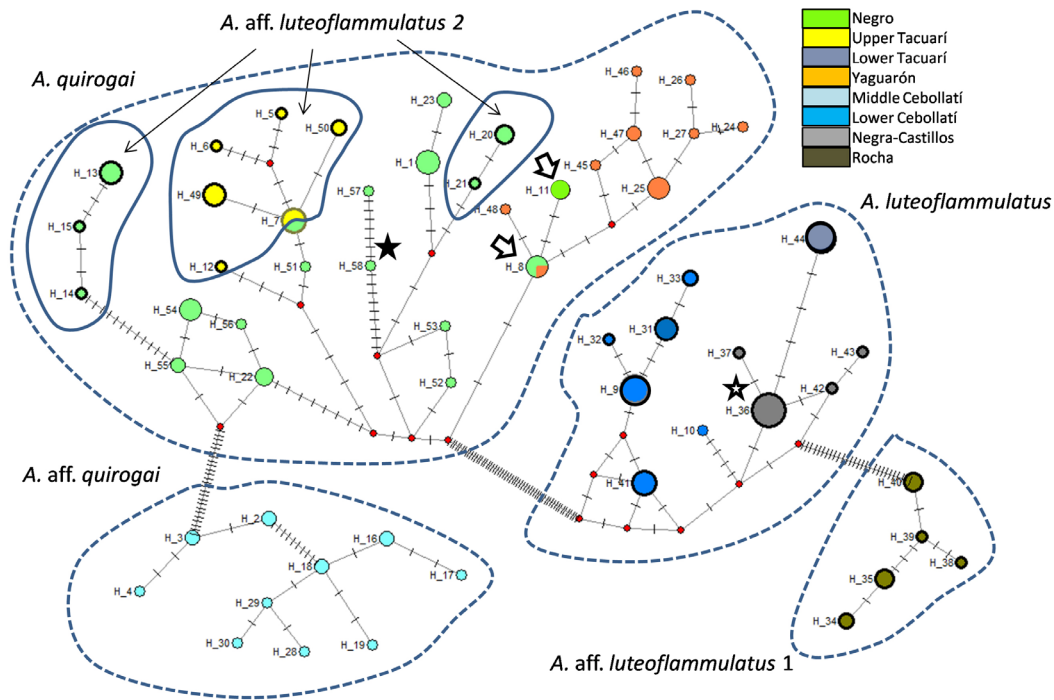


**FIGURE 1** | Haplotype network of a fragment of cytochrome b of the sequences of *Acantholebias luteoflammulatus*, *A. aff. luteoflammulatus* 1, *A. aff. luteoflammulatus* 2, *A. quirogai*, and *A. aff. quirogai*. Colours of haplotypes represent hydrographic basins (see reference chart in the figure). Continuous blue line encircles *A. aff. luteoflammulatus* 2 haplotypes. Black star = *A. quirogai* topotype; white star = *A. luteoflammulatus* topotype. White arrows = Aceguá populations.

**TABLE 1** | Estimates of evolutionary divergence over sequence pairs between and within species of *Acantholebias* based on Kimura-2-parameter model (Kimura, 1980). DNA polymorphism: nH = number of Haplotypes; Hd = Haplotype diversity; Nd = Nucleotide diversity; Ss = Segregating sites; N = number of individuals sampled.

|                                   | <i>A. quirogai</i> | <i>A. aff. quirogai</i> | <i>A. luteoflammulatus</i> | <i>A. aff. luteoflammulatus</i> 1 | nH | Hd   | Nd    | Ss | N  |
|-----------------------------------|--------------------|-------------------------|----------------------------|-----------------------------------|----|------|-------|----|----|
| <i>A. quirogai</i>                | 0.016              | 0.043                   | 0.099                      | 0.112                             | 25 | 0.96 | 0.015 | 56 | 67 |
| <i>A. aff. quirogai</i>           |                    | 0.009                   | 0.096                      | 0.098                             | 7  | 0.86 | 0.008 | 16 | 14 |
| <i>A. luteoflammulatus</i>        |                    |                         | 0.008                      | 0.049                             | 7  | 0.87 | 0.007 | 17 | 37 |
| <i>A. aff. luteoflammulatus</i> 1 |                    |                         |                            | 0.004                             | 9  | 0.81 | 0.004 | 8  | 20 |

present. On the other hand, *Acantholebias quirogai* was recovered as the sister clade of *A. aff. quirogai* with a divergence time of 0.9 million years before present; *A. quirogai* populations diverged 0.5 million years before present, while *A. aff. quirogai* populations diverged around 0.37 million years before present.

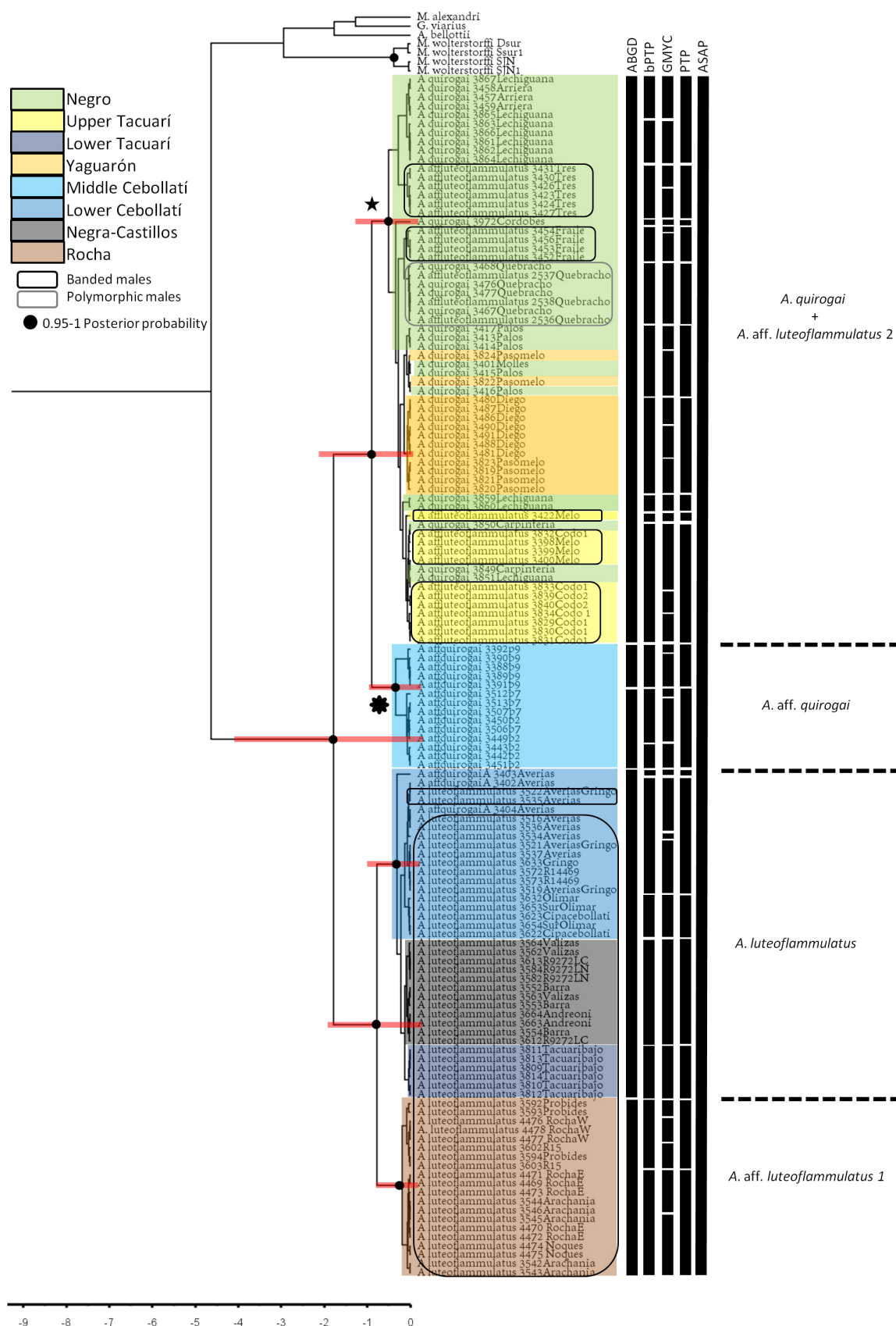


**FIGURE 2 |** Species delimitation analyses and time calibrated tree of *Acantholebias* species. Time bar in million years. Red bars = 95% confidence. Star = reduction in squamation; Asterisk = Absence of Parietal Neuromast.

Species delimitation methods showed different discrimination criteria (Fig. 2). While ASAP considered the whole clade as single “taxon”, bPTP, PTP, and GYMC clearly overestimated the number of species indicating almost every monophyletic cluster as a different species. On the other hand, ABGD identified five OTUs, which corresponded with geographic areas of distribution: lower Laguna Merín basin (Tacuarí, Cebollatí, Laguna Negra) + Laguna Castillos (*A. luteoflammulatus*); Laguna de Rocha basin (*A. aff. luteoflammulatus* 1); Río Negro and upper Yaguarón and Tacuarí river basins (*A. quirogai* with *A. aff. luteoflammulatus* 2 nested within); middle Cebollatí basin (*A. aff. quirogai* to the north of the Cebollatí River); middle Cebollatí basin (*A. aff. quirogai* to the south Cebollatí River).

Geometrical morphometry analyses showed that in males, Root 1 of the Canonical Analysis separated *A. quirogai* + *A. aff. luteoflammulatus* 2 from *A. aff. quirogai* + *A. aff. luteoflammulatus* 1, with changes associated with head shape and position of dorsal fin origin (Fig. 3); *A. luteoflammulatus* variation overlapped the variation of all of the populations considered. Less discrimination was observed among female populations (Fig. 4), although Root 1 of the Canonical Analysis also slightly discriminated *A. quirogai* and *A. aff. luteoflammulatus* 2 from the others.

