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Two new seasonal killifishes of the *Austrolebias adloffi* group from the Lagoa dos Patos basin, southern Brazil (Cyprinodontiformes: Aplocheilidae)

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Abstract

Morphological characters and phylogenetic trees generated by analyses of a segment of the mitochondrial gene cytochrome b support two new species from the Lagoa dos Patos basin, in southern Brazil. The phylogenetic analyses indicate that the two new species are each other's respective closest relatives among species of *A. adloffi* group. The clade comprising the two new species is supported as being more closely related to *A. adloffi* than to *A. nigrofasciatus* that is endemic to the same area. *Austrolebias pelotapes* Costa & Cheffe n. sp. is distinguished from all other species of the *A. adloffi* group by having the urogenital papilla base attached by a thin membrane to the anterior margin of the anal fin in males; it is endemic to an area containing temporary pools and swamps associated to small streams tributaries to the northern margin of the São Gonçalo channel, just about 10 km from the area inhabited by *A. nigrofasciatus*. *Austrolebias pongondo* Costa & Cheffe n. sp., from temporary pools and swamps in the floodplains south of the São Gonçalo channel, and *A. pelotapes* are distinguished from all other species of the *A. adloffi* group by a combination of character states, including the presence of a transverse row of small spots on the middle portion of the dorsal fin in males, unpaired fins with a single row of light blue spots on their basal portion in males, a distinctive dark grey zone on the posterior portion of the dorsal and anal fins, and caudal peduncle in males predominantly dark brownish grey or dark grey to black, with narrow vertical light blue zones.

Key words

Biodiversity, Cryptic species, Molecular phylogeny, Systematics, Taxonomy.

Introduction

An astonishing diversity in number of species and eco-morphological specializations has been recently reported for the aplocheiloid seasonal killifishes inhabiting temporary pools in grassland and savannah zones of Africa and South America (*e.g.*, Costa, 2009, 2011). The genus *Austrolebias* Costa, 1998 is a typical example, with over 40 valid species occurring in southern Brazil, southern Bolivia, Paraguay, Uruguay and north-eastern Argentina (*e.g.*, Costa, 2006). The highest species diversity is concentrated in the grassland region comprising southern Brazil and adjacent parts of Uruguay, where several species are often found in sympatry (*e.g.*, Costa, 2006, 2010). However, whereas sympatric species are easily recognizable by morphological features often related to feeding specializations (Costa, 2009), the genus contains some assemblages of allopatric cryptic species only identifiable by details of their morphology (Costa, 2006). Among these assemblages is the *A. adloffi* species group, first recognised by Costa & Cheffe (2001). It is easily diagnosed by a pair of black blotches vertically arranged on the posterior portion of the caudal peduncle in females and is distributed along a vast area of southern Brazil and

eastern Uruguay including the Patos-Mirim lagoon system and adjacent isolated coastal rivers basins (Costa & Cheffe, 2001; Costa, 2006).

The *A. adloffi* species group comprises seven nominal species: *A. adloffi* (Ahl, 1922), *A. charrua* Costa & Cheffe, 2001, *A. minuano* Costa & Cheffe, 2001, *A. nachtigalli* Costa & Cheffe, 2006, *A. nigrofasciatus* Costa & Cheffe, 2001, *A. reicherti* (Loureiro & García, 2004), and *A. salviai* Costa, Litz & Laurino, 2006 (Costa, 2006). All these species have been considered valid, except *A. salvia* recently placed in the synonymy of *A. reicherti* (Loureiro & García, 2008).

During ichthyological surveys in the area near the town of Pelotas, from where *A. nigrofasciatus* was described in the past (Costa & Cheffe, 2001), new populations exhibiting a different colour pattern were found, suggesting the existence of two new species. The objective of the present paper is to check the status of Pelotas populations diagnosed by colour patterns through phylogenetic trees generated by the analysis of a segment of the mitochondrial gene cytochrome b (cytb).

