Original article

Review of the family Rivulidae (Cyprinodontiformes, Aplocheiloidei) and a molecular and morphological phylogeny of the annual fish genus *Austrolebias* Costa 1998

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The family Rivulidae is the fourth most diverse clade of Neotropical fishes. Together with some genera of the related African family Nothobranchiidae, many rivulids exhibit a characteristic annual life cycle, with diapausing eggs and delayed embryonic development, which allows them to survive in the challenging seasonal ponds that they inhabit. Rivulidae also includes two species known as the only the self-fertilizing vertebrates and some species with internal fertilization. The first goal of this article is to review the systematics of the family considering phylogenetic relationships and synapomorphies of subfamilial clades, thus unifying information that is dispersed throughout the literature. From this revision, it is clear that phylogenetic relationships within Rivulidae are poorly resolved, especially in one of the large clades that compose it, the subfamily Rivulinae, where conflicting hypotheses of relationships of non-annual and annual genera are evident. The second goal of this work is to present an updated phylogenetic hypothesis (based on mitochondrial, nuclear, and morphological information) for one of the most speciose genus of Rivulidae, *Austrolebias*. Our results confirm the monophyly of the genus and of some subgeneric clades already diagnosed, but propose new relationships among them and their species composition, particularly in the subgenus *Acrolebias*.

Keywords: Cynolebiidae, Killifish, Neotropical, Total evidence.

La familia Rivulidae es el cuarto clado más diverso dentro de los peces Neotropicales. Junto con algunos géneros de la familia Nothobranchiidae, muchos rivulidos presentan un característico ciclo de vida anual, con huevos resistentes a la desecación y embriones con diapausas que les permiten sobrevivir en los ambientes estacionales donde habitan. Los Rivulidae presentan también dos especies consideradas como los únicos vertebrados hermafroditas suficientes y algunas especies con inseminación interna. El primer objetivo de este artículo es actualizar la sistemática de la familia considerando las relaciones filogenéticas y las sinapomorfías de los clados que la componen, reuniendo información que se encuentra dispersa en la literatura. De esta revisión surge que las relaciones filogenéticas dentro de Rivulidae están todavía sin resolver, especialmente en uno de los grandes clados que la componen, la subfamilia Rivulinae, donde relaciones conflictivas entre géneros anuales y no anuales son evidentes. El segundo objetivo de este trabajo es presentar una hipótesis filogenética, basada en datos morfológicos, mitocondriales y nucleares, de uno de los géneros más diversos de la familia, el género *Austrolebias*. Nuestros resultados confirman la monofilia del género y de algunos clados subgenéricos previamente definidos, y propone nuevas relaciones entre ellos, particularmente de las especies del subgénero *Acrolebias*.

Palabras Clave: Cynolebiidae, Evidencia total, Neotropical, Peces anuales.

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Introduction

Killifishes of the family Rivulidae are among the most biologically, ecologically, and evolutionary diverse clades of the Neotropical Ichthyofauna. Rivulids are sexually dimorphic, secondary freshwater fishes, with small adult body sizes (max. SL 200 mm), and unique reproductive characteristics among vertebrates. For example, two species of *Kryptolebias* Costa are the only known self-fertilizing vertebrates (Costa *et al*., 2010; Avise, Tatarenkov, 2015). Many rivulids have an annual or seasonal life cycle, shared with some genera of the African sister family Nothobranchiidae, which has attracted the attention of researchers in diverse areas (*e.g.* Arim *et al*., 2010; Furness *et al*., 2015; Berois *et al*., 2016; Casanova-Larrosa *et al*., 2016; Podrabsky *et al*., 2016; Reichard, 2016; Thompson *et al.*, 2017). Furthermore, two genera of Cynopoecilini have evolved internal fertilization (Costa *et al*., 2015). Adaptations associated with an annual life-history include drought resistant eggs and embryonic development with up to three diapauses, phases where development and growth are reversibly suspended (Wourms, 1972; Berois *et al*., 2016; Podrabsky *et al*., 2016). Diapause allows fishes to inhabit seasonal ponds that dry out completely during the dry season. Seasonal habitats also impose other challenges including, limited energy, space, and time for sexual maturation and reproduction (Loureiro *et al*., 2016). Annual rivulids exhibit many adaptations for survival in seasonal habitats, including accelerated growth (Errea, Danulat, 2001; Blazek *et al*., 2013), specialized trophic strategies (Arim *et al*., 2010), and reproductive behaviors (Haas, 1976; Belote, Costa, 2003; Garcia *et al*., 2008; Passos *et al.*, 2013). Despite these many developmental, ecological, and evolutionary challenges, phylogenetic evidence supports multiple origins of annualism within Aplocheiloidei, with six putative independent origins, four of them within Rivulidae (Furness *et al*., 2015). Seasonal ponds and wetlands also affect the dispersal abilities of annual fishes, potentially favoring endogamy and founder effects, which may facilitate morphological and local genetic variation, differentiation, and eventually speciation.

These attributes of a seasonal life-history strategy have contributed to the diversification of rivulids such that it is the fourth-most diverse family of Neotropical freshwater fishes, currently with 450 valid species (Eschmeyer *et al*., 2018). Rivulids are, therefore, an excellent model to study biogeographic patterns in the Neotropics at both small (Garcia *et al*., 2012; Ponce del León *et al*., 2014) and large spatial scales (Costa, 2010, 2013; Loureiro *et al*., 2016; Costa *et al*., 2017a). Nonetheless, these same characteristics elevate their endemism and probability of extinction. There are already four species included in the IUCN Red List as critically endangered and two as endangered (IUCN, 2017), and many more with clear need of conservation (Costa, 2012; Nascimento *et al*., 2015; Bertaco *et al*., 2016; Volcan *et al*., 2016) or included in regional conservation lists (FZBRS, 2014).

Rivulidae *vs*. Cynolebiidae *vs*. Aplocheilidae. The name Rivulidae was proposed by Parenti (1981), based on the tribe name Rivulini by Myers (1925). However, van der Laan *et al*. (2014) questioned the validity of this name since it was preoccupied by a tribe of the Lepidoptera family Noctuidae Grote, 1895. Eschmeyer *et al*. (2018) noticed this and began to use the name Cynolebiidae in the Catalogue of Fishes, apparently because the next available root comes from Cynolebiatidi (Hoedeman, 1965, in van der Laan *et al*., 2014). Costa (2016a), suggested the name Aplocheilidae instead, proposing a change in the current status of families of Aplocheiloidei to a subfamilial level. In any case, according to article 55.3.1 of the International Code of Zoological Nomenclature (ICZN, 1999), the definition of the name must wait for a decision from the Commission; until then, we will maintain Rivulidae as the family name of Neotropical aplocheiloids.

Origin, fossils and biogeography. The origin of Rivulidae and other clades of Neotropical freshwater fishes has been assumed to have occurred as a consequence of the separation of Africa and South America in the Upper Cretaceous about 100 million years ago (Costa 2013; Furness *et al*., 2015). However, using a fossil calibrated tree Costa *et al*. (2017a), estimated that the split of South American Rivulidae and African Nothobranchiidae could have been occurred around 49 (+/- 12 my) million years ago (early to middle Eocene). This time roughly coincides with Friedman's *et al*. (2013) hypothesis of the split between Neotropical and African cichlids. However, other hypotheses for the origin of Cichlinae (Genner *et al*., 2007; McMahan *et al*., 2013; Matschiner *et al*., 2017) push back the split from African cichlids back to the late Cretaceous. In all cases, these hypotheses support the idea of transoceanic dispersal for the origin of the New World taxa.

Noteworthy, Altner, Reichenbacher (2015) reported for the first time a fossil of Aplocheiloidei from the upper Miocene Lukeino Formation dated to about six million years ago in the Central Rift Valley of Kenya. Based on osteological and phylogenetic analyses, the authors described a new family, Kenyaichthyidae, which unexpectedly seems related to Rivulidae. However, the authors urge caution when interpreting this finding, considering the relatively young age and a dearth of synapomorphies for Rivulidae, Nothobranchiidae and Aplocheilidae.

Within Rivulidae, several dispersal-vicariance analyses (*sensu* Ronquist, 1997) by Costa (2010, 2013, 2014a) and a dispersal-extinction-cladogenesis analysis by Costa *et al*. (2017a), indicate alternative historical scenarios for the modern distribution patterns. These include vicariance events that split ancient (early Oligocene) Brazilian Shield populations, and younger, long-distance dispersal events. Indeed, the dates estimated (middle Miocene) for the divergence between *Cynolebias* Steindachner and

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Simpsonichthys Carvalho (Cynolebiatinae) coincide with the uplift of the Central Brazilian Plateau (Costa *et al*., 2017a, 2018). Interestingly, Costa *et al*. (2018) found that species of *Cynolebias* and *Hypsolebias* Costa that inhabit the Catinga in the São Francisco river basin, diversified synchronically during late Miocene to early Pliocene. The authors attributed this diversification to drainage reorganizations caused by continental drift and climate changes. On the other hand, at smaller spatial scales, there is evidence that some annual fishes of *Austrolebias* Costa (Cynolebiatinae) could be "geodispersed" by river drainage rearrangements connecting the lower Uruguay ecoregion and the Patos ecoregion (Loureiro *et al*., 2011).

Systematics of Rivulidae. The family Rivulidae was proposed by Parenti (1981) to accommodate the new world aplocheiloids; its monophyly was supported by seven synapomorphies. Subsequent morphological (Costa, 1990, 1998, 2004a; Hertwig, 2008) and molecular (Murphy, Collier, 1997; Hrbek, Larson, 1999; Murphy *et al*., 1999; Costa, 2013) phylogenetic analyses have supported a monophyletic Rivulidae; although the proposed synapomorphies have changed since Parenti (1981; see Loureiro, de Sá, 2016). In Tab. 1, we show the currently recognized synapomorphies of the family considering the analyses of Costa (2004) and Hertwig (2008). Costa (2004) suggested the existence of three clades within Rivulidae, and although he proposed eight synapomorphies supporting the Rivulinae + Cynolebiasinae sister group relationship, other phylogenetic analyses (combined and molecular) showed conflicting results: a basal polytomy at the origin of these clades (Hrbek, Larson, 1999; Murphy *et al*., 1999; Costa, 2011a, 2013); a sister group relationship between Kryptolebiasinae + Rivulinae (Vermeulen, Hrbek, 2005; Furness *et al*., 2015); and a sister group relationship between Kryptolebiasinae + Cynolebiasinae (Pohl *et al*., 2015; Costa *et al*., 2017a).

Kryptolebiasinae. This is a clade composed by a single genus with seven valid non-annual species, distributed in the Atlantic coastal basins and estuaries from Florida (USA) to southern Brazil. Three androdioecius species conform a monophyletic unit within the genus (Vermeulen, Hrbek, 2005; Costa *et al*., 2010) that inhabit mangroves; two of them with clear evidence of self-fertilization (Tatarenkov *et al*., 2011). Costa (2004) proposed two synapomorphies for *Kryptolebias*: a dorsally expanded flange on dorsal tip of the autopalatine (Fig. 1a) and a vestigial or absent anterodorsal process of urohyal (Fig. 2a).