External morphology analysis showed that *A. aff. luteoflammulatus* 2 (Fig. 5B) exhibited the reduction in abdominal squamation previously reported for *A. quirogai* (Fig. 5A), while *A. aff. quirogai*, *A. aff. luteoflammulatus* 1 presented the complete abdominal

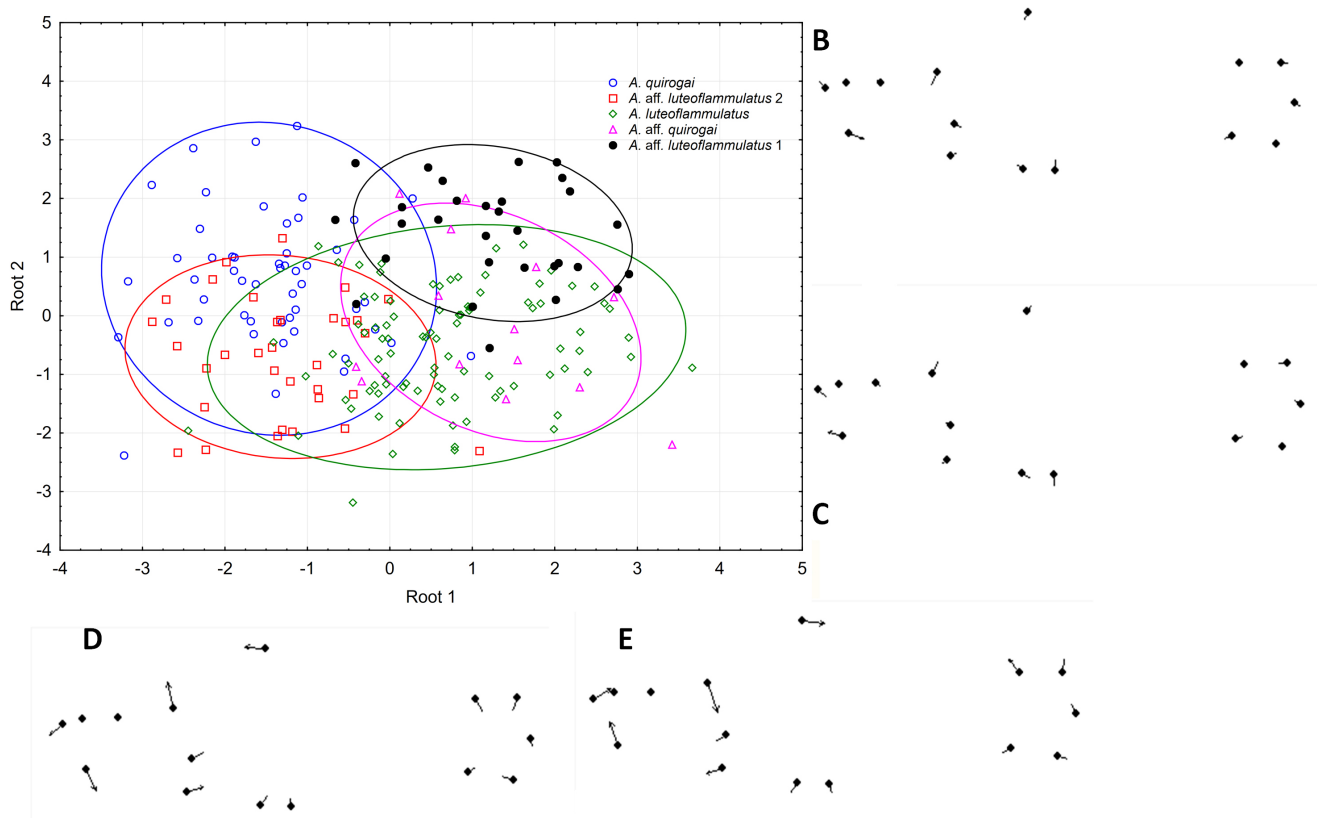


**FIGURE 3** | Geometric morphometry analysis of males of *Acantholebias*. **A.** Scatter plot of specimens in the Canonical Analysis (CA); **B.** Deformation of consensus in the positive values of Root 2 of the CA; **C.** Deformation of consensus in the negative values of Root 2 of the CA; **D.** Deformation of consensus in the negative values of Root 1 of the CA; **E.** Deformation of consensus in the positive values of Root 1 of the CA.

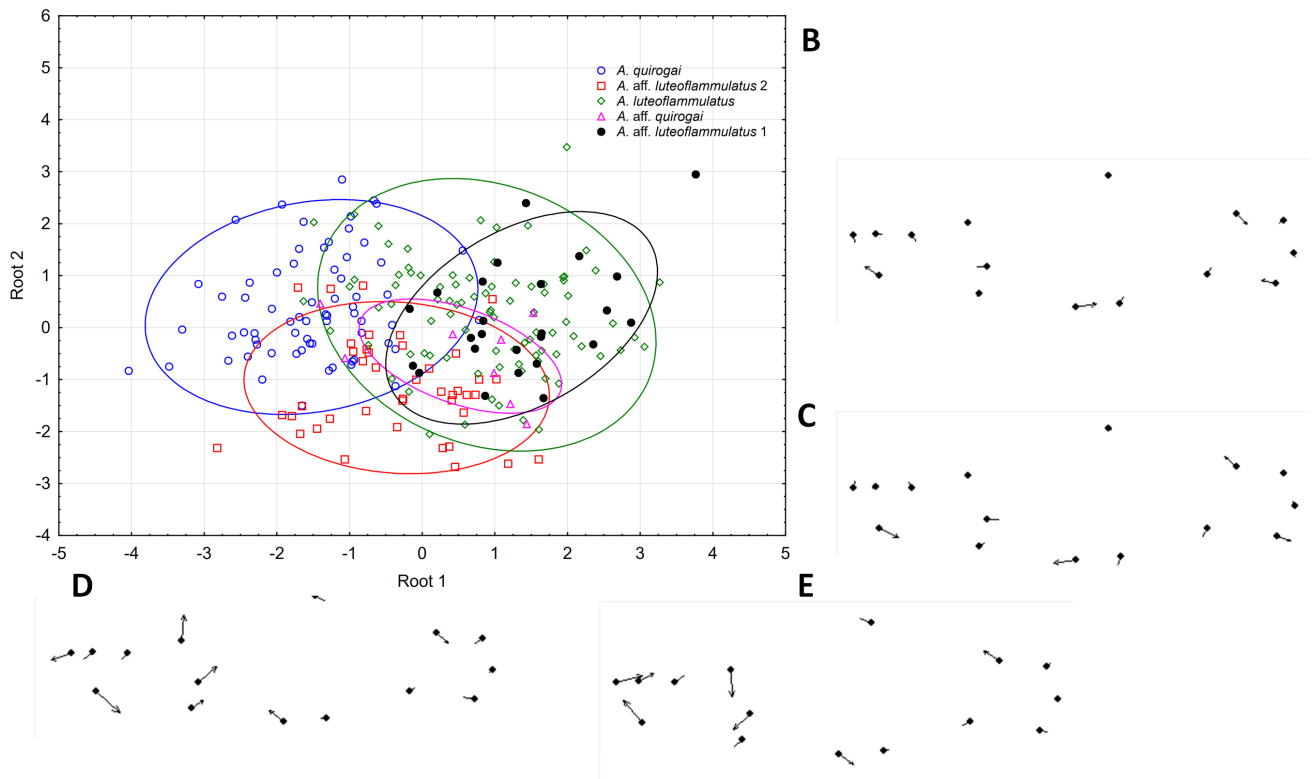


**FIGURE 4** | Geometric morphometry analysis of females of *Acantholebias*. **A.** Scatter plot of specimens in the Canonical Analysis (CA); **B.** Deformation of consensus in the positive values of Root 2 of the CA; **C.** Deformation of consensus in the negative values of Root 2 of the CA; **D.** Deformation of consensus in the negative values of Root 1 of the CA; **E.** Deformation of consensus in the positive values of Root 1 of the CA.