Material and methods

Methodology for species delimitation follows and integrative approach (*e.g.,* Costa *et al.*, 2012, 2013), using both a morphological character-based method (Davis & Nixon, 1992) and a molecular tree-based method (Wiens & Penkrot, 2002). Morphological characters were obtained from specimens fixed in formalin just after collection, for a period of 10 days, and then transferred to 70 % ethanol. Data on colour patterns were taken from adult individuals only, since in juveniles (below 25 mm SL) the colour pattern is not well established. Descriptions of colour patterns were based on colour photographs, including seven live males and two live females of *A. pelotapes* and four males and three females of *A. pongondo* at the moment of fixation in formalin; data on melanophore colour patterns were also taken from preserved specimens. Morphometric and meristic data were taken following Costa (1988); measurements are presented as percent of standard length (SL), except for those related to head morphology, which are expressed as percent of head length. Fin-ray counts include all elements. Number of vertebrae and gill-rakers, and data on skeleton structures were recorded from cleared and stained (C&S) specimens prepared according to Taylor & Van Dyke (1985). Terminology for frontal squamation follows Hoedeman (1958) and for cephalic neuromast series Costa (2001). Material is deposited in the ichthyological collection of the Biology Institute, Federal University of Rio de Janeiro, Rio de Janeiro (UFRJ) and Ichthyological Collection Morevy Cheffe, Grupo Especial de Estudo e Proteção do Ambiente Aquático do Rio Grande do Sul (CIMC).

In the tree-based approach, trees were generated through the analysis of a 600 pb sequence of the partial mitochondrial cytochrome b (cytb) gene, using both maximum parsimony (MP) and maximum likelihood (ML) methods. Statistical support for clade was assessed by bootstrapping (Felsenstein, 1985), considering bootstrap values equal or higher than 70 % as significant (Hillis & Bull, 1993). MP was performed with TNT 1.1 (Goloboff *et al.*, 2008), using the 'traditional' search and setting random taxon-addition replicates to 1000, tree bisection-reconnection branch swapping, multitrees in effect, collapsing branches of zero-length, and a maximum of 100,000 trees saved in each replicate. Branch support of the MP tree was assessed by bootstrap analysis, using a heuristic search with 1,000 replicates and the same settings used in the MP search, but saving a maximum of 1,000 trees in each random taxon-addition replicate. ML was run in MEGA 5 (Tamura *et al.*, 2013), under the Tamura 3-parameter model, which was previously determined by MEGA as the best nucleotide substitution model of sequence evolution. The ML analysis was performed with random-starting parameters and using a random-starting tree; branch support was calculated with 1000 nonparametric bootstrap replicates using the same settings. In species delimitation analyses, the term exclusive lineage is used instead of monophyletic, since the term monophyly is considered not applicable below the species level (*e.g.*, de Queiroz & Donoghue, 1990). Genetic distances, herein used only to illustrate genetic diversity among taxa, were calculated with the Kimura 2-parameter (K2P) model (Kimura, 1980) in MEGA 5. Lists of unique nucleotide substitutions shared by all analysed specimens of each new species were used to diagnose them. Optimization of nucleotide substitutions among lineages of *Austrolebias* were obtained from the MP tree described above, using TNT 1.1. Each unique substitution is represented by its relative numeric position determined through sequence alignment with the complete mitochondrial genome of *Kryptolebias marmoratus* (Lee *et al*., 2001), followed by the specific nucleotide substitution in parentheses.