Rivulinae. Monophyly of Rivulinae is supported by eight synapomorphies (Costa, 2011a): 1. ventral process of the angulo-articular narrow and triangular shaped (Fig. 3b-c); 2. interhyal bone rudimentary or absent; 3. first epibranchial twisted (Fig. 4a); 4. subdistal process of the second epibranchial absent (Fig. 4a); 5. uncinate process

of the third epibranchial forming an angle of about 45–60° (Fig. 4a); 6. paired process on the first vertebra anteriorly directed; 7. membrane between preopercular laterosensory series and isthmus continuous; 8. E-scales not overlapped. Other combined (Costa, 2013); and molecular phylogenies (Murphy, Collier, 1996; Murphy *et al*., 1999; Hrbek *et al*., 2004; Vermeulen, Hrbek, 2005; Furness *et al*., 2015; Pohl *et al*., 2015) support the monophyly of Rivulinae.

Tab. 1. Synapomorphies of the family Rivulidae proposed by Costa (2004) and Hertzwig (2008).

Costa (2004)
First postcleithrum absent
Branchiostegal and opercular membranes united
Infraorbital, preopercular, and mandibular canals open
Pronounced retrorse process of lateral ethmoid
Frontal scales circularly arranged around scale A
Ventral process of maxilla bent and directed posteriorly
Thin C-shaped preopercle
Posterior distal border of distal premaxilla straight
Coronoid process of dentary narrow
Ventral tip of autopalatine shortened
Uncinate process of third epibranchial median
Shortened lateral rim of frontal lacrimal very twisted and narrow
Minute dermosphenotic
Posterior tip of ascending process of premaxilla curved medially
Ventral process of angulo-articular median
Proximal edge of first hypobranchial bifid
Supracleithrum and postcleithrum separated
Hertzwig (2008)
A1 Adductor mandibulae with a completely separated medial portion
A1 Adductor mandibulae inserts caudally on the maxillary
A1 Adductor mandibulae origin expanded posteriorly and ventrally at the preopercle
Origin of the *m. intermandibularis* expanded posteriorly
Presence of a separate *foramen mandibulare* in the dorsal part of the hyomandibula

Plesiolebiasini. This tribe, revised by Costa (2011b), is diagnosed by seven unambiguous synapomorphies: 1. absence of an anterior expansion on the ventral process of the maxilla; 2. anterior portion of the entopterygoid not overlapping the autopalatine; 3. anterior retrorse process of lateral ethmoid contacting the main axis of the bone; 4. first two proximal radials of anal fin fused (Fig. 5a); 5. pelvic bones medially overlapped; 6. increase in number of neuromasts (between five and seven in the anterior, and four or more in the posterior sections) of supraorbital series; and 7. anal fin with basal region dark gray to black with a row of white to yellow spots. Besides these, Costa (2011b) mentions other synapomorphies that have independently arose in *Rachovia maculipinnis* Radda (Rachoviini): bony flange on the posterior margin of the autopalatine, dorsal margin of the symplectic with an expansion towards entopterygoid, anterior portion of basihyal narrow, anterior proximal radials of the anal fin widened.
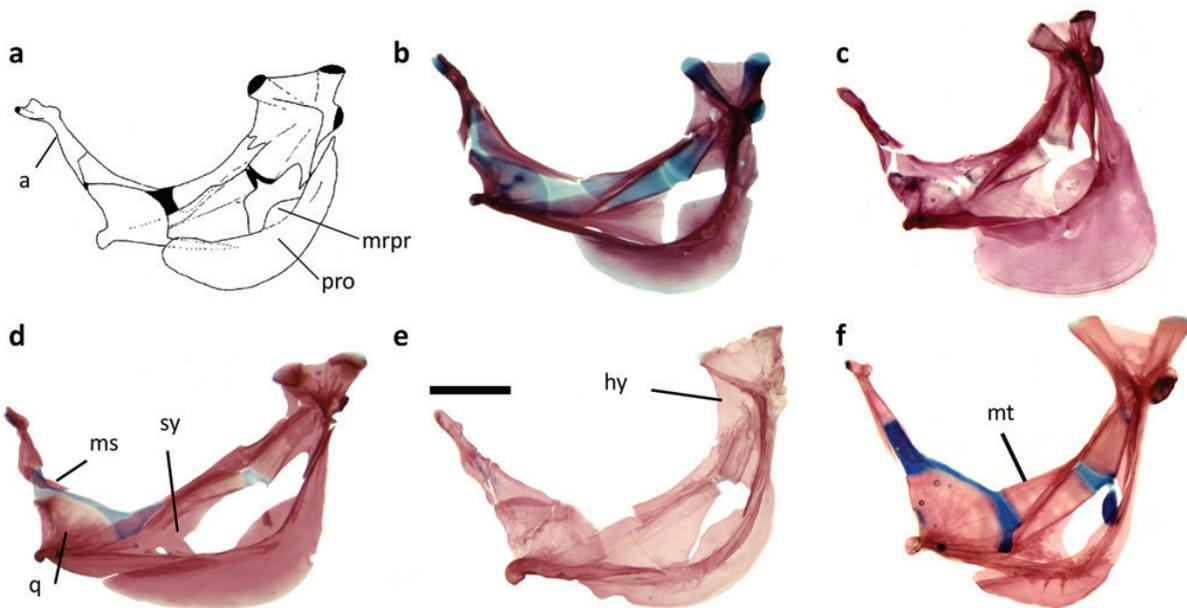
Fig. 1. Suspensorium of **a.** *Kryptolebias caudomarginatus* (Seegers) (modified from Costa, 1998), **b.** *Pterolebias longipinnis* Garman, **c.** *Papiliolebias bitteri* (Costa), **d.** *Cynopoecilus melanotaenia* (Reagan), **e.** *Ophthalmolebias constanciae* (Myers), **f.** *Austrolebias vazferreirai* (Berkenkamp, Etzel, Reichert & Salvia). "a" = autopalatine, hy = hyomandibula, "mrpr" = median rim of preopercle, "ms" = mesopterygoid, "mt" = metapterygoid, "pro" = preopercle, "q" = quadrate, "sy" = symplectic. Scale bar = 1 mm.

Fig. 2. Urohyal bone of **a.** *Kryptolebias brasiliensis* (modified from Costa, 2004), **b.** *Atlantirivulus* aff. *paranaguensis*, **c.** *Cynopoecilus melanotaenia*, **d.** *Ophthalmolebias constanciae*, **e.** *Austrolebias juanlangi*, **f.** *Austrolebias wolterstorffi*; **adp** = anterodorsal process. Scale bar = 1 mm.
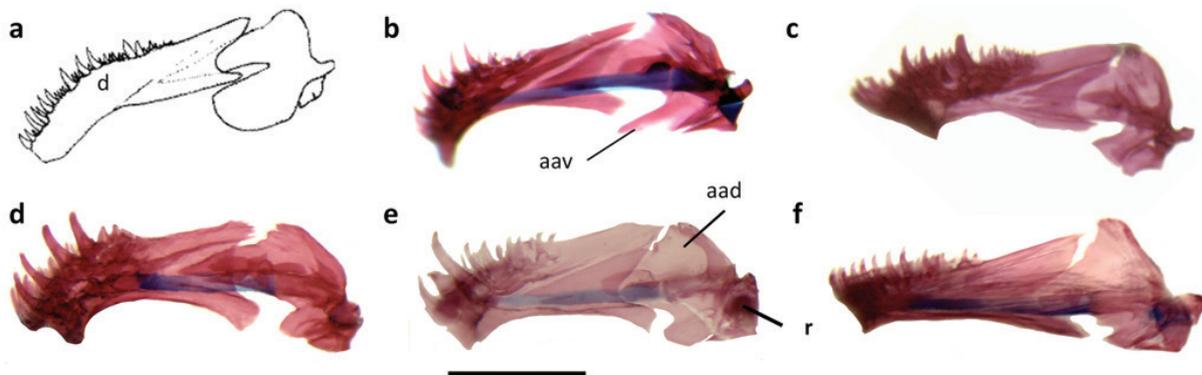
Fig. 3. Lower jaw of **a.** *Kryptolebias brasiliensis* (modified from Costa, 2004), **b.** *Pterolebias longipinnis,* **c.** *Papiliolebiass bitteri,* **d.** *Cynopoecilus melanotaenia,* **e.** *Austrolebias juanlangi,* **f.** *Austrolebias wolterstorffi;* **aad** = anguloarticular dorsal process, **aav** = anguloarticular ventral process, **d** = dentary, **r** = retroarticular. Scale bar = 1 mm.
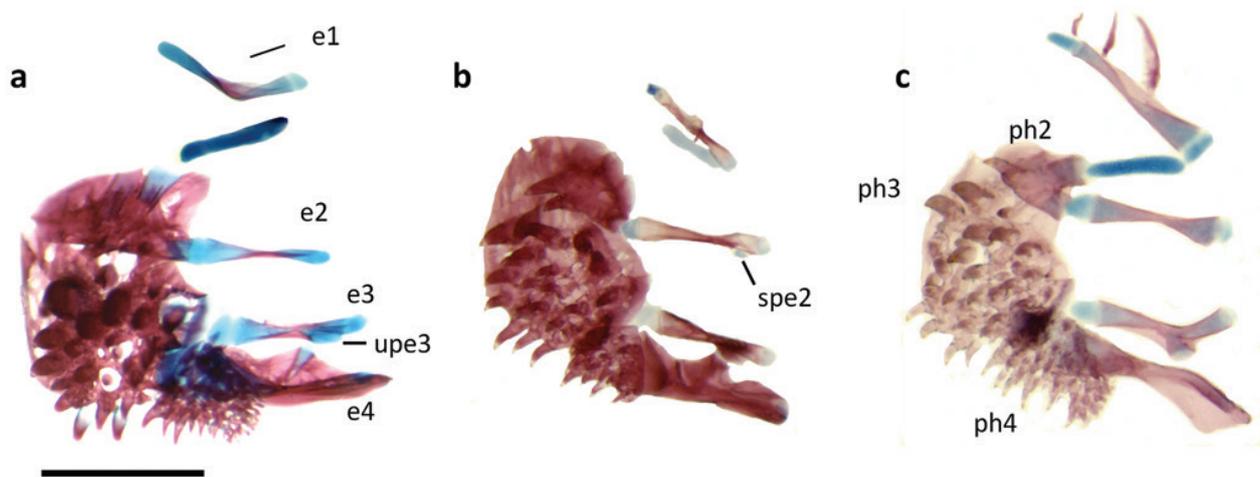
Fig. 4. Ventral view of dorsal gill arches of **a.** *Pterolebias longipinnis*, **b.** *Cynopoecilus melanotaenia*, **c.** *Austrolebias juanlangi*; **e** = epibranchial series, **ph** = pharyngobranchial series, **spe2** = epibranchial subdistal process, **upe3** = uncinate process of epibranchial 3. Scale bar = 1 mm.
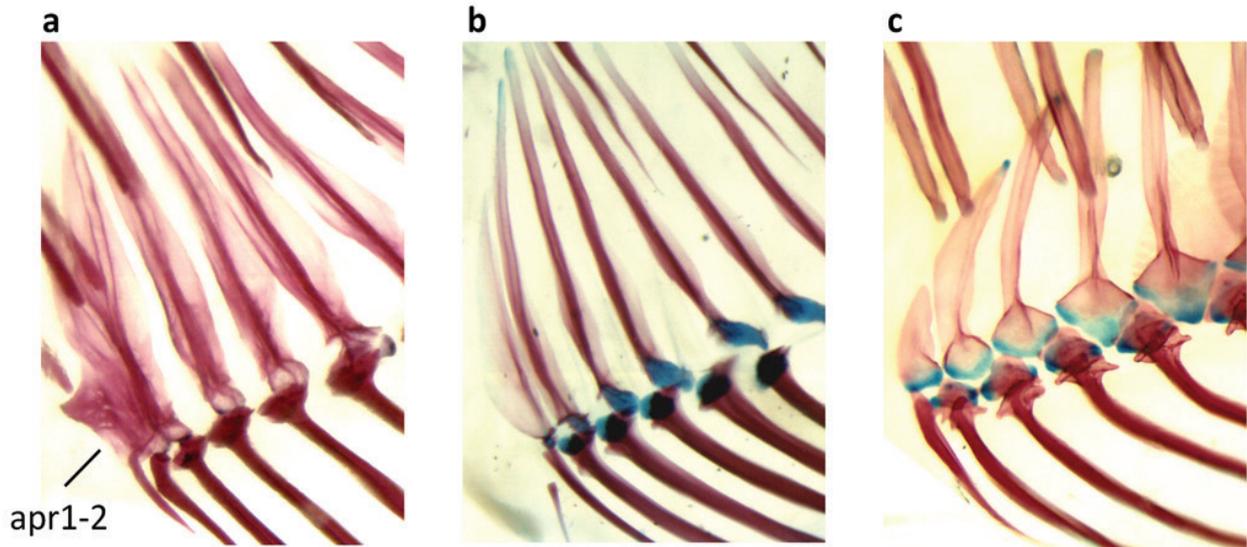
Fig. 5. Anterior section of anal fin of **a.** *Papiliolebias bitteri*, **b.** *Pterolebias longipinnis*, **c.** *Ophthalmolebias constanciae*; **apr1-2** = proximal radials fused. Scale bar = 1 mm.