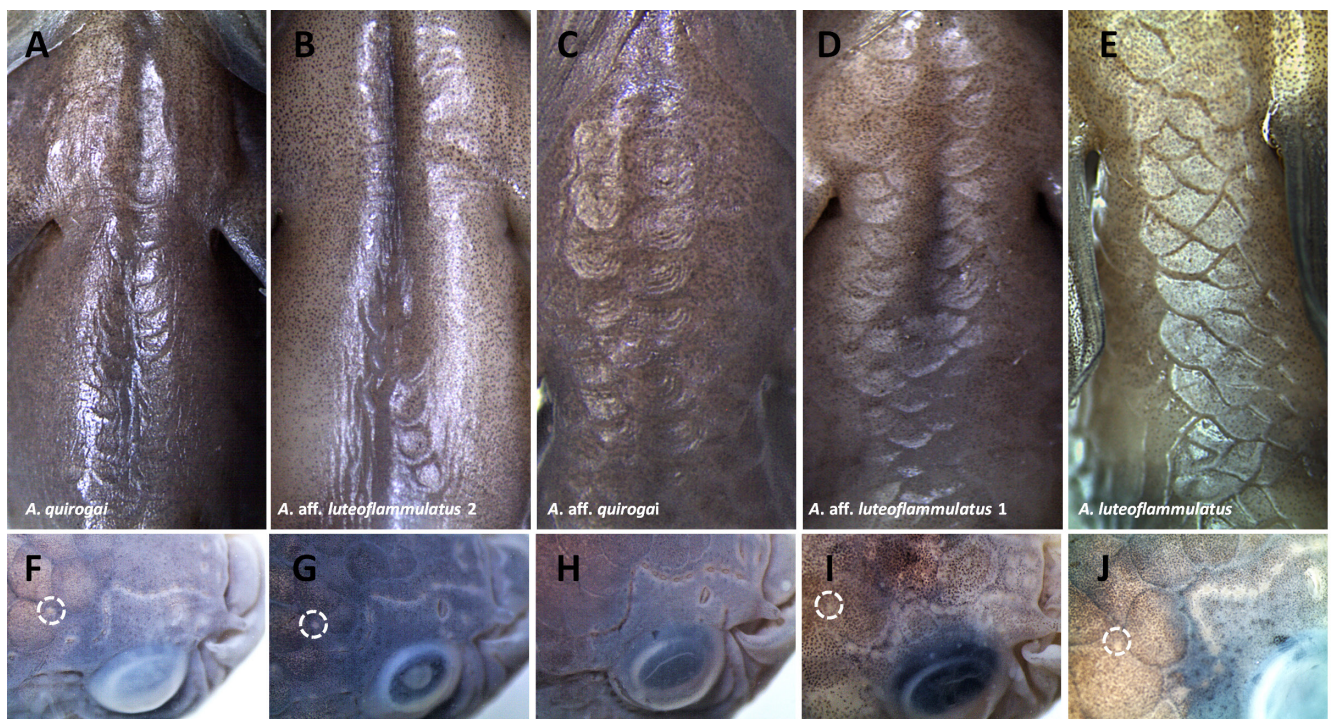


**FIGURE 5** | Abdominal squamation (above) and right dorsal view of cephalic pores (below) in *Acantholebias* species. **A, F.** = *A. quirogai*; **B, G.** = *A. aff. luteoflammulatus 2*; **C, H.** = *A. aff. quirogai*; **D, I.** = *A. aff. luteoflammulatus 1*; **E, J.** = *A. luteoflammulatus*. White dotted circle = Parietal Neuromast.

squamation previously observed in *A. luteoflammulatus* (Figs. 5C–E). *Acantholebias* aff. *quirogai* lacked the parietal neuromasts (Fig. 5H) which are present in the remaining groups (Figs. 5F, G, I, J).

Apart from the pigmentation variation already reported, males of *A. luteoflammulatus* (including the uniform pigmented males from lower Cebollati) exhibited, in most individuals analyzed, a dark grey vertical bar on posterior portion of the dorsal fin preceded by two vertically aligned clear dots (Figs. 6A, B); in *A. aff. luteoflammulatus* 2 the bar was diffusely present in 57 % of individuals analyzed (n = 21); in *A. aff. luteoflammulatus* 1, *A. quirogai*, and *A. aff. quirogai* the bar was absent (Figs. 6C, D, F). Female pigmentation presented two states: dots and elongated dots/bars (Figs. 6G–J). In *A. luteoflammulatus*, both variants were equally represented (n = 58); in *A. aff. luteoflammulatus* 1, 12% presented dots while 88% presented elongated dots/bars; *A. quirogai*, *A. aff. luteoflammulatus* 2, and *A. aff. quirogai* only presented dots.

Based on all the analyses we consider *A. aff. luteoflammulatus* 1 and *A. aff. quirogai* to be two new species of *Acantholebias*. The first diagnosed by cytochrome b specific mutations (Tab. 2), variation in pigmentation patterns, and osteological characters; the second by a combination of morphological and pigmentation characters.

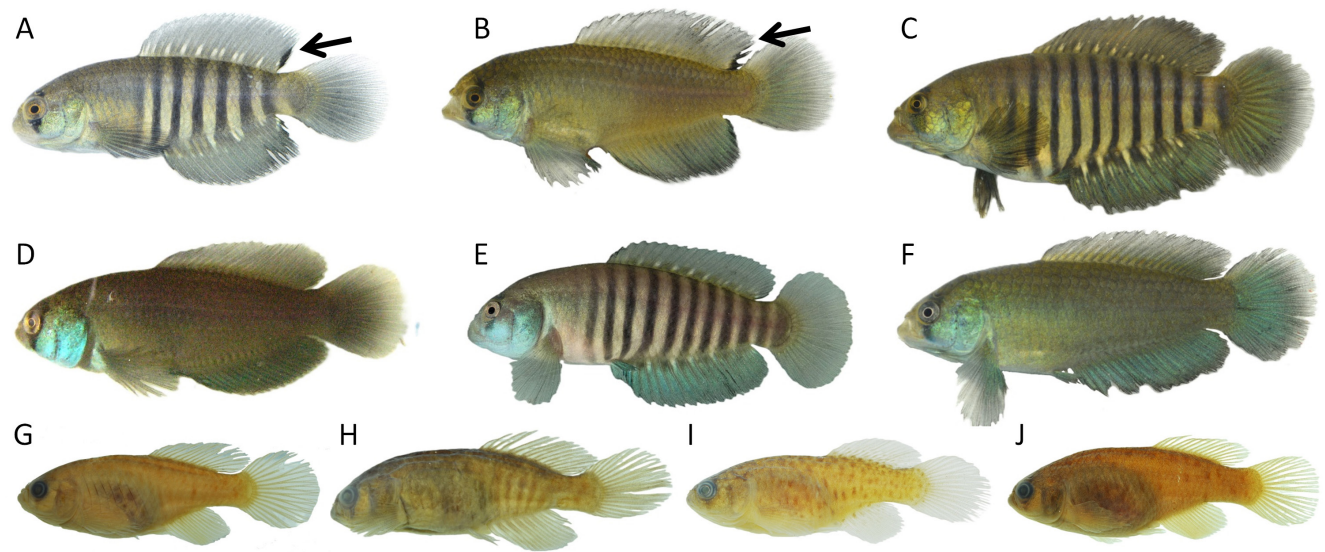


**FIGURE 6 |** Pigmentation pattern in *Acantholebias* males. **A.** *A. luteoflammulatus* banded (ZVC-P 16444); **B.** *A. luteoflammulatus* uniform (ZVC-P 16444); **C.** *A. aff. luteoflammulatus* 1 (ZVC-P 16445); **D.** *A. quirogai* (ZVCP 8742); **E.** *A. aff. luteoflammulatus* 2 (ZVCP 14462); **F.** *A. aff. quirogai* (ZVCP 16449). Pigmentation patterns in *Acantholebias* females. **G.** *A. luteoflammulatus* (ZVC-P 4105); **H.** *A. aff. luteoflammulatus* 1 (ZVC-P 15359); **I.** *A. quirogai* (ZVC-P 9718); **J.** *A. aff. quirogai* (ZVC-P 13502). Arrow = dark grey vertical bar.

**TABLE 2 |** Diagnostic Nucleotide for *Acantholebias tuireae* within the genus *Acantholebias* (bold and \*) and those that differentiate it from *A. luteoflammulatus*. Positions were numbered according to the complete cytochrome b sequence of *Garcialebias charrua* (GenBank accession number: NC028510) used as reference.

| Cytochrome b position      | 45 | 81 | 125       | 133 | 210 | 264 | 267 | 327       | 348 | 475 | 510 | 564 | 574       | 583 | 585       | 624       | 631       | 635       | 669 | 696 |
|----------------------------|----|----|-----------|-----|-----|-----|-----|-----------|-----|-----|-----|-----|-----------|-----|-----------|-----------|-----------|-----------|-----|-----|
| <i>A. tuireae</i>          | T  | T  | <b>T*</b> | A   | T   | T   | C   | <b>C*</b> | A   | G   | G   | A   | <b>G*</b> | A   | <b>C*</b> | <b>G*</b> | <b>T*</b> | <b>G*</b> | T   | T   |
| <i>A. luteoflammulatus</i> | C  | C  | C         | G   | C   | C   | T   | T         | G   | A   | A   | G   | A         | G   | A         | C         | C         | A         | C   | C   |

## Taxonomic account

### *Acantholebias tuireae*, new species

urn:lsid:zoobank.org:act:8B624542-7010-41CA-998D-D676E0B71DC4

(Figs. 7A, B; Tab. 3)

**Holotype.** ZVCP-P 16238, male, 36.2 mm SL, Uruguay, Departamento de Rocha, Laguna de Rocha basin, Camino Vecinal, 34.563°S 54.331°W, 5 Sep 2020, M. Loureiro, A. Duarte & W. Serra.

**Paratypes.** All from Uruguay, Departamento de Rocha, Laguna de Rocha basin. ZVC-P 15359, 13 males, 23.4–36.0 mm SL (2 c&s), 20 females, 20.7–29.9 mm SL (2 c&s), collected with the holotype. ZVC-P 4077, 9 males, 45.3–59.5 mm SL (3 c&s), 11 females, 39.9–46.1 mm SL (3 c&s), Ruta 10 km 225, 34.612°S 54.158°W, 3 Oct 1998, M. Loureiro, J. García & C. Piccini. ZVC-P 4078, 20 males, 35.0–47.5 mm SL (2 c&s), 20 females, 31.9–37.9 mm SL (2 c&s), Ruta 9 km 205, 34.504°S 54.335°W, 3 Oct 1998, M. Loureiro, J. García & C. Piccini. ZVC-P 4085, 7 males, 30.2–44.1 mm SL, 1 female, 29.6 mm SL, Camino Puerto de los Botes, 34.509°S 54.333°W, 5 Oct 1998, M. Loureiro, J. García & C. Piccini. ZVC-P 4104, 3 males, 29.9–37.4 mm SL, 6 females, 29.4–31.7 mm SL, Camino Puerto de los Botes, 34.509°S 54.333°W, 1 Oct 1987, C. Ríos. ZVC-P 4105, 6 males, 22.6–39.6 mm SL, 2 females, 27.2–31.6 mm SL, Camino Puerto de los Botes, 34.509°S 54.333°W, 9 Jul 1982, R. Vaz-Ferreira. ZVCP 16445, 2 males, 30.4–37.0 mm SL, 1 female, 28 mm SL, Camino Vecinal, 34.563°S 54.331°W,