Terminals of the analyses were sequences for 52 individuals representing all nominal species of the *A. adloffi* group and two outgroups, *A. viarius* (Vaz-Ferreira, Sierra-de-Soriano & Scaglia-de-Paulete, 1964) and *A. vazferrerai* (Berkenkamp, Etzel, Reichert & Salvia, 1994). Thirty sequences for *A. adloffi*, *A. charrua*, *A. reicherti*, *A. salvia*, and outgroups, previously published by García *et al.* (2004), were downloaded from GenBank; the remaining 22 sequences, listed in Appendix 1 with their respective GenBank accession numbers, were obtained from specimens fixed in absolute alcohol just after collection and later preserved in the same solution. Total genomic DNA was extracted from muscle tissue of the caudal peduncle using the DNeasy Blood & Tissue Kit (Qiagen), following manufacturer instructions. To amplify the fragment of the mitochondrial DNA were used the primers L14724 and CB3-H (Meyer & Wilson, 1990; Palumbi *et al*., 1991), specific for the mitochondrial gene cytochrome b (cytb). Polymerase chain reaction (PCR) was performed in 15 µl reaction mixtures containing 5x

Green GoTaq Reaction Buffer (Promega), 3.6 mM $MgCl_2$, 1 µM of each primer, 75 ng of total genomic DNA, 0.2 mM of each dNTP and 1U of Taq polymerase. The thermocycling profile was: (1) 1 cycle of 5 minutes at 94 °C; (2) 35 cycles of 40 seconds at 92 °C, 1 minute at 48–54 °C and 1 minute at 72°C; and (3) 1 cycle of 4 minutes at 72°C. In all PCR reactions, negative controls without DNA were used to check contaminations. Amplified PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing reactions were made using the BigDye Terminator Cycle Sequencing Mix (Applied Biosystems). Cycle sequencing reactions were performed in 10 µl reaction volumes containing 1 µl BigDye 2.5, 1.55 µl 5x sequencing buffer (Applied Biosystems), 2 µl of the amplified products (10–40 ng), and 2 µl primer. The thermocycling profile was: (1) 35 cycles of 10 seconds at 96°C, 5 seconds at 54°C and 4 minutes at 60°C. The sequencing reactions were purified and denatured and the samples were run on an ABI 3130 Genetic Analyzer. Sequences were edited using MEGA 5.

Results

The MP (not depicted) and the ML (Fig. 1) analyses generated identical single trees. The two new species are congruently supported as exclusive sister lineages, closely related to *A. adloffi* and members of a clade that includes both *A. adloffi* and *A. nigrofasciatus* the first one exhibiting a unique colour pattern and below recognised as a new species. This clade is sister to a clade comprising the remaining species of the *A. adloffi* group. The two new species are formally described below.

Austrolebias pelotapes Costa & Morevy sp. nov.

Fig 2–3, Table 1

Holotype: UFRJ 8601, male, 31.0 mm SL; Brazil: Estado do Rio Grande do Sul: Município de Pelotas: temporary pool in the Sanga Funda drainage, tributary of Arroio Pelotas, 31°43′46″S 52°19′07″W; L. Lanés *et al.*, 5 Nov. 2005.

Paratypes: All from Município de Pelotas, Estado do Rio Grande do Sul, southern Brazil: UFRJ 8600, 5 males, 25.9–31.4 mm SL, 5 females, 22.8–36.9 mm SL; collected with holotype. – UFRJ 8599, 2 males, 23.7–26.4 mm SL, 4 females, 27.3–37.5 mm SL; CIMC 8578, 1 male, 34.6 mm SL, 1 female, 34.4 mm SL; swamp close to Arroio Pelotas, near Cotuvelo bridge; L. Lanés *et al*., 19 Sep. 2004. – UFRJ 8597, 7 males, 19.7–26.4 mm SL, 3 females, 22.4–23.6 mm SL; CIMC 8590, 2 males, 22.8–24.5 mm SL; UFRJ 8598, 3 males, 24.6–26.6 mm SL, 3 females, 20.7–23.8 mm SL (C&S); temporary pool at the end of the Plácido de Castro street, Sanga da Barbuda drainage, tributary of Arroio Santa Bárbara, Lindóia, 31°42′29″S 52°21′40″W, M. Cheffe *et al.*, 24 Sep. 2004. – CIMC 13668, 2 males, 27.5–31.4 mm SL, 2 females, 24.0–24.5 mm SL (C&S); same locality as UFRJ 8599; M. Cheffe & F. Silveira, 14 Oct. 2004. – UFRJ 8800, 1 male, 23.4 mm SL, 1 female, 24.0 mm SL (DNA); same locality as UFRJ 8599; M. Volcan & A.C. Gonçalves, 6 Jul. 2011. – CIMC 17345, 10 males, 20.3–29.2 mm SL, 12 females, 21.5–30.4 mm SL; temporary pool near Ildefonso Simões Lopes avenue, Sanga Funda drainage, tributary of Arroio Pelotas, 31°42′47″S 52°18′04″W; L. Lanés & M. Volcan, 4 Oct. 2010. – UFRJ 8885, 3 males, 18.1–27.2 mm SL, 3 females, 19.4–21.9 mm SL (DNA); same locality as CIMC 17345; M. Volcan & A.C. Gonçalves, 6 Jul. 2011.