The clade is composed by five small (max. 50 mm SL) annual genera: *Plesiolebias* Costa, with eight species distributed in the Araguaia, Canabrava, Tocantins, and Xingú rivers in Brazil and eastern Paraguay; *Papiliolebias* Costa, with four species distributed in the western chacoan region in the Pilcomayo and Bermejo river basins (La Plata) and Mamoré and Madeira river basin (Amazonas); *Maratecoara* Costa, with four species distributed in the Tocantins, Xingú, and Canabrava river basins; *Stenolebias* Costa, with two species distributed in the Paraguay river basin; *Pituna* Costa, with six species distributed in the Araguaia, Tocantins, Paranaiba, and Canabrava basins. Costa (2011b) phylogenetic analysis supports *Plesiolebias* as the sister group to the rest of plesiolebiasines, while relationships among these are not clear due to low bootstrap support.

Rachoviini. This tribe, revised by Costa (2014b), is diagnosed by the presence of a prominent urogenital papilla in adult males (Costa 1998), and is well supported by molecular analyses (Hrbek *et al.*, 2004; Hrbek, Taphorn, 2008). Rachoviini is composed by eleven small to large (from 47 to 130 mm SL) annual genera: *Moema* Costa, with twenty species (including the recently synonymized *Aphyolebias* Costa, Costa, 2014b) distributed in the rainforest of the Amazon river basin and the Pantanal region in the Paraguay river basin; *Trigonectes* Myers, with six species distributed in the Paraguay, Guaporé, Mamoré, Araguaia, and Tocantins river basins; *Neofundulus* Myers, with seven species distributed in the Bermejo, Pilcomayo, Guaporé, Mamoré, and Das Velhas river basins; *Pterolebias* Garman, with two species distributed in the eastern and southern Amazon and the Paraná-Paraguay

river basins; *Renova* Thomerson & Taphorn, with one species distributed in the Orinoco river basin in Venezuela; *Micromoema* Costa with one species with similar distribution; *Terranatos* Taphorn & Thomerson, with one species with similar distribution; *Gnatholebias* Costa, with two species distributed in the Orinoco river basin; *Llanolebias* Hrbek & Taphorn, with one species in Los Llanos of the Orinoco river basin in Venezuela and Colombia; *Rachovia* Myers, with four species distributed in the Magdalena, Orinoco (Venezuela and Colombia) river basins and in Lago Maracaibo basin. The very well studied genus *Austrofundulus* Myers was synonymized to *Rachovia* by Costa (2014b), based on the paraphyletic nature of the latter, results that were corroborated by Furness *et al.* (2015). Relationships within rachovines are not confidently resolved, and the clade composed by the genera distributed in the Orinoco, Maracaibo, and Magdalena basins (*Renova*, *Micromoema*, *Terranatos*, *Llanolebias*, *Gnatholebias*, *Rachovia*) is the most supported.

The following taxa have conflicting relationships among one another, and with Plesiolebiasini and Rachoviini, and in the case of Melanorivulini, even its monophyly is questioned (Furness *et al.*, 2015).

***Rivulus* Poey.** It comprises at least two valid species of non-annual fishes (max. 50 mm SL) distributed in freshwater systems of Cuba (Ponce de León *et al.*, 2014). The genus is distinguished from other non-annual rivulines by the following synapomorphies: all hypurals fused (single plate); neural prezygapophysis of caudal vertebrae rudimentary; fourth ceratobranchial teeth absent; about 50% of anterior half of caudal fin covered by scales; four neuromasts on the anterior supraorbital series; and black round spot with white margin on the dorsoposterior portion of caudal peduncle in females (Costa, 2011a).

"*Rivulus*". This taxon is composed by a single non-annual species endemic of the Hispaniola Island, "*Rivulus roloffi*" Roloff, which according to Hrbek *et al.* (2004) would be the sister species of all rivulines except *Rivulus sensu stricto*.

***Prorivulus* Costa.** A monotypic non-annual genus, *P. auriferus* Costa, Lima & Suzart, from the coastal drainages of northeastern Brazil, is diagnosed by the presence of soft anal fin rays in females, minute urogenital papilla in males, and absence of a dark bar on iris. It has some morphological characteristics similar to *Kryptolebias*, but other characters more closely align with the rest of Rivulidae. According to the original description it would be the sister taxa of all Rivulidae except *Kryptolebias* (Costa, 2004b, 2006a). Unfortunately, it has never been included in any subsequent phylogeny or revision, and its position remains uncertain.

***Laimosemion* Huber.** Composed by ca. twenty-nine small to medium-sized species (20-40 mm SL), widely distributed in northwestern South America. The genus lacks synapomorphies, and can be distinguished from other

rivulines only by combination of characters, in particular an intense yellow or orange pigmentation of the anal fin in adult females. Monophyly is supported by molecular analyses (Hrbek *et al.*, 2004; Costa 2011a, 2013). This is considered a non-annual genus; however, Furness *et al.* (2018) have found diapause stages in some species similar to those found in annual species, which could represent intermediates steps in the evolution of the annual life cycle.

Melanorivulini. Costa (2011a) proposed this non-annual tribe based on a single synapomorphy related to the pigmentation pattern of the caudal fin of females. However, molecular analyses suggest that this may be a polyphyletic group (Hrbek *et al.*, 2004), with the sister taxa *Anablepsoides* Huber and *Atlantirivulus* Costa more closely related to Plesiolebiasine than to *Cynodonichthys* Meek and *Melanorivulus* Costa. *Anablepsoides* consists of ca. forty-two small to medium-sized species (30-60 mm SL) distributed in the Antilles, small tributaries of northeastern South America, and the Amazon and Orinoco river basins. It is diagnosed by the presence of scales on the whole ventral surface of the head. *Atlantirivulus* consists of eleven small to medium-sized (25-45 mm SL) species, distributed in coastal rivers of eastern to southern Brazil, diagnosed by disposition in zig-zag of the neuromasts of the infraorbital series and by the presence of a curved ventral process of the angulo-articular. *Cynodonichthys* consists of ca. twenty-seven medium to large-sized species (50-80 mm SL) distributed in the Caribbean and Pacific drainages of Central America and northwest South America. This genus lacks synapomorphies. *Melanorivulus* consists of ca. thirty-seven small to medium-sized species (20-40 mm SL), mainly distributed in central South America including La Plata, São Francisco, and Amazon river basins, and small coastal basins of northeast Brazil and it is diagnosed by the presence of black pigmentation on the margin of the caudal fin and on the distal margin of the dorsal and anal fins in females.

***Millerichthys* Costa.** Monotypic ((*M. robustus* (Miller & Hubbs)) small-sized (30 mm SL), annual genus, that is distributed in southern Mexico. The species is diagnosed by the following apomorphies: reduced mesopterygoid; conical and elongate ventral process of posttemporal; long posterior series of supraorbital neuromasts; absence of transverse dark bar crossing the eye; three transverse yellow stripes on the anal fin of males; and black spots along the caudal-fin base of females (Costa, 1998). Its phylogenetic relationship with other Rivulidae remains unclear. Costa (1998), proposed three synapomorphies to support the sister relationship of *Millerichthys* Costa with Rachoviini: a dorsally pointed preopercle, reduced neural prezygapophyses of caudal vertebrae, and a reduced dark pigmentation in females. However, Costa (2004) hypothesized the genus as the sister taxon of Cynolebiasinae based on three different synapomorphies: mesopterygoid small, posterior tip not contacting metapterygoid; hypurals completely fused; frontal scales in transverse pattern without distinct central scale.

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Cynolebiasinae. The monophyly of this subfamily, composed by small to large-sized annual species, is well supported presenting the following synapomorphies: dorsally positioned dorsomedial process of autopalatine; reduced mesopterygoid; thin C-shaped preopercle with reduced dorsal tip, median rim, and sensory canal (Fig. 1f); elongate epibranchials and interarcual cartilage (Fig. 4c); rounded second hypobranchial (Fig. 6d); absence of teeth in the fourth ceratobranchial; absence of a posteroventral process on the anterior portion of the fourth ceratobranchial; absence of teeth in vomer; thin lachrymal, with narrow lower portion and vestigial sensory canal; absence of dermosphenotic; absence of anterior processes of first vertebra; vestigial or absent neural prezygapophysis of caudal vertebrae (Fig. 7c-d); narrow and anteriorly curved epural and parahypural (Fig. 7c-d); anteriorly placed dorsal fin origin; reduced first proximal anal-fin radial; reduced ossification of medial anal-fin radials; vestigial or absent ventral process of posttemporal; elongate supracleithrum; scale-like pectoral radials (Fig. 8b-c); cylindrical urogenital papilla in males; prominent pocket-like urogenital papilla in females; reduced supraorbital squamation; reduced caudal-fin squamation; continuous supraorbital series of neuromasts; long posterior series of supraorbital neuromasts; minute neuromast on dermosphenotic; and vertical bars on body sides of juveniles (Costa, 1998); Molecular phylogenies support this clade (Murphy *et al.*, 1999; Hrbek, Larson, 1999; Costa, 2013; Furness *et al.*, 2015; Pohl *et al.*, 2015). The two clades that composed the subfamily, Cynopoecilini and Cynolebiasini, are also very well supported by morphological and molecular analyses.

Cynopoecilini. Costa (2016a) proposed seven synapomorphies for Cynopoecilini, based on a previous total combined (morphological and mitochondrial) phylogenetic analysis (Costa, 2016b). Five of them already described in Costa (2008a): 1. vomer teeth absent; 2. ventral process of angulo-articular vestigial or absent; 3. posterior process of quadrate wide (Fig. 1d); 4. symplectic elongate (Fig. 1d), 5. supraorbital scales absent (according to Ferrer *et al.*, 2014, reversed in some *Cynopoecilus* Reagan species); 6. distinctive postero-ventral process on autopalatine; and 7. E-scales not overlapped "(reversed in *Cynopoecilus feltrini*)".