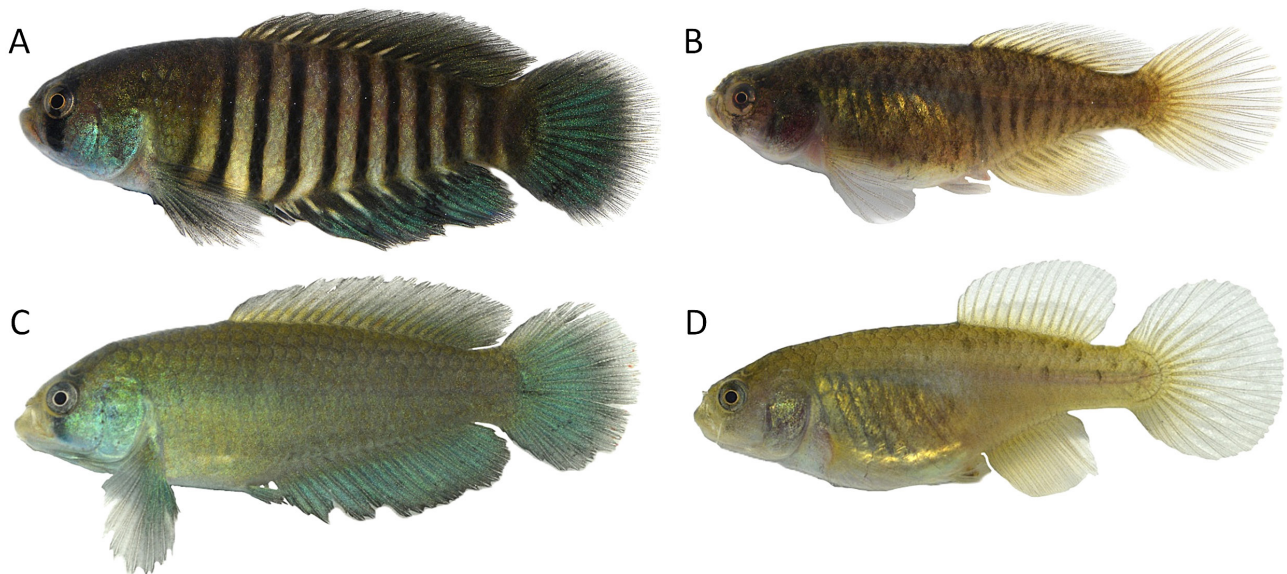


**FIGURE 7** | **A.** Holotype of *Acantholebias tuireae*, ZVC-P 16238, 31.6 mm SL; **B.** Female paratype of *A. tuireae*, ZVC-P 16445. **C.** Holotype of *Acantholebias bertae*, ZVC-P 16449, 31.6 mm SL; **D.** Female paratype, ZVC-P 16443.

25 Aug 2025, M. Loureiro, J. Bessonart & J. Sampayo. MHNM 4526, 1 male, 26.7 mm SL, 1 female, 24.3 mm SL, ciudad de Rocha, camino de Ejido, 34.499°S 54.318°W, 12 Jul 2020, W. S. Serra. MHNM 4628, 1 male, 40.4 mm SL, 1 female, 32.2 mm SL, PROBIDES, 34.504°S 54.341°W, 13 May 2021, W. S. Serra. MHNM 4632, 6 males, 19.2–31.4 mm SL, Camino Agustín de la Rosa, 1400 m al sur de la Ruta 9, 34.486°S 54.297°W, 12 Jul 2020, W. S. Serra. MHNM 4664, 2 females, 34.5–36.6 mm SL, ciudad de Rocha, 34.492°S 54.325°W, 1 Sep 2021, W. S. Serra. MHNM 4853 (GR2184 to GR2189), 6 males, 30.2–39.7 mm SL, PROBIDES, 34.504°S 54.341°W, 11 Jul 2011, G. García, N. Papa & N. Ríos. MHNM 4933, 5 males, 20.9–26.9 mm SL, 1 female, 21.5 mm SL, charco asociado a Cañada del Ejido, 34.494°S 54.294°W, 12 Jul 2020, W. S. Serra. MHNM 4934, 4 males, 24.6–28.4 mm SL, 1 female, 24.1 mm SL, Ruta 15 frente al CURE, 34.489°S 54.319°W, 25 Jul 2020, W. S. Serra. MHNM 6079, 3 males, 18.6–21.6 mm SL, 3 females, 13.7–26.0 mm SL, Camino Tropas, 34.523°S 54.301°W, 9 Aug 2020, W. S. Serra. MHNM 7634, 4 females, 27.3–35.6 mm SL, PROBIDES, 34.504°S 54.341°W, 30 Oct 2017, W. S. Serra.

**Diagnosis.** *Acantholebias tuireae* can be differentiated from *A. luteoflammulatus* by the absence of the dark grey vertical bar on the posterior portion of the dorsal fin, preceded by two vertically aligned clear dots (*vs.* present). It is further differentiated by the number of caudal vertebrae in males (16 *vs.* 16–18 mode 17 in *A. luteoflammulatus* and 17–19 mode 18 in *A. quirogai*) (Fig. 8); number of ventral gill rakers (8–9 mode 8 *vs.* 7–9 mode 7 in *A. luteoflammulatus*); number of teeth in second pharyngobranchial (3–6 mode 4 *vs.* 0–4 mode 2 in *A. luteoflammulatus* and *A. quirogai*, 2–3 mode 3 in *A. bertae*, new species). *Acantholebias tuireae* also presents 6 unique mutations of cytochrome b within the genus and 20 mutations that differentiate it from *A. luteoflammulatus* (Tab. 2).

**Description.** Morphometric data in Tab. 3. Body elongate, laterally compressed. Dorsal profile from dorsal tip of mouth to back of head straight, slightly convex to origin of dorsal fin, straight below dorsal fin, and straight on caudal peduncle. Ventral profile arched from tip of lower jaw to origin of anal fin, straight over anal fin, and straight on caudal peduncle. Mouth slightly prognathous. Dorsal-fin rays in males 23–25 (mode 24); in females 16–20 (mode 17). Dorsal and posterior tip of dorsal fin of males rounded. Anal-fin rays in males 19–23 (mode 22); in females 15–18 (mode 17). Anal-fin tip rounded in males. Anal-fin contour rounded in females. Caudal fin distal margin rounded, 21–24 (mode 22) rays in males and 21–24 (mode 22) fin rays in females. Caudal-fin rays supported by last 4 or 5 (mode 4) vertebrae. Pectoral fin rays 11–12 (mode 12) in males, 11–12 (mode 11) in females; pectoral-fin margin rounded in males, slightly pointed in females. Three pectoral-fin radials. Pelvic fin rays 5 or 6 (mode 5). Pelvic fins separated from each other. Total number of vertebrae 28–29 (mode 29), 12–13 (mode 13) precaudal, 16 caudal. Scales cycloid, 27–30 (mode 29) in lateral series; transverse scales 13–15 (mode 14) in males, 13–14 (mode 14) in females; scales around caudal peduncle 14–18 (mode 16). Preopercular and opercular region completely scaled. Abdominal region completely scaled (Fig. 3D). Contact organs on scales throughout most of body, more conspicuous on abdominal and opercular regions. Contact organs on inner surface of pectoral rays with bony support from first to sixth ray. Contact organs on 1st to 6th anal-fin rays, in some males with bony support from 2nd to 4th. Cephalic

**TABLE 3** | Morphometric data of *Acantholebias tuireae*. H = Holotype, N = number of specimens, Min = Minimum, Max = Maximum, SD = Standard deviation.

|                                    | H    | Males (N = 16) |      |      |      | Females (N = 14) |      |       |      |
|------------------------------------|------|----------------|------|------|------|------------------|------|-------|------|
|                                    |      | Mean           | Min  | Max  | SD   | Mean             | Min  | Max   | SD   |
| Standard length                    | 35.9 | 42.9           | 30.4 | 58.7 | –    | 34.6             | 24.8 | 46.1  | –    |
| <b>Percents of standard length</b> |      |                |      |      |      |                  |      |       |      |
| Predorsal length                   | 41.3 | 41.4           | 29.3 | 54.9 | 7.1  | 57.1             | 44.3 | 73.6  | 9.8  |
| Dorsal fin base                    | 53.2 | 44.7           | 33.3 | 60.6 | 8.4  | 32.0             | 22.4 | 41.0  | 5.3  |
| Preanal length                     | 49.3 | 45.5           | 32.4 | 68.3 | 9.5  | 57.7             | 37.4 | 78.0  | 13.3 |
| Anal fin base                      | 41.9 | 36.7           | 26.7 | 49.6 | 7.2  | 22.4             | 26.9 | 17.9  | 3.1  |
| Prepelvic length                   | 39.8 | 38.8           | 28.0 | 58.2 | 8.3  | 49.6             | 34.5 | 66.8  | 10.8 |
| Body depth                         | 32.6 | 28.5           | 19.8 | 41.6 | 6.0  | 30.0             | 20.8 | 43.9  | 7.2  |
| Peduncle depth                     | 18.1 | 14.2           | 9.0  | 19.8 | 3.2  | 13.7             | 10.0 | 20.6  | 3.6  |
| Peduncle length                    | 9.9  | 12.1           | 6.5  | 15.2 | 2.7  | 17.2             | 11.3 | 24.9  | 3.7  |
| Head length                        | 28.3 | 23.2           | 17.3 | 28.6 | 3.2  | 25.4             | 20.4 | 32.3  | 3.8  |
| Pectoral fin length                | 29.7 | 21.8           | 15.1 | 42.9 | 6.5  | 22.9             | 16.9 | 29.6  | 4.1  |
| Pelvic fin length                  | 13.0 | 10.0           | 7.4  | 14.3 | 2.1  | 13.2             | 9.3  | 19.6  | 2.8  |
| Caudal fin length                  | 27.8 | 21.9           | 18.8 | 27.8 | 2.2  | 26.5             | 22.4 | 33.9  | 4.5  |
| <b>Percents of head length</b>     |      |                |      |      |      |                  |      |       |      |
| Head width                         | 63.0 | 63.0           | 45.1 | 82.4 | 12.5 | 84.7             | 57.5 | 120.3 | 19.6 |
| Orbital diameter                   | 33.4 | 25.2           | 19.8 | 33.4 | 3.7  | 31.9             | 23.1 | 41.0  | 5.5  |
| Interorbital distance              | 38.6 | 40.5           | 30.4 | 51.5 | 5.9  | 45.3             | 36.4 | 68.4  | 9.9  |
| Snout length                       | 19.7 | 21.7           | 16.6 | 29.2 | 3.7  | 23.1             | 18.4 | 30.4  | 3.7  |

neuromast series: Anterior rostral 2; posterior rostral 2; supraorbital 14–18; parietal 0–2 (mode 1); infraorbital 15–24; orbital 1–2; preorbital 1–3; preopercular 11–20; lateral mandibular 3–5; mandibular 8–13; lateral supratemporal 0–1; posttemporal 1–2; postotic 1–4; median opercular 0–1; ventral opercular 0–4. Ventral gill rakers 8–9 (mode 8), dorsal gill rakers 2–3 (mode 3). Anterior cartilaginous basihyal 25 to 33% of total length; 3–6 teeth on second pharyngobranchial. Unicuspid rakers on ceratobranchials. Urohyal deep. Ventral process of anguloarticular reduced. Dermosphenotic absent. Urogenital papila of males not attached to first anal-fin ray.

**Coloration in alcohol.** Males with brown body, darker in anterior third, with black vertical bands in the posterior 2/3. Head dark brown, preopercular region lighter. Dark brown supra and suborbital band. Supraorbital band reaching supraorbital neuromast series. Dorsal and anal fins dark brown with white vertical marks between fin rays. Caudal, pectoral, and pelvic fins dark brown, lighter distally. Females light brown body background with scattered darker rounded or slightly elongate spots on whole body. Fins hyaline.

**Coloration in life.** Males with bluish gray body in the first third, light gray with vertical dark bars on the last two thirds in most males (some individuals can present the bars also in the first third); isthmus and opercular region bright light blue. Dorsal-fin dark grey with bluish hues, darker at the base which presents light gray elongated marks among fin rays that are continuous with lighter areas of body flank; anal-fin bluish in

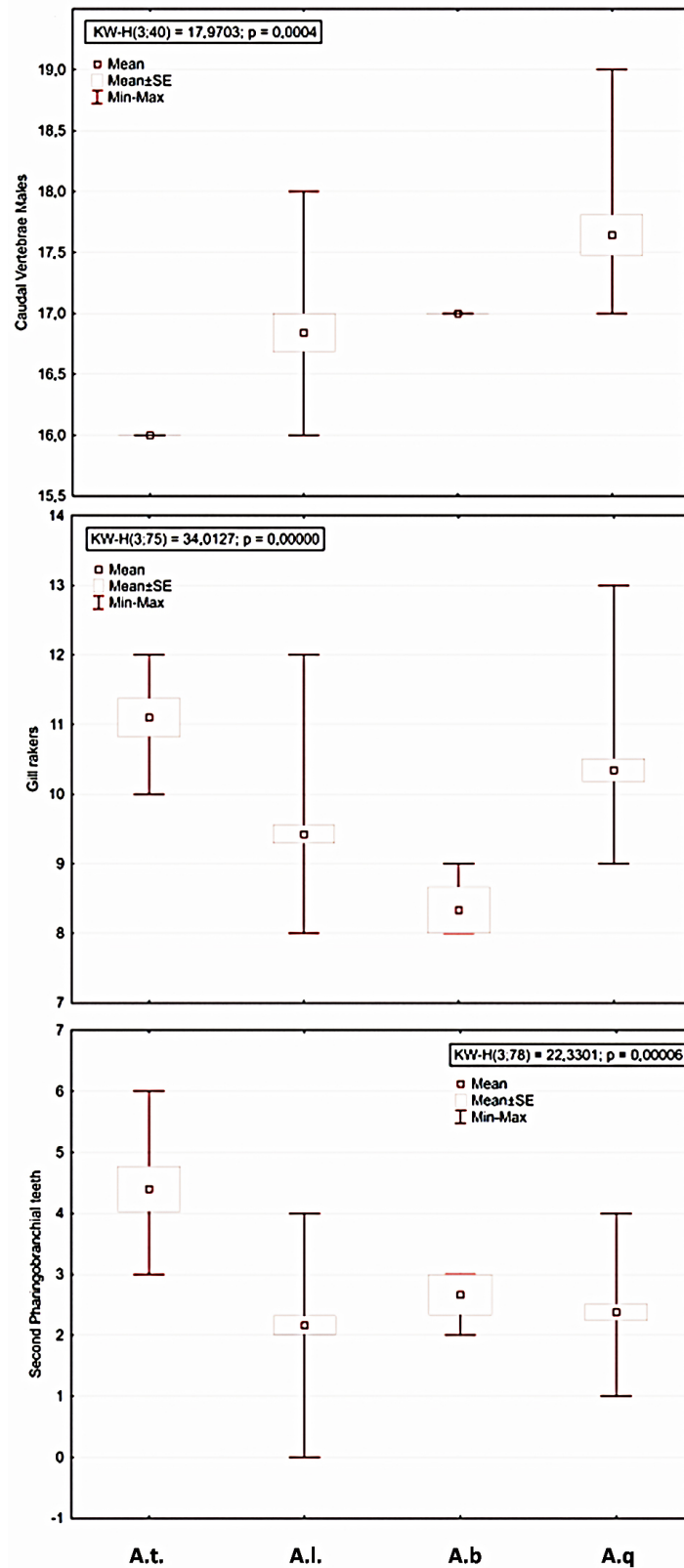


**FIGURE 8** | Box-plot of osteological characters with significant differences (Kruskall-Wallis test) in *Acantholebias* species.

distal 2/3 portion, darker at the base with light gray elongated marks between fin rays which are continuous with lighter areas of body flank; caudal fin bluish in proximal 2/3, light gray in the distal 1/3; pelvic fin bright light blue; pectoral fin similar coloration than caudal fin. Suborbital and supraorbital bands dark gray. Female's body brownish with scattered and sometimes faint dark gray or dark brown vertically elongate spots; abdominal region lighter and yellowish. Fins hyaline. Supra and suborbital band light gray, inconspicuous (Figs. 7A, B).

**Geographical distribution.** The new species is distributed in temporary ponds from the Atlantic coastal Laguna de Rocha basin (Laguna dos Patos freshwater ecoregion) (Fig. 9A).

**Ecological notes.** *Acantholebias tuireae* inhabits seasonal ponds that are mainly associated with flood plains of large wetlands or streams (Fig. 9C); particularly it can be found in suburban artificial ditches. Currently, is the only annual fish species in Laguna de Rocha basin.