Previously proposed synapomorphies for Cynopoecilini (Costa 2008a; Ferrer *et al.*, 2014), changed after the description of a new genus (*Mucurilebias* Costa) created by Costa (2014a) to include "*Leptolebias leitaoi* (da Cruz & Peixoto)" in the phylogenetic analysis (Costa, 2016a). The muscular fibers circularly arranged around the base of the urogenital papilla forming an ejaculatory pump became synapomorphies for the sister genera *Campellolebias* Vaz-Ferreira & Sierra de Soriano and *Cynopoecilus*; the minute contact organs along margin of flank scales in males became a synapomorphy of the clade composed by *Leptolebias* Myers, *Leptopanchax* Costa, *Campellolebias* and *Cynopoecilus*. A green iris (blue to yellowish-green in males, in Ferrer *et al.*, 2014) remains the only synapomorphy for *Cynopoecilus*. A reticulated egg envelope (chorion) with mushroom-like projections and a supraorbital series of neuromasts anteriorly interrupted are synapomorphies of a clade composed by all genera except *Mucurilebias*. Additional proposed synapomorphies are a short mesopterygoid (Costa, 2008a), dorsal and anal fins twisted in males during courtship behavior and four to seven neuromasts on the caudal-fin base (Ferrer *et al.*, 2014).
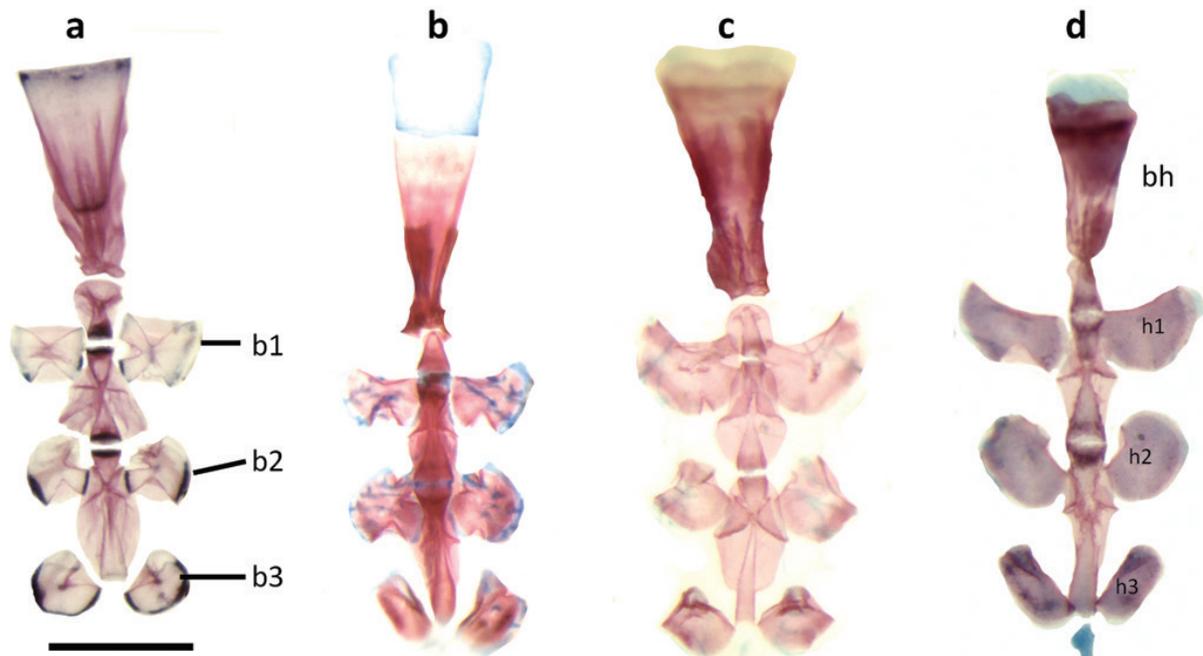
Fig. 6. Dorsal and partial view of ventral gill arches of **a.** *Papiliolebias bitteri*, **b.** *Cynopoecilus melanotaenia*, **c.** *Ophthalmolebias constanciae*, **d.** *Austrolebias juanlangi*; **b** = basibranchial series, **bh** = basihyal, **h** = hypobranchial series. Scale bar = 1 mm.
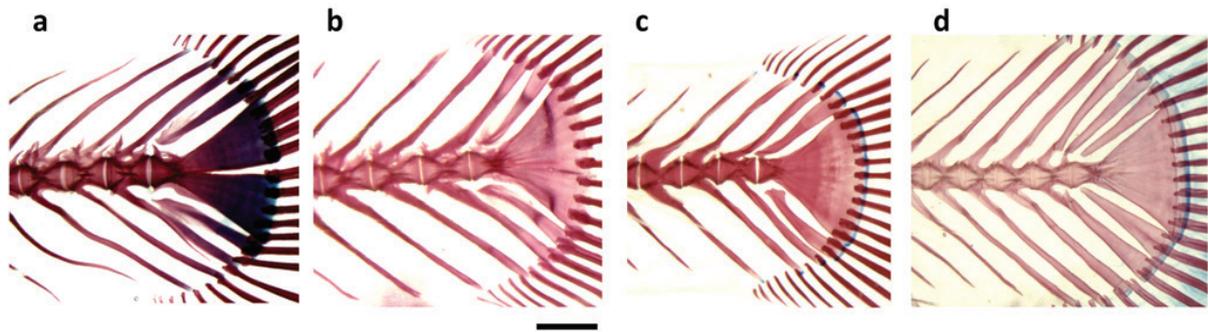
Fig. 7. Caudal skeleton of **a.** *Pterolebias longipinnis*, **b.** *Papiliolebias bitteri*, **c.** *Cynopoecilus melanotaenia*, **d.** *Austrolebias wolterstorffi*. Scale bar = 1 mm.
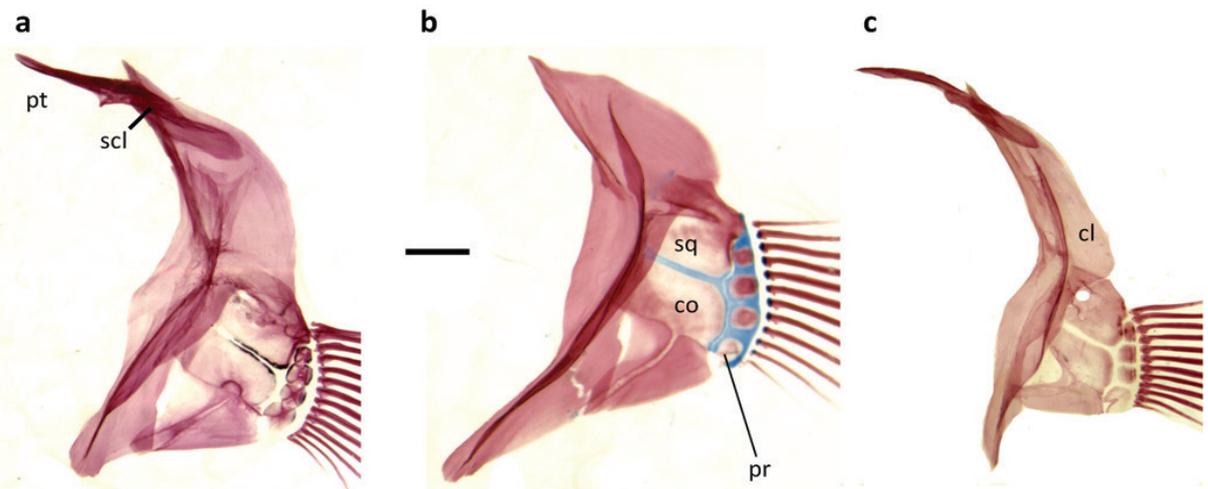
Fig. 8. Pectoral girdle of **a.** *Papiliolebias bitteri*, **b.** *Cynopoecilus melanotaenia*, **c.** *Ophthalmolebias constanciae*; **cl** = cleithrum, **co** = coracoid, **pr** = pectoral radial, **pt** = postemporal, **scl** = supracleithrum, **sq** = scapula. Scale bar = 1 mm.

The tribe is composed of seven genera distributed in Atlantic coastal drainages of southeastern and south Brazil and eastern Uruguay (Costa, 2016a): *Notholebias* Costa, with four small-sized species (max. 30 mm SL) from southeastern Brazil; *Mucurilebias*, monotypic and small-sized (max. 30 mm SL) genus with similar distribution; *Leptolebias*, with one small-sized species (max. 29 mm SL) with similar distribution; *Leptopanchax*, a genus created by Costa (2016a) to fix the paraphyly of *Leptolebias* found in Costa (2016b), composed of five species with similar distribution; *Campellolebias*, four small-sized species (max. 32 mm SL) with similar distribution and with the development of pseudogonopodium involved in internal fertilization; *Cynopoecilus*, six small-sized (max. 40 mm SL), distributed in floodplains of the Jacuí river basin and coastal basins of southern Brazil and eastern Uruguay. This genus also presents internal insemination.

Cynolebiasini. This tribe is diagnosed by twelve synapomorphies (Costa, 1998): a broad posteroventral process of dentary; concave anterodorsal margin of opercle; anterior position of anal-fin origin in males; elongated filamentous rays on dorsal and anal fins of males; sexual dimorphism in number of dorsal- and anal fin rays; numerous anal-fin rays in males (19–32); elongated dorsal portion of cleithrum (Fig. 8c); absence of posteroventral flange of cleithrum (Fig. 8c); ventral tip of cleithrum ventrally directed (Fig. 8c); broad anteroventral tip of coracoids; numerous supraorbital neuromasts (12–25); and spawning within substrate (Costa, 1998). Its monophyly is supported also by molecular analyses (Costa *et al.*, 2017a; Furness *et al.*, 2015).

The tribe is composed of eight genera, with small to large adult body sizes (30 to 150 mm SL): *Nematolebias* Costa, with three medium-sized species (max. 80 mm SL) distributed in coastal basin of southeastern Brazil; *Xenurolebias* Costa, with four species with small sizes (max. 30 mm SL) endemic to the Atlantic Forest in southeastern Brazil; *Ophthalmolebias* Costa, with six species with small sizes (max. 65 mm SL) distributed in eastern Brazil (Costa, 2010); *Simpsonichthys*, with nine small-sized species (max. 55 mm SL) distributed in the central Brazil Plateau (Costa, 2010); *Spectrolebias* Costa & Nielsen, with nine small-sized species (max. 32 mm SL) distributed in the Paraguay, Guaporé, Xingu, Araguaia, and Tocantins river basins (Costa, 2010); *Hypsolebias,* with forty-nine medium-sized species (max. 80 mm SL) distributed in Jequitinhonha, São Francisco, and Urucuia river basins (Costa, 2010); *Cynolebias*, with twenty two medium to large-sized

species (50–120 mm SL) distributed in the São Francisco and Urucuia river basins (Costa, 2010); *Austrolebias*, with forty-eight small to large-sized species (35–150 mm SL) distributed in the La Plata, Patos-Merin, and southwestern Amazon basins (Loureiro, de Sá, 2016; Costa *et al*., 2017a; Volcan *et al*., 2017; Alonso *et al*., 2018).