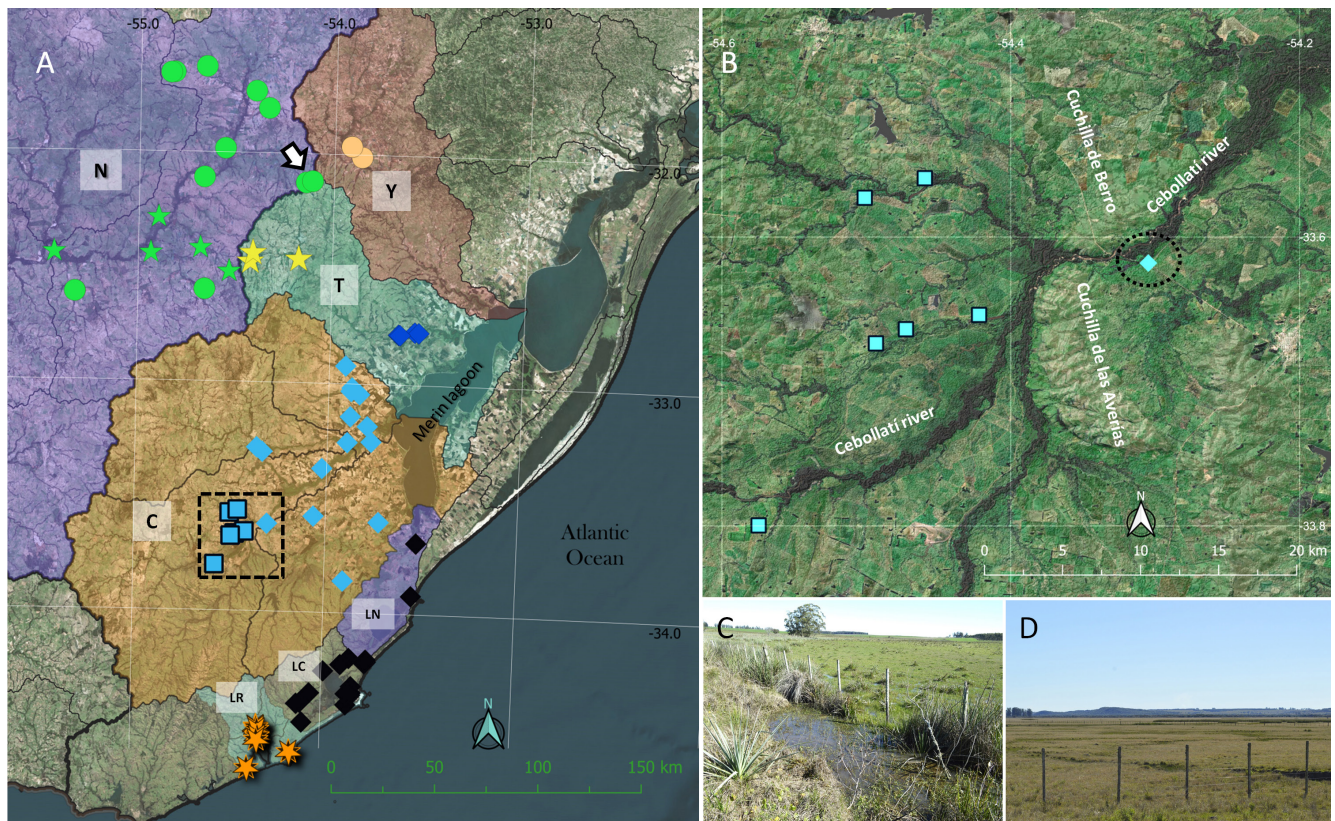


**FIGURE 9 |** A. Distribution of *Acantholebias luteoflammulatus* (light blue, blue, and black diamonds); *A. tuireae* (ten pointed orange stars); *A. quirogai* (uniform pigmented males: green and orange circles), *A. quirogai* (banded males: green and yellow five pointed stars); *A. bertae* (light blue squares). N = Negro River basin (LU); Y = Yaguarón River basin (LP); T = Tacuarí River basin (LP); C = Cebollatí River basin (LP); LN = Laguna Negra basin (LP); LC = Laguna Castillos basin (LP); LR = Laguna de Rocha basin (LP). White Arrow = Aceguá populations; thick black line = limit between Laguna dos Patos (LP) and Lower Uruguay (LU) ecoregions. B. Detail of dotted square from A, showing the topography which divides middle from lower Cebollatí River; dotted black circle = Paso Averías population. C. Type locality of *A. tuireae* (Laguna de Rocha basin; Laguna dos Patos freshwater ecoregion); D. Type locality of *A. bertae* (Middle Cebollatí River basin; Laguna dos Patos freshwater ecoregion).

**Conservation status.** Its Extension of Occurrence (IUCN, 2012) is 63 km<sup>2</sup>, and its Area of Occupancy (IUCN, 2012) is 48 km<sup>2</sup>. The area where it is distributed is subjected to intensive soy crops, an increase in urban areas, and tourism infrastructure. Annual killifishes are also subjected to non-regulated collection activities by fish hobbyists. Its populations are highly fragmented, with the quality of their habitat negatively affected by the human activities mentioned. For these reasons we consider the new species as Critically Endangered (CR) according to B1 IUCN criteria (IUCN, 2012).

**Etymology.** We name this species to honor the late Tuíre Kayapó, warrior and chief of the Kayapó people, who actively fought against the construction of Belo Monte Dam. The specific name is an eponym.

*Acantholebias bertae*, new species

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(Figs. 7C, D; Tab. 4)

**Holotype.** ZVC-P 16449, male 31.6 mm SL, Uruguay, Departamento de Lavalleja, Cebollatí River basin, A° del Sauce, Camino a Paso del Bote, 33.799°S 54.574°W, 26 Aug 2025, M. Loureiro, J. Bessonart & J. Sampayo.

**Paratypes.** Uruguay, Departamento de Lavalleja, Cebollatí River basin. ZVC-P 11665, 4 males, 26.2–29.8 mm SL, 4 females, 22.2–25.3 mm SL, Rincón del Cebollatí, 33.663°S 54.472°W, 12 Set 2012, M. Loureiro, J. Bessonart & D. Díaz. ZVC-P 13502, 5 males, 34.3–40.5 mm SL (1 c&s), 3 females, 29.6–33.2 mm SL (1 c&s), Rincón del Cebollatí, 33.663°S 54.472°W, 27 Jun 2011, M. Loureiro, A. Duarte, J. Bessonart & W. Serra. ZVC-P 14240, 2 males, 28.8–30.5 mm SL (1c&s), Rincón del Cebollatí, 33.653°S 54.422°W, 28 Jun 2011, M. Loureiro, A. Duarte, J. Bessonart & W. Serra. ZVC-P 15071, 3 males, 25.0–27.1 mm SL, 3 females, 20.6–24.0 mm SL, A° del Sauce, Camino a Paso del Bote, 33.799°S 54.574°W, 3 Oct 2020, M. Loureiro, J. Bessonart, A. Duarte & V. Pinelli. ZVC-P 15074, 3 males, 27.6–30.5 mm SL, 3 females, 22.3–27.7 mm SL, Rincón del Cebollatí, 33.673°S 54.493°W, 3 Oct 2020, M. Loureiro, J. Bessonart, A. Duarte & V. Pinelli. ZVCP 16443, 4 males, 39.3–40.3 mm SL, 3 females, 26.1–29.4 mm SL, A° Gutiérrez, 33.559°S 54.459°W, 26 Aug 2025, M. Loureiro, J. Bessonart & J. Sampayo. ZVCP 16452, 4 males, 29.8–30.2 mm SL, 4 females, 26.3–28.0 mm SL, A° del Sauce, Camino a Paso del Bote, 33.799°S 54.574°W, 14 Set 2012, M. Loureiro, J. Bessonart & D. Díaz. MHNM 7714, 1 male, 25.0 mm SL, 1 female, 20.6 mm SL, A° del Sauce, Camino a Paso del Bote, 33.799°S 54.574°W, 3 Oct 2020, M. Loureiro, J. Bessonart, A. Duarte & V. Pinelli. MHNM 7715, 1 male, 34.3 mm SL, 1 female, 29.6 mm SL, Rincón del Cebollatí, 33.663°S 54.472°W, 27 Jun 2011, M. Loureiro, A. Duarte, J. Bessonart & W. Serra.

**Diagnosis.** *Acantholebias bertae* can be differentiated from all other species of the genus by the unique absence of the parietal neuromast series (Fig. 5H). It can also be differentiated by the combination of the bluish gray uniform pigmentation of the male

**TABLE 4** | Morphometric data of *Acantholebias bertae*. H = Holotype, N = number of specimens, Min = Minimum, Max = Maximum, SD = Standard deviation.

|                                    | H    | Males (N = 17) |      |      |     | Females (N = 14) |      |      |     |
|------------------------------------|------|----------------|------|------|-----|------------------|------|------|-----|
|                                    |      | Mean           | Min  | Max  | SD  | Mean             | Min  | Max  | SD  |
| Standard length                    | 31.6 | 30.8           | 25.0 | 40.5 | –   | 25.6             | 20.6 | 33.2 | –   |
| <b>Percents of standard length</b> |      |                |      |      |     |                  |      |      |     |
| Predorsal length                   | 42.7 | 45.8           | 40.0 | 52.2 | 4.3 | 58.6             | 54.3 | 65.1 | 3.0 |
| Dorsal fin base                    | 51.7 | 49.3           | 41.9 | 54.6 | 3.5 | 33.0             | 23.6 | 37.6 | 3.6 |
| Preanal length                     | 47.5 | 49.6           | 46.0 | 53.4 | 2.1 | 59.2             | 55.2 | 63.0 | 2.6 |
| Anal fin base                      | 46.2 | 42.9           | 38.5 | 47.0 | 2.6 | 25.5             | 18.4 | 30.0 | 3.0 |
| Prepelvic length                   | 42.3 | 43.6           | 40.7 | 49.0 | 2.4 | 50.6             | 48.0 | 52.7 | 1.6 |
| Body depth                         | 30.1 | 30.4           | 24.8 | 34.5 | 2.7 | 30.6             | 25.8 | 37.2 | 3.3 |
| Peduncle depth                     | 16.7 | 15.7           | 13.6 | 17.5 | 1.1 | 13.8             | 10.6 | 15.3 | 1.2 |
| Peduncle length                    | 9.7  | 13.7           | 9.7  | 16.9 | 1.7 | 17.6             | 12.6 | 21.7 | 2.9 |
| Head length                        | 27.6 | 27.2           | 23.8 | 30.7 | 1.9 | 28.6             | 24.8 | 33.5 | 2.4 |
| Pectoral fin length                | 25.6 | 25.2           | 21.3 | 29.3 | 2.4 | 23.5             | 19.8 | 28.2 | 2.3 |
| Pelvic fin length                  | 13.3 | 10.2           | 8.1  | 13.3 | 1.0 | 11.4             | 8.5  | 14.5 | 1.6 |
| Caudal fin length                  | 26.6 | 27.4           | 21.7 | 30.6 | 2.4 | 29.1             | 22.1 | 34.1 | 3.6 |
| <b>Percents of head length</b>     |      |                |      |      |     |                  |      |      |     |
| Head width                         | 64.9 | 62.9           | 56.1 | 68.6 | 3.9 | 66.9             | 59.2 | 77.0 | 5.2 |
| Orbital diameter                   | 31.8 | 29.8           | 24.3 | 34.5 | 3.1 | 31.0             | 28.1 | 34.2 | 1.9 |
| Interorbital distance              | 38.0 | 43.2           | 35.0 | 53.6 | 5.4 | 38.7             | 29.5 | 45.5 | 5.0 |
| Snout length                       | 17.8 | 20.1           | 15.4 | 24.9 | 2.9 | 18.8             | 12.5 | 23.9 | 3.4 |

flank vs. males with vertical bands in all individuals of *A. luteoflammulatus* (except a few individuals from lower Cebollatí basin) and in all individuals of *A. tuireae*, polymorphic in *A. quirogai*; abdominal squamation complete vs. reduced in *A. quirogai*. This species also has a lower number of gill rakers in the first branchial arc (8–9) (vs. 9–13 in *A. quirogai*) (Fig. 8).