According to a recent molecular phylogenetic analysis (Costa *et al*., 2017a), *Nematolebias* + *Xenurolebias* are the sister clade of the remaining genera, which relate to each other as follows: *Spectrolebias*, (((*Austrolebias*, ((*Simpsonichthys*, *Cynolebias*), (*Opthalmolebias*, *Hypsolebias*)))). However, Ponzetto *et al*. (2016), in a phylogenetic analysis based on one mitochondrial marker, had recently challenged the monophyly of *Hypsolebias*, with some species more closely related to *Ophtlamolebias* than to other *Hypsolebias*.

Morphological and molecular phylogeny of *Austrolebias*. Species of the genus *Austrolebias* were originally included in *Cynolebias*, that was described just five years earlier by the same author from a single species, *C. porosus* Steindachner from Pernambuco, northeastern Brazil (Steindachner, 1876). In the first phylogenetic analysis of Cyprinodontiformes, Parenti (1981) provided one synapomorphy to diagnose *Cynolebias* (caudal fin without scales) and synonymized this genus with several annual genera that had been described during the 20th century (*Cynopoecilus* Regan, 1912, *Leptolebias* Myers, 1952, *Simpsonichthys*, Carvalho, 1959, *Campellolebias* Vaz-Ferreira & Sierra, 1974, and *Terranatos* Taphorn & Thomerson, 1978). Costa's (1990) phylogenetic analysis of Rivulidae elevated *Cynolebias* sensu Parenti (1981) to a subfamily level, revalidated those genera previously synonymized, except *Simpsonichthys*, and rediagnosed *Cynolebias* based on five synapomorphies.

Costa (1998), in a new phylogenetic analysis of Rivulidae, described *Austrolebias* and *Megalebias* Costa (both from La Plata and Patos Merin basins), including some species previously located in *Cynolebias*. *Austrolebias* was diagnosed by three synapomorphies and included species previously diagnosed as the "*C. bellottii* Steindachner" species complex (Costa, 1995); *Megalebias* was also diagnosed based on three synapomorphies and included species previously diagnosed as the "*C. elongatus* Steindachner" species complex (Costa, 1995). *Cynolebias* sensu stricto (diagnosed by three synapomorphies) included species previously diagnosed as the "*C. porosus*" species complex (Costa, 1995), distributed in northeastern Brazil. These three genera were included in the Subtribe Cynolebiatina with *Austrolebias* as the sister clade of *Megalebias* + *Cynolebias* (Costa, 1998). Costa (2002a), changed this relationship and proposed that *Cynolebias* was the sister genus of *Austrolebias* + *Megalebias*. Costa (2006b), considered *Megalebias* as a synonym of *Austrolebias* based on a morphological phylogenetic analysis in which *Megalebias* was nested within *Austrolebias*. Cynolebiatina was diagnosed by nine synapomorphies (Costa, 1998). However, as was mentioned before, Costa *et al*. (2017a), proposed *Austrolebias* as the sister genus of a clade with

the following relationships: ((*Cynolebias*, *Simpsonichthys*), (*Ophthalmolebias*, *Hypsolebias*)).

Monophyly of *Austrolebias* is supported by three exclusive synapomorphies (Costa, 2006b): absence of scales between the corner of the mouth and anterior portion of the preopercular region and ventral portion of the opercular region, a deep urohyal (Fig. 2e), a dark gray to black infraorbital bar and supraorbital spot; and three synapomorphies independently arose in other cynolebiatines: males with dorsal and anal fins rounded, males with long urogenital papilla, and reduced ventral process of the angulo-articular. However, according to relationships proposed by Costa *et al*. (2017a), these non-exclusive synapomorphies may represent plesiomorphic states of Cynolebiasini.

Costa (2008b), divided the genus in several subgenera according to the phylogenetic relationships obtained in Costa (2006b): *Acantholebias* Costa ((type species *A. luteoflammulatus* (Vaz-Ferreira, Sierra & Scaglia)), *Acrolebias* ((type species *A. carvalhoi* (Myers)), *Argolebias* ((type species *A. nigripinnis* (Regan); also known as the "*A. alexandri*" species group), *Austrolebias* (type species *A. bellottii*; includes "*A. bellotti*" + "*A. adloffi*" species groups), *Cypholebias* ((type species *A. robustus* (Günther)), *Gymnolebias* ((type species *A. gymnoventris* (Amato)), *Megalebias* ((type species *A. wolterstorffi* (Ahl)). According to a morphological phylogeny (Costa, 2010) the subgenus *Argolebias* could be paraphyletic.

Relationships within *Austrolebias* are still unresolved; conflicting phylogenetic hypotheses have been proposed for this genus using morphological (Costa, 2006b, 2010; Loureiro, 2004) and molecular (mitochondrial) analyses (Garcia *et al*., 2014). Most subgeneric clades remain stable in species composition; however, relationships among clades and position of some species are still unresolved.

In this study, we performed a phylogenetic analysis of *Austrolebias sensu* Costa (2006b), using the most complete species sampling to date and a total evidence approach, including morphological, and molecular (mitochondrial and nuclear) data.

Material and Methods

Specimens analyzed, and tissue samples belong to: Universidade Federal do Rio Grande do Sul (UFRGS), Facultad de Ciencias (ZVCP), Universidade de Taubaté (UNITAU), and Sección Genética Evolutiva (GP, GR). Additional fish were collected on a field trip to Rio Grande do Sul (Brazil) done under the Collection Permit "Portaria No- 909, de 17 de setembro de 2013". For molecular analyses, we extracted total DNA using an extraction kit (Qiagen/ DNeasy Blood & Tissue Kit) and amplified two segments of mitochondrial genes (16s Ribosomal Unit and Cytochrome b) and two segments of nuclear genes (Recombination activating protein 1, RAG1 and Glycosyltransferase, Glyt) using standard PCR amplification techniques. Primers used were: 16s Ar-L (CGCCTGTTTATCAAAAACAT), Br-H (CCGGTCTGAACTCAGATCACGT) (Palumbi

et al., 1991); Cytb CB3-H (GGCAAATAGGAARTAT-CATTC), Gludg-L (TGACTTGAARAACCAYCGTTG) (Palumbi et al. 1991); Rag1-Pachyp-F1 (TGAAAArGCT-GTTCGCTTCT) (Pohl et al., 2015), H3405_RAG1ex3 (GCNGAGACTCCTTTGACTCTGTC) (Near et al., 2012); Glyt_F577 (ACATGGTACCAGTATGGCTTTGT), Glyt_R1562 (CCCAAGAGGTTCTTGTT(AG)AAGAT) (Li et al., 2007). Additional (64) sequences were obtained from GenBank. PCR Cycle details were: *Cytochrome b*: initial denaturalization at 94 ºC/3 min; 4 cycles with denaturalization at 94 ºC/1min, annealing at 45 ºC/1 min and extension at 72 ºC/1 min; 29 cycles with denaturalization at 94ºC/ 1min, annealing at 50 ºC/1 min and extension at 72 ºC/1 min; and final extension at 72 ºC/1min; *16s RU*: initial denaturalization at 94 ºC/3 min; 4 cycles with denaturalization at 94 ºC/1min, annealing at 45 ºC/1 min and extension at 72 ºC/1 min; 29 cycles with denaturalization at 94ºC/ 1min, annealing at 50 ºC/1 min and extension at 72 ºC/1 min; and final extension at 72 ºC/1min; *RAG1*: initial denaturalization at 95 ºC/4 min; 35 cycles with denaturalization at 95ºC/ 40 sec, annealing at 53 ºC/40 sec, and extension at 72 ºC/90 sec; and final extension at 72 ºC/10min; *Glyt*: initial denaturalization at 94 ºC/4 min; 35 cycles with denaturalization at 92ºC/1 min, annealing at 55 ºC/1 min, and extension at 72 ºC/1 min; and final extension at 72 ºC/4min. PCR products were sequenced in Eurofins Genomics Sequencing Data and MACROGEN (Seoul, Korea). Sequences were edited and aligned with MEGA (v. 7.018); nuclear sequences were aligned with Saté (v2.2.7) using the ClustalW2 and MUSCLE options; final edition and concatenation were done with Sequence Matrix. Genbank accession numbers for sequences collected in this study are given in **S1**. Total and partial number of Bp, variable, and phylogenetic informative sites are shown in Tab. 2.

Tab. 2. Mitochondrial and nuclear markers amplified and used in the phylogenetic analyses. Bp = total number of base pairs amplified; VS = number of variable sites, PS = number of phylogenetic informative sites.

Marker	Bp	VS	PS
16s	612	377	261
Cytb	996	537	410
RAG1	477	130	77
GLYT1	1016	360	192
Total	3101	1404	940

A 173 character morphological matrix was constructed based on modifications from Costa (2010b) and Loureiro (2004) (**S2**, **S3**, **S4**).

Forty described, plus one undescribed species of *Austrolebias* were included in the analyses. We used species of **Cynolebiatinae** (Cynolebiasini: *Cynolebias* (2), *Simpsonichthys* (1), *Spectrolebias* (2), *Hypsolebias* (6), *Ophthalmolebias* (1), *Nematolebias* (1); Cynopoecilini: *Cynopoecilus* (1), *Campellolebias* (1), *Leptopanchax* (2)), **Rivulinae** (*Pterolebias* (1), *Papiliolebias* (1),

Atlantirivulus (1)), and **Kryptolebiatinae** (*Kryptolebias* (1)) as outgroups.

Phylogenetic analyses. Parsimony analyses were implemented in PAUP 4 with the following settings: Heuristic search (stepwise addition, random additional sequence, branch-swapping TBR, Max trees = 1000, branches collapsed if maximum length is zero, optimization ACCTRAN) and Bootstrap analyses with 1000 replicates. Bayesian analyses were performed with MrBayes (v3.2) with two independent runs of 20 million generations (MCMC) with the following molecular evolution models (estimated with jModelTest 2.0 run on Cypress): 16s (GTR+I+G), Cytb (GTR+I+G), Rag1 (TPM3+I+G), Glyt (TVM+I+G); the MKV evolution model was used for morphological characters. Tracer v1.6 was used to check for convergence of parameter estimates; (ESS) values of all parameters above 200. The first 25% of each run was discarded as burn-in. Phylogenetic analyses were performed on the concatenated sequences alone and combined with morphological characters.

Results

Concatenated sequences. Parsimony and Bayesian analyses of molecular data failed to recover a monophyletic *Austrolebias* (Figs. 9, 10). Parsimony yielded 1,325 trees with 5,255 steps, and the node supporting *Austrolebias* had low support (Bootstrap value < 80%). Considering that low support also appeared in more inclusive and nested nodes, the resulting topology can be interpreted as a polytomy with seven branches: 1. *Ophthalmolebias constanciae*, 2. a clade composed by *Simpsonichthys*, *Spectrolebias*, and *Hypsolebias*, 3. *Cynolebias*, 4. *A. nigripinnis*, 5. the "*A. alexandri*" species group, 6. the clade conformed by *A. wichi* and *A. varzae*, and 7. remaining *Austrolebias* species. Within this latter clade another polytomy resulted: *A. wolterstorffi*, the remaining "*A. elongatus*" species group, the clade *A. jaegari* and *A. gymnoventris*, the "*A. bellottii*" species group, the clade *A. luteoflammulatus* + *A. quirogai*, the "*A. robustus*" species group, and the "*A. adloffi*" species group (including *A. araucarianus*).

Bayesian analysis resulted in *A. nigripinnis* as the sister taxon of the clade conformed by *Cynolebias*, the clade *Simpsonichthys*, *Spectrolebias*, and *Hypsolebias*, and the remaining *Austrolebias*. Within these, the topology recovered was (*A. wichi*, *A varzeae*) (the "*A. alexandri*" species group, (((*A. jaegari*, *A. gymnoventris*) (the *A. bellottii* species group)) (part of the "*A. elongatus*" species group), (*A. wolterstorffi*), ((*A. luteoflammulatus*, *A quirogai*) ("*A. robustus*" species group, "*A. adloffi*" species group, including *A. araucarianus*)))).

Other interesting results come from within the species groups. In the "*A. alexandri*" species group, species from the Jacuí river basin (*A. litzi*, *A. paucisquama*, *A. cyaneus*) composed a clade. The enigmatic *A. toba*, is the sister species to the clade *A. ibicuiensis*, *A. alexandri*. In both

analyses, *A. periodicus* appears as a polyphyletic entity, with some specimens more related to *A. affinis* and others to *A. duraznensis* and *A. juanlangi*. In the "*A. robustus*" species group, the topology recovered questions the validity of *A. vazferreirai*, which could be a junior synonym of *A. cinereus*, and the validity of *A. nonoiuliensis*, which could be a junior synonym of *A. robustus*.

In the "*A. adloffi*" species group, the analyses show that *A. nachtigalli* from Arroio Grande could be a different entity from those from Yaguarón river, which are more closely related to *A. reicherti*. In the parsimony analysis, *A. araucarianus* is nested in this group, however the Bayesian analysis suggests this species is the sister to the remaining species excluding *A. viarius*.

Fig. 9. Maximun parsinony phylogenetic tree of *Austrolebias*, based on the molecular markers (ribosomal unit 16s, Citochrome b, RAG1, Glyt). Colored areas same as Fig. 12.
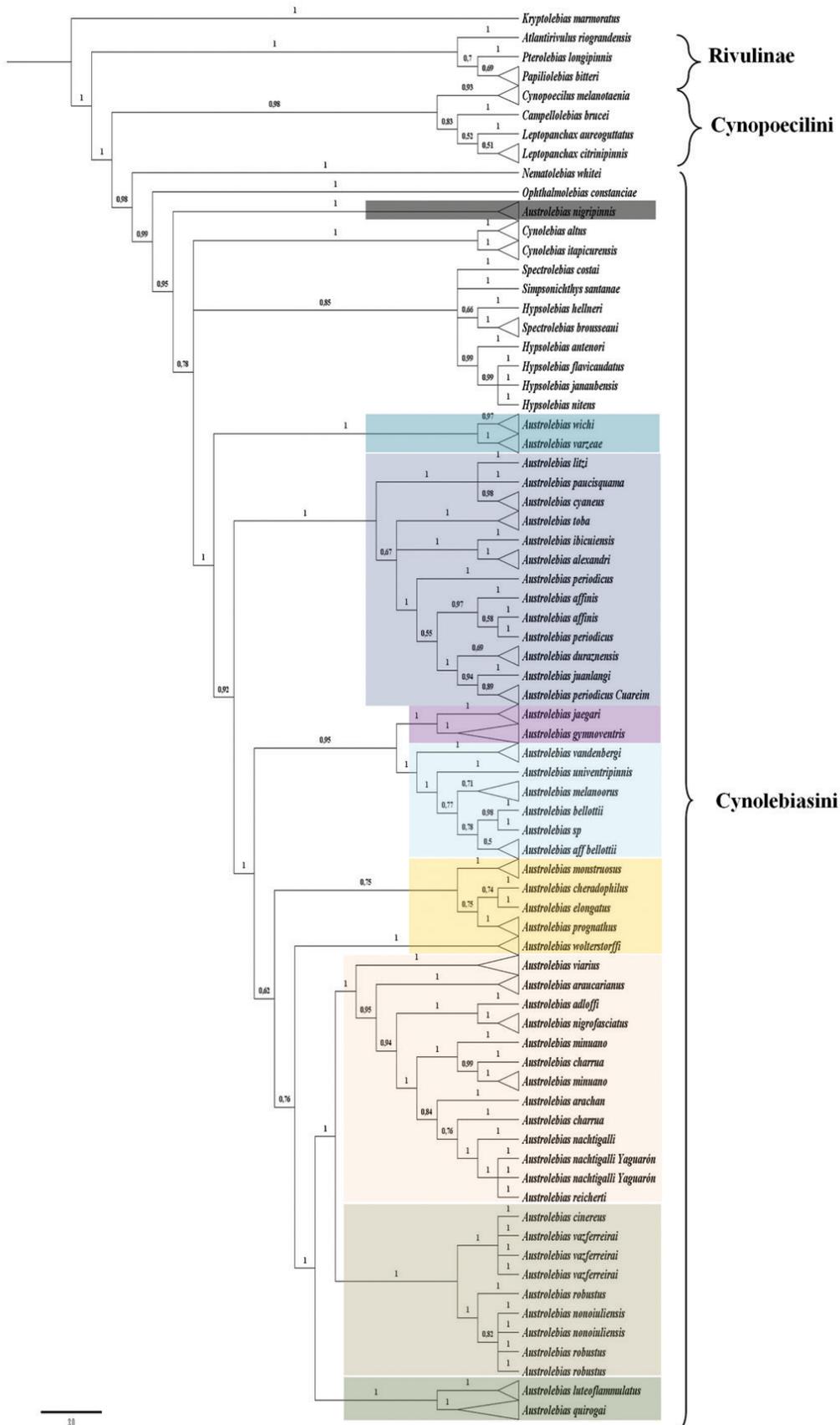
Fig. 10. Bayesian phylogenetic tree of *Austrolebias*, based on the molecular markers (ribosomal unit 16s, Cytochrome b, RAG1, Glyt). Values above branches are posterior probabilities. Colored areas same as Fig. 12.

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Total evidence (Figs. 11, 12). Both approaches (Parsimony and Bayesian) supported a monophyletic *Austrolebias*, with *A. nigripinnis* as the sister taxon to the remaining species. Due to the low support of many nodes, Parsimony analysis did not resolve relationships among species groups which collapsed in a basal polytomy. Bayesian analysis resulted in better support for some nodes, but also could not resolve relationships among several species groups within the genus.
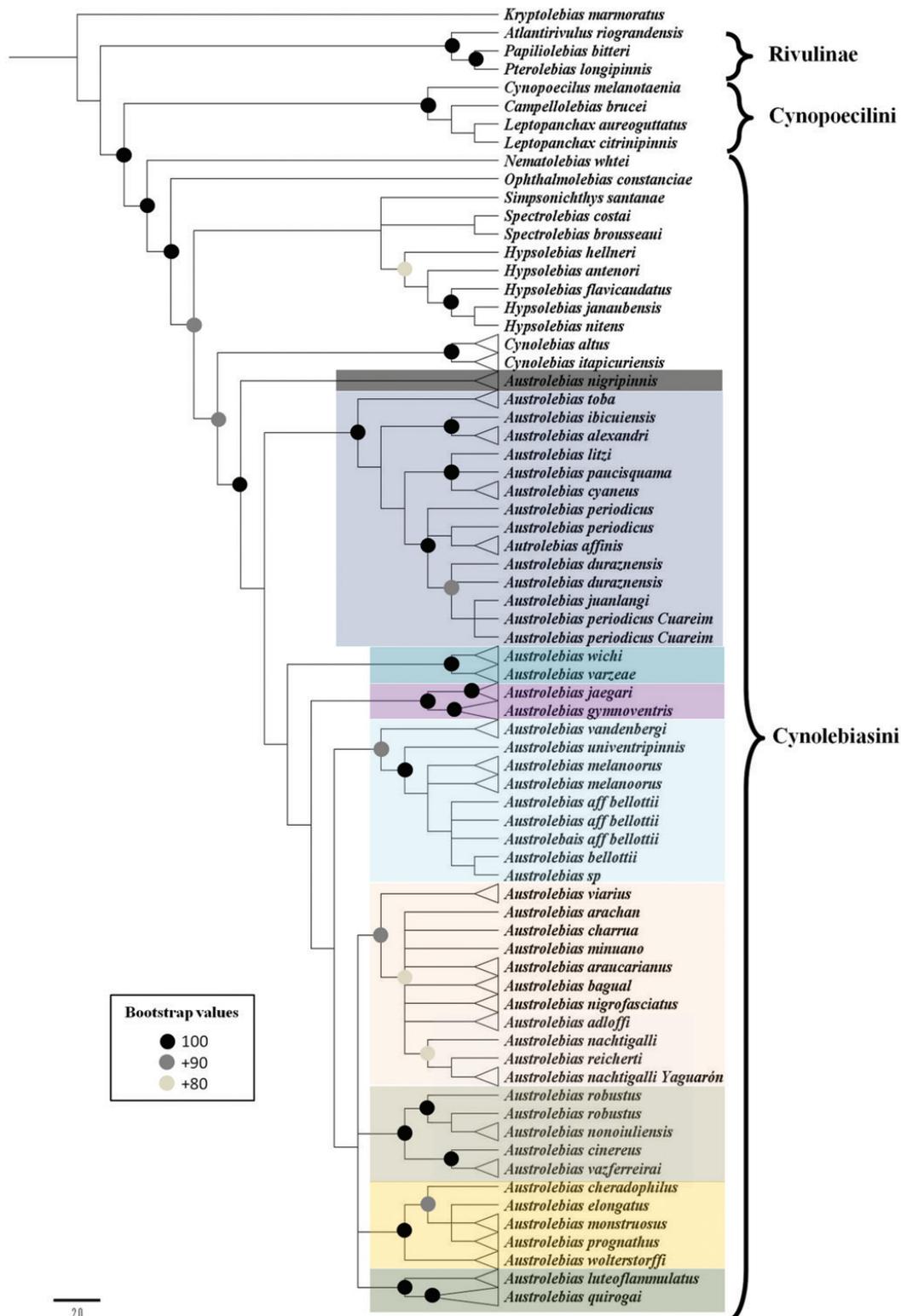
Fig. 11. Maximun parsimony phylogenetic tree of *Austrolebias*, based on molecular (ribosomal unit 16s, Cytochrome b, RAG1, Glyt) and morphological characters. Colored areas same as Fig. 12.
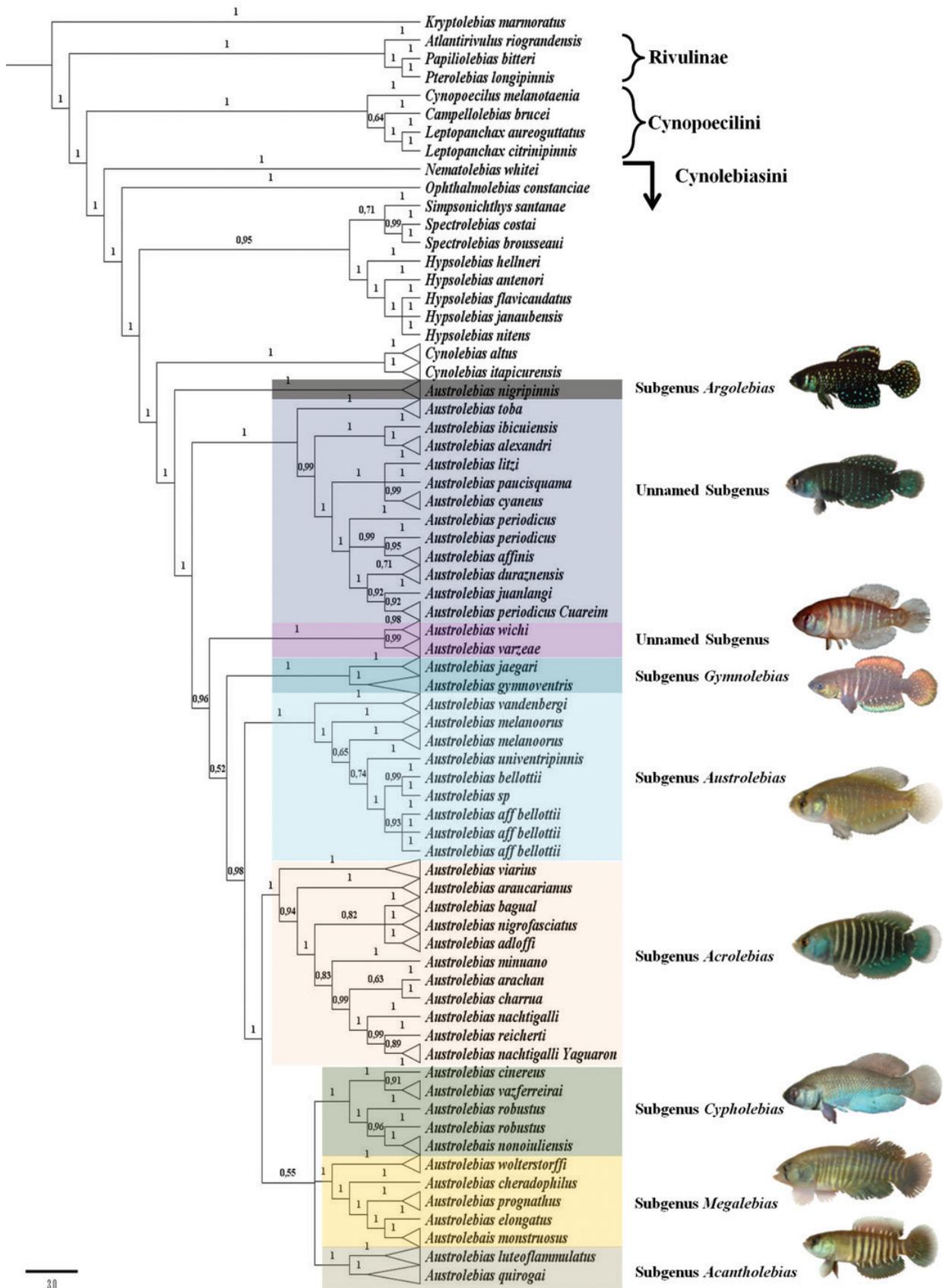
Fig. 12. Bayesian phylogenetic tree of *Austrolebias*, based on molecular (ribosomal unit 16s, Cytochrome b, RAG1, Glyt) and morphological characters. Values above branches are posterior probabilities.