**Description.** Morphometric data in Tab. 3. Body elongate, laterally compressed. Dorsal profile from dorsal tip of mouth to back of head straight, slightly convex to origin of dorsal fin, straight below dorsal fin, and straight on caudal peduncle. Ventral profile arched from tip of lower jaw to origin of anal fin, straight over anal fin, and straight on caudal peduncle. Mouth slightly prognathous. Dorsal-fin rays in males 19–25 (mode 23); in females 16–21 (mode 17). Dorsal and posterior tip of dorsal fin of males rounded. Anal-fin rays in males 18–23 (mode 21); in females 16–20 (mode 16). Anal-fin tip rounded in males. Anal-fin contour rounded in females. Caudal fin distal margin rounded, 16–23 (mode 20) rays in males and 16–23 (mode 21) fin rays in females. Caudal-fin rays supported by last 4 or 5 (mode 4) vertebrae. Pectoral fin rays 9–14 (mode 11) in males, 10–12 (mode 11) in females; pectoral-fin margin rounded in males, slightly pointed in females. Three pectoral-fin radials. Pelvic fin rays 4 to 6 (mode 5). Pelvic fins separated from each other. Total number of vertebrae 29–30 (mode 29), 12–13 (mode 12) precaudal. Scales cycloid, 27–33 (mode 29) in lateral series; transverse scales 12–17

(mode 14) in males, 12–14 (mode 13) in females; scales around caudal peduncle 12–17 (mode 14). Preopercular and opercular region completely scaled. Abdominal region completely scaled (Fig. 3D). Contact organs on scales throughout most of body, more conspicuous on abdominal and opercular regions. Contact organs on inner surface of pectoral rays with bony support from first to sixth ray. Contact organs on 1st to 6th anal-fin rays, in some males with bony support from 2nd to 4th. Cephalic neuromast series: anterior rostral 2; posterior rostral 2; supraorbital 12–18; parietal absent; infraorbital 14–25; orbital 1–4; preorbital 1–2; preopercular 12–19; lateral mandibular 3–6; mandibular 8–14; lateral supratemporal 0–1; posttemporal 1–2; postotic 1–4; median opercular 0–1; ventral opercular 0–4. Ventral gill rakers 7, dorsal gill rakers 1–2 (mode 1). Anterior cartilaginous basihyal approximately 1/3 of total length; 2–3 (mode 3) teeth on second pharyngobranchial. Unicuspid rakers on ceratobranchials. Urohyal deep. Ventral process of anguloarticular reduced. Dermosphenotic absent. Urogenital papilla of males not attached to first anal-fin ray.

**Coloration in alcohol.** Males with brown body, darker in anterior third. Head dark brown, preopercular region lighter. Dark brown supra and suborbital band. Supraorbital band reaching supraorbital neuromast series. Dorsal and anal fins dark brown with basal margin light brown. Caudal, pectoral, and pelvic fins dark brown. Females light brown body background with scattered darker rounded or slightly elongate spots on whole body. Fins hyaline.

**Coloration in life.** Males with bluish gray body, darker on dorsal half; abdominal region light pale blue; dorsal portion of head bluish gray with some green hues laterally; isthmus and opercular region bright light blue. Dorsal-fin base bluish gray, distal portion ochre; anal-fin base bluish gray, distal 2/3 portion bright light blue; caudal fin same color as body, gray; pelvic fin bright light blue. Suborbital band dark gray, supraorbital dark gray but less conspicuous. Female's body brownish with scattered and sometimes faint dark gray or dark brown dots; abdominal region lighter and yellowish. Fins hyaline. Supra and suborbital band light gray, inconspicuous (Figs. 8C, D).

**Geographical distribution.** The new species is distributed in the middle Cebollatí River basin (Laguna dos Patos freshwater ecoregion) (Figs. 9A, B).

**Ecological notes.** As in all annual fishes, *Acantholebias bertae* inhabits seasonal ponds mainly associated with floodplains of large wetlands or streams (Fig. 9D); particularly this species can also be found in artificial ditches of rice crops, which have substituted natural wetlands in the region. It is sympatric with other annual fishes, such as *Garcialebias charrua* (Costa & Cheffe, 2001), *Gymnolebias gymnoventris* (Amato, 1986), and *Titanolebias prognathus* (Amato, 1986).

**Conservation status.** Its Extension of Occurrence (IUCN, 2012) is 65 km<sup>2</sup> and its Area of Occupancy (IUCN, 2012) is 28 km<sup>2</sup>. The area where it is distributed is subjected to intensiverice crops, while the headwaters of the Cebollatí basin are subject to *Eucalyptus* spp. forestry. Annual killifishes are also subject to non-regulated collection activities by fish hobbyists. Its populations are highly fragmented, with the quality of

their habitat negatively affected by the human productive activities mentioned. For these reasons, we consider the new species as Critically Endangered (CR) according to B1 IUCN criteria (IUCN, 2012).

**Etymology.** We name this species to honor the late Berta Cáceres, a Lenca environmental and social activist murdered because of her fight to protect rivers and their people from dam building. The specific name is an eponym.

## DISCUSSION

Morphological, molecular, and geographic information show that the genus *Acantholebias* is a more complex clade than previously thought. In this context, we described two new species and discovered the more extreme chromatic polymorphism within the *Austrolebias* genus group, a clade already proposed as having high morphological and chromatic intraspecific variation (D'Anatro, Loureiro, 2005; García *et al.*, 2014). Our results also support previous findings about river rearrangement processes in the area studied (Loureiro *et al.*, 2011; Ramos-Fregonezi *et al.*, 2017; Souza *et al.*, 2020; Loureiro *et al.*, 2023).

Although using single locus data sets for phylogenetic analyses presents some difficulties, recent comparative studies suggested that a combination of single locus with various delimitation methods and the inclusion of other sources of information (morphological and geographical) provide well supported results representing evolutionary lineages (Luo *et al.*, 2018; Serrano *et al.*, 2019). In fact, these approaches have allowed the detection of new taxa and populations in species of the *Austrolebias* genus group (García *et al.*, 2012, 2019a,b, 2020). Among the species delimitation methods, ABGD was the most conservative and most congruent with geography, morphological variation, and phylogeny. All species analyzed, showed an intraspecific distance value between 0.4% and 1.6%, which falls in the range of most intraspecific variation among other species of the *Austrolebias* genus group previously reported (García *et al.*, 2012, 2019a). Interspecific divergence, ranging from 4.0 to 11.0%, is also congruent with previous studies in other genera within the *Austrolebias* genus group (García *et al.*, 2012, 2019a).

**Morphological and chromatic variation in the genus *Acantholebias*.** A high level of differentiation in body morphology and pigmentation pattern in males was previously reported by D'Anatro, Loureiro (2005) for *Acantholebias luteoflammulatus* and was also observed among species of other genera of the *Austrolebias* genus group (García *et al.*, 2012, 2019a). The highest morphometric variation in *A. luteoflammulatus* males could be related to its wider distribution range and concomitant variation in local and regional environmental conditions. However, male pigmentation variation in *A. quirogai* was the most striking finding. Although Loureiro *et al.* (2011) had already pointed out that some populations of this species exhibited the two observed patterns, with most individuals being uniform, in this study, we have found that the banded pattern in this species is predominant or even unique in several localities. Furthermore, at least based on the mitochondrial marker, these banded populations are more related

to uniform populations than to each other and present a marginal position within the haplotype network. On the other hand, banded populations present, although in different basins, a central and continuous spatial distribution within *A. quirogai*. This raises interesting questions about the nature of the evolution of the pigmentation pattern in these taxa, which could be explained by extrinsic factors (environmental and historical) and/or intrinsic such as genomic features. Among the environmental factors, it could be proposed the local action of natural selection (predation or sexual selection). Changes in coloration may have negative fitness consequences if they elevate the likelihood of predation (Ruiz-Rodríguez *et al.*, 2013), or aggression from conspecifics (Korzan, Fernald, 2006). In this context, *Cypholebias cinereus* (Amato, 1986) is another annual fish species that is a potential predator (due to its larger size). However, this species is syntopic with *A. quirogai* across its entire distribution, sharing ponds with both banded and uniform populations. The male chromatic patterns have been proposed as a robust driver of sexual selection in many fish groups (Stelkens *et al.*, 2008) as well as in other related species of the genus *Garcialebias* (Passos *et al.*, 2014, 2016). Further studies in this subject are needed to assess whether female preferences are selecting the different chromatic forms in different localities in this species.

Historical processes could also explain the distribution of these chromatic variants. It has been hypothesized that the area was subjected to Pleistocene river rearrangements (Loureiro *et al.*, 2011, 2023) connecting the Negro river basin (Lower Uruguay ecoregion) with effluents of Laguna Merín basin (Laguna dos Patos ecoregion). Strikingly, the population structure obtained for *A. quirogai* is topologically very similar to the sympatric *G. arachan* (Loureiro, Azpelicueta & García, 2004) (Loureiro *et al.*, 2023), and also points to two events of capture, one associated with the Tacuarí River and the other with the Yaguarón River. In this case, the similarity is astonishing since in both species, the Río Negro localities (Aceguá) closest to Yaguarón River, share haplotypes that are nested within Paso de Melo (Yaguarón basin) haplotypes. This pattern, and the migration estimate by Loureiro *et al.* (2023) indicates a recent connection (river capture) between both basins and it suggests that the direction of the dispersion could be also from the coastal basin (LM) to the “upland” basin (RN), which is contrary to the general tendency proposed by Ribeiro (2006) for the passive margin of South America. This complex scenario of historical connectivity could be influencing the chromatic complexity and high genetic intraspecific variation of *A. quirogai*.

Among intrinsic factors, genome instability caused by large scale chromosomal rearrangements detected in species of this genus could be taken into account (García *et al.*, 2014). Furthermore, recent whole genome sequencing in *Garcialebias charrua*, which has a similar genome size to those of *Acantholebias* species ( $\approx 6$  Gb), revealed that a genome increase resulted from the indiscriminate amplification of transposable elements (TEs,  $\approx 76.5\%$  of the total), particularly from the LINE and DNA transposon orders (Gajardo-Escobar *et al.*, 2026). It is well documented that TEs contribute to cichlid diversity by inducing mutations, chromosome rearrangements such as inversions and translocations, and alteration of gene regulation, which can lead to changes in pigmentation and other phenotypic traits (Galbraith, Hayward, 2023). Increased TE mobility may be associated with interspecific hybridization, which disrupts the mechanism of TE suppression. In this context, chromatic polymorphism in *A. quirogai* could also be a consequence of complex connectivity dynamics associated with the

capture events and concomitant secondary contact among populations. Hybridization events have also been documented between *Garcialebias charrua* and *G. reicherti* (Loureiro & García, 2004) in the lower Cebollatí River (Laguna dos Patos ecoregion) (García *et al.*, 2019b, 2020). These studies documented changes in chromatic patterns within the hybrid swarms. Finally, retention of ancestral characters may also be responsible for this pattern. All these potential processes cannot be addressed with mitochondrial markers alone, since gene mixing, hybridization, and incomplete lineage sorting may result in misinterpretation of the populations/species relationships analyzed (Xing *et al.*, 2025). Particularly in the case of *A. quirogai* and its pigmentation polymorphism, where mitochondrial DNA may not fully capture its evolutionary history when gene flow and hybridization are present. Further analysis including new genomic approaches could clarify the variable chromatic patterns in the genus *Acantholebias*.