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The general topology of the total evidence analysis was similar to the molecular analyses, however, some noteworthy differences could be observed. Within the "*A. alexandri*" species group, *A. toba* was found to be the sister taxon of the remaining species. The polytomy within the genus was composed by three clades: (*A. wichi, A. varzeae*), (*A. jaegari, A. gymnoventris*), and the remaining species. Within the latter, the "*A. bellottii*" species group is the sister taxon of a clade composed by the "*A. adloffi*" species group, *A. luteoflammulatus + A. quirogai*, the "*A. robustus*" species group, and the "*A. elongatus*" species group. Bayesian analysis supports the position of *A. araucarianus* as the sister species of the "*A. adloffi*" species group (except *A. viarius*) and grouped species from the Laguna dos Patos in a single clade and species from the Laguna Merin as its sister taxon.

Discussion

Although monophyly of the genus *Austrolebias* was supported in most analyses, the position of *A. nigripinnis* in the analyses of the genetic markers generates a doubt about the generic identity of this species. Previous morphological (Costa, 2010) and morphological plus cytochrome b (Alonso *et al*., 2018) phylogenetic analyses, had already proposed *A. nigripinnis* as the sister taxon of the remaining *Austrolebias*. The strong support of this hypothesis in the total evidence analyses of this study confirms that this species may have originated from the earliest split of the genus. The affinity of this species to other Cynolebiasini, and its changing position in the tree depending on the genetic marker considered (data not shown) may be the result of incomplete lineage sorting, retention of ancestral polymorphisms (Garcia *et al*., 2009), or the effect of introgressive events; events that have already been proposed to occur in the genus (Garcia, 2006). However, more detailed analyses with more genetic data and more populations sampling will be needed to address this hypothesis.

In order to avoid paraphyly, *A. nigripinnis* should not be considered as part of the "*A. alexandri*" species group and should be considered the only species in the subgenus *Argolebias*. Unfortunately, sequences of *A. paranaensis*, a species very similar to *A. nigripinnis* (Costa 2006b), were not available for this study and its phylogenetic position remains unknown. Consequently, the remaining species of the "*A. alexandri*" species group lose its single synapomorphy of dark gray pectoral fins with bright blue iridescence in males (Costa, 2006), and can only be diagnosed by combination of characters. This study confirms the position of *A. toba* within this species group as suggested by Calviño (2005), possibly as the sister taxon of the remaining species, and the position of *A. paucisquama* (Ferrer *et al*., 2008), which is closely related to the other species of the group distributed in the Jacuí river basin (Patos drainage). In all analyses, the monophyly of *A. periodicus* is questioned, suggesting that it could be a junior synonym of *A. affinis*, a species with unclear diagnostic characters (Costa, 2002b, 2006b). Furthermore, populations

of the upper Cuareim River (middle Uruguay basin), originally described as *A. luzardoi* (Perujo *et al*., 2005), and later synonymized with *A. periodicus* (Costa, 2006b), seem to be more closely related to *A. duraznensis* and *A. juanlangi*, species from the Negro river basin. Analyses at the species and population levels will eventually elucidate the identity and validity of these taxa. As mentioned by Loureiro *et al*. (2011), this clade presents a distribution pattern that suggests dynamic interconnections among basins of the lower Uruguay (Ibicuy and Negro river basins) and the Patos (Jacuí, Yaguarón, and Tacuarí river basins) ecoregions. The recent description of a species of this group (Volcan *et al*., 2017, not included in these analyses) from the Camaquã river basin (Patos Ecoregion), completes this scenario. In this sense, results of these analyses show a strong biogeographic relationship between the upper and middle Ibicuí and the upper Negro river basins. This pattern is similar to that of other freshwater fish taxa, such as the cichlid *Gymnogeophagus* (Malabarba *et al*., 2015). Interestingly, the upper parts of both basins present stream configurations such as elbows (see Bishop, 1995) that suggest past river capture events.

Austrolebias wichi is a species recently described and closely related to *A. patriciae* (Alonso *et al*., 2018). The position of *A. patriciae* has always been difficult to resolve. Costa (2006b) proposed it as the sister taxon of the clade composed by the "*A. bellottii*" + "*A. adloffi*" species groups. Costa (2010) included it as the sister taxa of the "*A. bellottii*" species group. A mitochondrial analysis placed it as the sister taxon of the "*A. alexandri*" species group (Garcia *et al*., 2014). In this study, we recovered this species as closely related to *A. varzeae*, a morphologically similar species that inhabits the upper Uruguay river basin (Alonso *et al*., 2018). This clade presents one synapomorphy: males with dark grey to bluish bands anterior to dorsal fin origin.

Costa (2014c) included *A. araucarianus* in the subgenus *Acrolebias*, originally composed by *A. varzeae* and *A. carvalhoi* (Costa, 2008b). According to all our analysis, *A. araucarianus* is closely related to the "*A. adloffi*" species group, and although we did not include *A. carvalhoi* in this work, the similarity of this species with *A. araucarianus* (Costa, 2014c) suggests their close phylogenetic affinity. If this is confirmed, the subgenus *Acrolebias* should exclude *A. varzeae* and include the "*A. adloffi*" species group.

These results show close relationships among species distributed in highlands of the Paraná (*A. araucarianus*), upper Negro (*A. arachan*) and Patos ecoregions ("*A. adloffi*" species group), conforming a clade with a disjunct distribution (Fig. 13). These spatial gaps in the distribution of members of this clade suggest the possibility of extinction in a large region located in the southwestern tip of the Brazilian shield. Such events could have been triggered by the peripheral depression that occurred from early to middle Miocene in this area (Ab'Saber, 1969). Splitting of species from Patos and Merin lagoons basins may also be associated with sea level changes that have occurred in the last four hundred thousand years (Sprechmann, 1978).
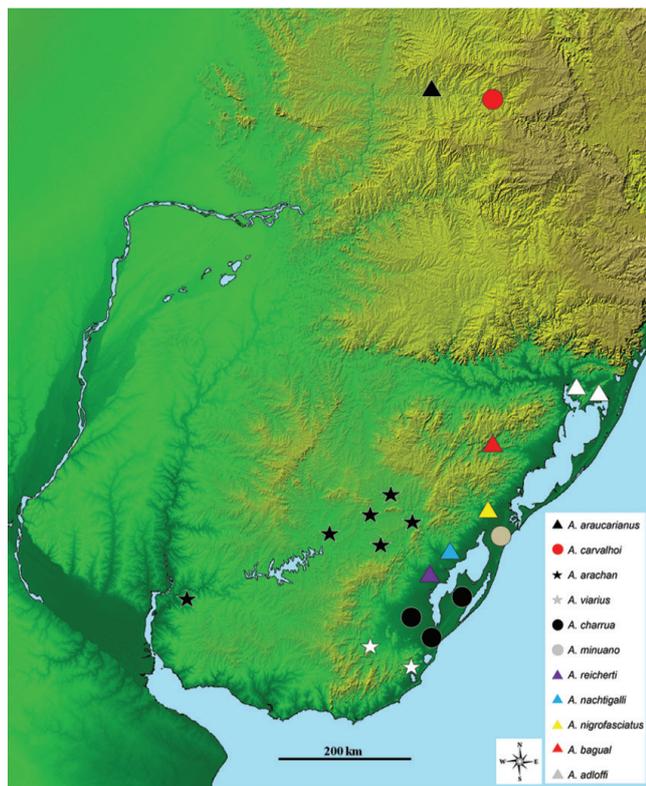
Fig. 13. Distribution map of the subgenus *Acrolebias* according to present phylogenetic analyses. Red dot = *A. carvalhoi*; Black triangle = *A. araucarianus*; Black star = *A. arachan*; White star = *A. viarius*; Black dot = *A. charrua*; White dot = *A. minuano*; Purple triangle = *A. reicherti*; Light blue triangle = *A. nachtigalli*; Yellow triangle = *A. nigrofasciatus*; Red triangle = *A. bagual*; White triangle = *A. adloffi*. Map modified from Shuttle Radar Topography Mission (SRTM), Courtesy NASA/JPL-Caltech.

Our analysis also questions the validity of the current limits of *A. nachtigalli*; populations of the northern bank of the Yaguarón river basin are more closely related to *A. reicherti*, distributed in the southern bank of the same river and in Tacuarí river basin, than to populations of *A. nachtigalli* from Arroio Grande basin (a northern tributary of Laguna Merin in Rio Grande do Sul). Loureiro, García (2008) reported no distinguishing characters between these species. Unfortunately, Costa's *et al*. (2017b) cytochrome b phylogenetic analysis of the "*A. adloffi*" species group did not includ sequences from the Yaguarón river basin. This analysis also suggested that *A. minuano* and *A. charrua* could represent a single species. Our analyses could not resolve the position of *A. minuano*, which, depending on the approach, related this species to a clade composed by *A. charrua* and *A. arachan*, or to *A. adloffi*. As was proposed for *A. nigripinnis*, changes in topologies according to the genetic marker suggest possible past events of hybridization or introgression, phenomena that were hypothesized by García (2006) to have influenced the evolution of the "*A. adloffi*" species group.

The absence or reduction of scales in the opercular and abdominal region (Loureiro *et al*., 2011) and cytochrome b phylogenetic analyses (García *et al*., 2014) suggested that the clade composed by *A. gymnoventris* and *A. jaegari* may be closely related to *A. luteoflamulatus* and *A. quirogai*. In this study, we show that these pairs of species may be distantly related, and that reduction of scales (also present in *A. cheradophilus* and *A. prognathus*) may have occurred independently.

The polytomy that joins the "*A. luteoflammulatus*" species group, the "*A. robustus*" species group, and the "*A. elongatus*" species group, could be a "soft" polytomy which needs to be solved with more data (especially molecular data). Alternatively, this could be a "hard" polytomy, the result of rapid simultaneous speciation of ancestors of these clades, a phenomenon which has been proposed as probable for this genus by Garcia *et al*. (2014). Furthermore, the "*A. elongatus*" species group presents a large set of synapomorphic characters probably associated with an increase in body size (many of these traits arose independently in the "giant" *Cynolebias*), and large prey acquisition. These features could indicate an ecological process of rapid phenotypic differentiation and speciation. Species of the "*A. elongatus*" group are restricted to seasonal environments associated with large wetlands or river floodplains of the Bermejo, La Plata estuary, lower Uruguay, and Patos-Merin basins, suggesting ecological connections among these areas possibly associated with sea level retractions during the Pleistocene. The most recent of these putative connections may have occurred ca. 18 thousand years ago when the Paraná River flowed directly to the Atlantic Ocean (Ayup-Zouain, 2006).

Despite the large amount of systematic work done in the family, especially by W. Costa, more work is still needed to reach a stable phylogenetic hypothesis for Rivulidae. At the subfamilial level, relationships among the Kryptolebiasinae, Rivulinae, and Cynolebiasinae remain unclear, and at the generic level, relationships within Rivulinae and Cynolebiasini are also incompletely resolved. Problems arise from the great morphological similarity among species and even genera, often diagnosed mainly by coloration patterns alone; and the great intraspecific variability, which has not even been incorporated in analyses of many taxa. Going forward, we must increase molecular sampling and analyses with conservative markers and variable markers to refine the species limits and relationships among closely related taxa. Morphological work also needs to be expanded to the analysis of intraspecific variability, since many diagnostic characters for species and genera rely on very few specimens, and the polymorphic nature of many species remains obscure.

Comparative material examined. Lots examined belong to: Facultad de Ciencias, Montevideo (ZVC-P); Museu de Ciências e Tecnologia da PUCRS, Pontifícia Universidade Católica do Rio Grande do Sul (MCP); National Academy of Sciences

of Philadelphia (ANSP); Museum of Zoology, University of Michigan (UMMZ); Muséum d'histoire naturelle de Genève (MHNG); C&S = Cleared and Stained. *Austrolebias adloffi*: Brazil: ANSP 168844, 6, 32.1-42.0 mm SL., ZVC-P 724, 25, 23.2-37.1 mm SL (8 C&S), ZVC-P 747, 25, 22.1-42.2 mm SL; ZVC-P 854, 16, 24.2-35.2 mm SL; ZVC-P 855, 13, 32.1-33.0 mm SL; MCP 10288, 12, 30.1-33.2 mm SL; MCP 15040, 2, 32.2-37.2 mm SL; MCP 10933, 20, 30.1-31.4 mm SL; UMMZ 168844, 4, 30.1-41.2 mm SL; UMMZ 181681, 16, 30.1-33.0 mm SL; UMMZ 181682, 22, 24.1-30.2 mm SL (2C&S). *Austrolebias affinis*: Uruguay. ZVC-P 4300, 4, 23.6-32.0 mm SL; ZVC-P 4301, 6, 22.1-25.0 mm SL, ZVC-P 4302, 10, 32.1-34.0 mm SL; ZVC-P 4305, 4, 35.4-38.0 mm SL; ZVC-P 4306, 6, 23.5-34.6 mm SL; ZVC-P 4308, 10, 31.2-33.1 mm SL.*Austrolebias alexandri*: Uruguay: ZVC-P 2760, 2, 31.2-36.0 mm SL (2C&S). *Austrolebias arachan*: ZVC-P 4336 (holotype), 1, 37.8 mm SL; ZVC-P 4574, 14, 32.4-40.2 mm SL (2 C&S); ZVC-P 4314, 3, 35.4-42.0 mm SL; ZVC-P 4317, 8, 34.1-42.0 mm SL (4 C&S); ZVC-P 4332, 15, 37.1-42.2 mm SL; ZVC-P 4333, 19, 35.4-41.0 mm SL (8 C&S); ZVC-P 4329, 4, 40.0-42.2 mm SL (4 C&S); ZVC-P 4331, 21, 31.5-39.8 mm SL (3 C&S). *Austrolebias bellottii*: Argentina: UMMZ 181683, 3, 42.1-45.0 mm SL (3C&S); ZVC-P 708, 10, 38.9-41.2 mm SL; ZVC-P 712, 1, 50.1 mm SL; ZVC-P 715, 1, 45.6 mm SL; ZVC-P 716, 3, 38.7-45.1 mm SL; ZVC-P 954, 5, 35.6-45.1 mm SL. *Austrolebias aff. bellottii*: Uruguay: ZVC-P 876, 61, 24.5-48.4 mm SL (19 C&S). *Austrolebias charrua:* Uruguay. ZVC-P 3882, 11, 32.4-35.0 mm SL (4C&S); ZVC-P 3883, 16, 34.5-41.0 mm SL (4C&S); ZVC-P 3884, 6, 32.1-37.9 mm SL; ZVC-P 3885, 4, 21.4-34.2 mm SL; ZVC-P 3948, 15, 31.1-36.0 mm SL (4C&S); ZVC-P 3886, 16, 32.1-35.1 mm SL (4C&S); ZVC-P 3911, 15, 29.8-34.1 mm SL (4C&S); ZVC-P 3947, 15, 38.4-42.1 mm SL; ZVC-P 3944, 20, 34.9-45.0 mm SL (4C&S). *Austrolebias cheradophilus*: Uruguay: ZVC-P 518, 1, 52.3 mm SL; ZVC-P 4283, 1, 49.9 mm SL; ZVC-P 2584, 1, 48.9 mm SL; ZVC-P 4282, 3, 45.8-54.9 mm SL (1C&S); ZVC-P 4287, 5, 40.5-48.9 mm SL (3C&S). *Austrolebias cinereus*: Uruguay; ZVC-P 658, 6, 24.5-38.1 mm SL (3C&S); ZVC-P 797, 14, 29.8-34.1 mm SL. *Austrolebias elongatus*: Argentina: ZVC-P 713, 6, 52.4-68.1 mm SL (2C&S); Uruguay: ZVC-P 12478, 3, 56.1-68.4 mm SL. *Austrolebias juanlangi*: Uruguay: ZVC-P 4310, 6, 28.9-36.5 mm SL; ZVC-P 4311, 4, 30.1-32.5 mm SL; ZVC-P 4313, 10, 25.9-38.2 mm SL.*Austrolebias duraznensis*: Uruguay: ZVC-P 4303 8, 34.1-42.6 mm SL (2C&S). *Austrolebias luteoflammulatus*: Uruguay: ZVC-P 4077 20, 35.1-37.8 mm SL (4C&S); ZVC-P 4101, 20, 22.3-33.9 mm SL (4C&S). *Austrolebias gymnoventris*: ZVC-P 4318, 2, 227.3-28.3 mm SL (1C&S); ZVC-P 4319, 2, 22.6-25.4 mm SL (1C&S). *Austrolebias melanoorus*: Uruguay: ZVC-P 4320 16, 38.4-45.2 mm SL; ZVC-P 4322, 10, 32.4-45.0 mm SL (2 C&S); ZVC-P 4323, 10, 34.2-47.1 mm SL (2C&S); ZVC-P 4325, 2, 32.1-35.0 mm SL. *Austrolebias nigripinnis*: Paraguay: UMMZ 207493, 30, 24.1-26.9 mm SL; UMMZ 207494, 4, 23.1-32.5 mm SL; Argentina: ZVC-P 718 3, 24.5-28.9 mm SL; Uruguay: ZVC-P 848, 6, 24.6-29.9 mm SL (2C&S). *Austrolebias prognathus*: Uruguay: ZVC-P 4273, 1, 56.1 mm SL; ZVC-P 4274 3, 49.9-59.4 mm SL (2C&S); ZVC-P 4275, 2, 58.6-59.3 mm SL (1C&S). *Austrolebias quirogai*: Uruguay: ZCV-P 9758 (Holotype), 36.8

mm SL; ZVC-P 8725, 69, 21.0-38.2 mm SL (5C&S). *Austrolebias reicherti*: Uruguay: ZVC-P 4362, 1, 25.5-34.2 mm SL; ZVC-P 4363, 18, 30.1-40.2 mm SL (9 C&S). *Austrolebias vandenbergi*: Paraguay: ANSP 175281, 3, 34.5-367 mm SL; ANSP 175282, 16, 34.6-40.1 mm SL (5C&S). *Austrolebias vazferreirai*: Uruguay: ZVC-P 4289 1, 54.1 mm SL; ZVC-P 4290 4, 35.6-58.1 mm SL (2C&S); ZVC-P 4295, 6, 42.1-61.0 mm SL (2C&S). *Austrolebias viarius*: Uruguay: ZVC-P 525, 50, 30.1-42.5 mm SL (8 C&S). *Austrolebias wolterstorffi:* Brazil: UMMZ 181684 25, 35.1-58.3 mm SL (3C&S); ZVC-P 3694 4, 50.1-52.3 mm SL (2C&S); Uruguay: ZVC-P 901, 1, 57.8 mm SL; ZVC-P 4277, 8, 42.1-53.5 mm SL (4C&S). *Cynopoecilus melanotaenia*: Brazil: ZVC-P 528 6, 23.5-29.9 mm SL (2C&S); Uruguay: ZVC-P 3946, 2, 29.8-35.1 mm SL. *Papiliolebias bitteri*: Paraguay: ANSP 170422 19, 25.5-30.1 mm SL; ZVC-P 2766, 20, 23.4-32.1 mm SL (5C&S). *Simpsonichthys chacoensis*: Paraguay: ANSP 170272, 3, 23.5-28.9 mm SL (1C&S); ANSP 170273, 2, 23.5-28.0 mm SL.

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