**Timing of cladogenesis and present species distribution.** Another striking similarity between the sympatric *Acantholebias quirogai* and *Garcialebias arachan* was found in their estimated time of divergence of around 500 k years. This suggests that processes that govern their diversification have been common to both species, and could have occurred during the Pleistocene glaciation cycles, which for this area and altitude should be associated with climatic changes, from dry to wet periods. Bessonart *et al.* (2021) identified this area as an Area of Endemism for freshwater fishes, with *A. quirogai* and *Matilebias juanlangi* (Costa, Cheffe, Salvia & Litz, 2006) as diagnostic species. Thus, analyzing the population structure of *M. juanlangi*, as well as other annuals and non-annuals co-distributed in the area could offer a more complete view of the processes that govern the evolution of this freshwater habitat and their concomitant demographic processes.

The newly described species are located in the margins of the distribution of *A. luteoflammulatus*. Although, at first glance this could represent an example of peripheral isolation, recently demonstrated in other Neotropical Atheriniformes (Berg *et al.*, 2025), the phylogenetic tree only supported that this is the case for *A. tuireae*. Interestingly, although the proposed split from *A. luteoflammulatus* occurred 800 k years before present, the new species has not clearly differentiated morphologically. *Acantholebias tuireae* is restricted to wetlands associated with the Atlantic coastal Laguna de Rocha, which is currently isolated from similar adjacent basins, in particular from Laguna Castillos. Our estimated time of divergence is twice the age of the Pleistocenic sea level changes that are reported for the area from the last 400k years. Interestingly, Castillos and Laguna Negra populations of *A. luteoflammulatus* seem to be more related (tree and haplotype network) to the distant lower Tacuarí populations than to the lower Cebollatí populations.

The case of *A. bertae* is somewhat more complex. Although, its pigmentation pattern suggests a relationship with *A. quirogai* (confirmed in this study), squamation and distribution point to a closer relationship with *A. luteoflammulatus*. The phylogenetic tree indicates that this species is more closely related to *A. quirogai*, a geographically distant species with no evidence of recent contact between the two areas of distribution. *Acantholebias bertae* is distributed in a small area of wetlands of the middle Cebollatí River (from 30 to 50 m above sea level), only separated from *A. luteoflammulatus* populations by a topographic barrier (Averias and Berro Hills, Fig. 9B) that forms a

funnel-like topography that isolates the middle from the lower Cebollatí wetlands (from 0 to 25 m above sea level). Despite finding some males without dark bands in the downstream pond closest to the funnel, a possible evidence of downstream dispersion and secondary contact (see above), the mitochondrial marker indicates that they are nested within *A. luteoflammulatus* variation. Furthermore, their estimated divergence time is approximately 2 million years. The low capacity of dispersion through river main channel is also indicated by the split of *A. bertae* haplotypes between northern and southern populations of the Cebollatí River. However, the use of nuclear markers could put light in the population dynamics in this area and how these populations are evolving. Thus, ancient hybridization events concurrent with historical river rearrangements in this area could not be ruled out as an alternative hypothesis to explain the high genetic and morphological diversity detected in species of the genus *Acantholebias*.

Finally, this study also has shown that at least two of the synapomorphies proposed for *Acantholebias* (dorsal fin with a dark grey vertical bar on the posterior portion in males and dark grey pigmentation pattern on the flank of females composed by dots) should be reevaluated in light of the pigmentation patterns found herein. Furthermore, the high intraspecific variation in chromatic characters we observed also cast a doubt over the validity of these kinds of characters to justify generic identities in the *Austrolebias* “genus group”.

**Comparative material examined.** **Uruguay.** *Acantholebias luteoflammulatus*. Departamento de Rocha. ZVC-P 526, 1 male, 31.9 mm SL. ZVC-P 3819, 6 males, 19.8–32.0 mm SL, 3 females, 20.0–21.2 mm SL. ZVC-P 3892, 1 male, 33.2 mm SL. ZVC-P 3942, 9 males (1 c&s), 29.8–36.3 mm SL, 8 females, 25.6–36.8 mm SL. ZVC-P 4082, 24 males (3 c&s), 23.4–37.6 mm SL, 12 females (2 c&s), 28.4–34.6 mm SL. ZVC-P 4084, 4 males, 25.9–38.9 mm SL, 15 females, 17.1–31.4 mm SL. ZVC-P 4088, 51 males, 17.0–31.9 mm SL, 38 females, 16.9–24.3 mm SL. ZVC-P 4092, 1 male, 27.6 mm SL. ZVC-P 4093, 10 males (2 c&s), 22.4–30.9 mm SL, 19 females (2 c&s), 18.7–26.9 mm SL. ZVC-P 4098, 2 females, 21.5–22.9 mm SL. ZVC-P 4995, 3 males, 31.4–40.8 mm SL, 2 females, 32.7–33.1 mm SL. ZVC-P 15720, 20 males, 28.4–41.8 mm SL, 14 females, 23.9–33.1 mm SL. ZVC-P 16444, 2 males, 30.5–30.7 mm SL. MHNM 2394, 3 males, 15.5–21.9 mm SL, 12 females 16.2–24.9 mm SL. MHNM 2402, 2 males, 23.2–27.3 mm SL. MHNM 2552, 1 male, 20.1 mm SL, 2 females, 15.2–17.6 mm SL. MHNM 4483, 1 male, 17.3 mm SL, 1 female, 16.9 mm SL. MHNM 4550, 16 males, 21.2–40.4 mm SL, 16 females, 26.3–33.4 mm SL. MHNM 4837, 2 females, 39.1–35.9 mm SL. MHNM 4856, 1 male, 29.3 mm SL. MHNM 4936, 2 males, 29.5–30.9 mm SL, 1 female, 26.0 mm SL. MHNM 6157, 1 female, 23.8 mm SL. MHNM 6161, 1 male, 15.7 mm SL. MHNM 7012, 2 males, 26.7–30.4 mm SL, 2 females, 21.0–23.3 mm SL. MHNM 7565, 6 males, 21.9–25.3 mm SL, 3 females, 21.4–22.9 mm SL. MHNM 7566, 13 males, 20.5–29.6 mm SL, 9 females, 15.9–23.9 mm SL. MHNM 7570, 24 males, 17.7–25.3 mm SL, 17 females, 15.8–20.6 mm SL. Departamento de Treinta y Tres. ZVC-P 4090, 4 females (2 c&s), 28.9–31.5 mm SL. ZVC-P 4248, 2 males c&s, 31.9–40.7 mm SL, 3 females, 26.5–33.6 mm SL. ZVC-P 4357, 1 male, 22.8–32.2 mm SL. ZVC-P 12465, 2 males, 22.8–32.2 mm SL. ZVC-P 14225, 46 males, 16.7–28.3 mm SL, 11 females, 16.7–18.3 mm SL. MHNM 4609, 2 males, 21.2–23.3 mm SL. MHNM 4613, 1 male, 21.0 mm SL. MHNM 4858, 1 female, 15.2 mm SL. MHNM 7633, 1 male, 32.3 mm SL. *Acantholebias quirogai*. Departamento de Cerro Largo. ZVC-P 6970, 3 females, 29.2–31.2 mm SL. ZVC-P 7745, 2 females, 26.7–29.2 mm SL. ZVC-P 7781, 4 females, 28.3–29.3 mm SL. ZVC-P 8699, 10 males, 37.3–48.2 mm SL (2 c&s), 28 females, 29.4–36.4 mm SL (6 c&s). ZVC-P 8725, 33 males, 24.7–38.2 mm SL (5 c&s), 36 females, 21.0–29.9 mm SL (5 c&s). ZVC-P 13462, 3 males, 26.3–30.6 mm SL, 3 females, 26.1–26.5 mm SL. ZVC-P 13475, 7 males, 25.5–31.0 mm SL, 4 females, 16.2–25.7 mm SL. ZVC-P 13486, 2 males, 35.4–36.1 mm SL, 2 females, 28.1–35.9 mm

SL. ZVC-P 13514, 1 male, 27.9 mm SL; 9 females, 21.2–26.8 mm SL. MHN 4575, 1 male, 29.1 mm SL, 1 female, 23.4 mm SL. MHN 4928, 1 male, 35.2 mm SL, 1 female, 30.5 mm SL. MHN 6070, 9 males, 15.6–22.5 mm SL, 1 female, 17.6 mm SL. MHN 6126, 19 males, 10.8–21.5 mm SL, 8 females, 12.1–16.7 mm SL. Departamento de Rivera. ZVC-P 7807, 2 males, 31.4–31.5 mm SL. ZVC-P 13505, 2 males, 36.9–38.8 mm SL, 3 females, 29.8–30.5 mm SL. ZVC-P 15748, 4 males, 26.1–37.8 mm SL, 3 females, 23.9–24.1 mm SL.

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**Alejandro Duarte:** Conceptualization, Data curation, Formal analysis, Investigation, Writing—original draft, Writing—review and editing.

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### DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

### AI STATEMENT

Artificial intelligence was not used in any section of the manuscript.

### COMPETING INTERESTS

The authors declare no competing interests.

### SUPPLEMENTARY MATERIAL

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