Diagnosis. *Austrolebias pelotapes* is distinguished from all other species of the *A. adloffi* group by having the urogenital papilla base attached by a thin membrane to the anterior margin of the anal fin in males (*vs.* urogenital papilla free). *Austrolebias pelotapes* also seems to be the smallest species of the *A. adloffi* group, not reaching 35 mm SL (*vs.* maximum adult size between 43 and 45 mm SL). It is distinguished from all other species of the *A. adloffi* group except *A. pongondo*, by the following combination of character states: a transverse row of small spots on the middle portion of the dorsal fin in males (*vs.* transverse row absent in all other species), unpaired fins with a single row of light blue spots on their basal portion in males, and with distinctive dark grey zone on the posterior portion of the dorsal and anal fins (*vs.* multiple rows of blue dots and distinctive dark grey zone absent in *A. charrua* and *A. minuano*), and caudal peduncle in males predominantly dark brownish grey or dark grey to black, with narrow vertical light blue zones in males (*vs.* alternating dark grey and light blue bars, dark grey bars slightly wider, equal or narrower than light blue bars, in *A. adloffi*, *A. nachtigalli*, *A. nigrofasciatus*, *A. reicherti*). *Austrolebias pelotapes* is distinguished from *A. pongondo* by the former having 14–19 neuromasts in the supraorbital series (*vs.* 20–21) and 22–23 neuromasts around orbit (*vs.* 24–27); also useful to distinguish them are details of the colour pattern in live males, including the grey bars on the flank barely contrasting with the light blue colour ground (*vs.* dark grey to black bars in deep contrast to light blue interspace in *A. pongondo*) and absence of a row of blue dots on the basal portion of the caudal fin (*vs.* presence).

Austrolebias pelotapes is also distinguished from all other species of the *A. adloffi* group by five unique nucleotide substitutions: cytb.219(A>G), cytb.243(T>C), cytb.480(T>C), cytb.501(G>A), cytb.663(C>T); it is similar to *A. pongondo* and distinguished from all other congeners of the *A. adloffi* group by four unique nucleotide substitutions: cytb.357(T>C), cytb.393(C>T), cytb.580(A>G), cytb.660(C>T); it is also distinguished from *A. pongondo* by the latter having six unique nucleotide substitutions: cytb.568(G>T), cytb.581(T>G), cytb.641(A>G), cytb.643(G>A), cytb.648(T>C), cytb.750(G>C). Other nucleotide loci useful to distinguish *A. pelotapes* from *A. pongondo* are: cytb.165(C), cytb.258(A), cytb.345(G), cytb.408(A), cytb.569(C) (*vs.* cytb.165(T), cytb.258(G), cytb.345(A), cytb.408(G), cytb.569(T) in *A. pongondo*).

Description. Morphometric data appear in Table 1. Largest male examined 34.6 mm SL; largest female examined 34.4 mm SL. Dorsal and ventral profiles convex

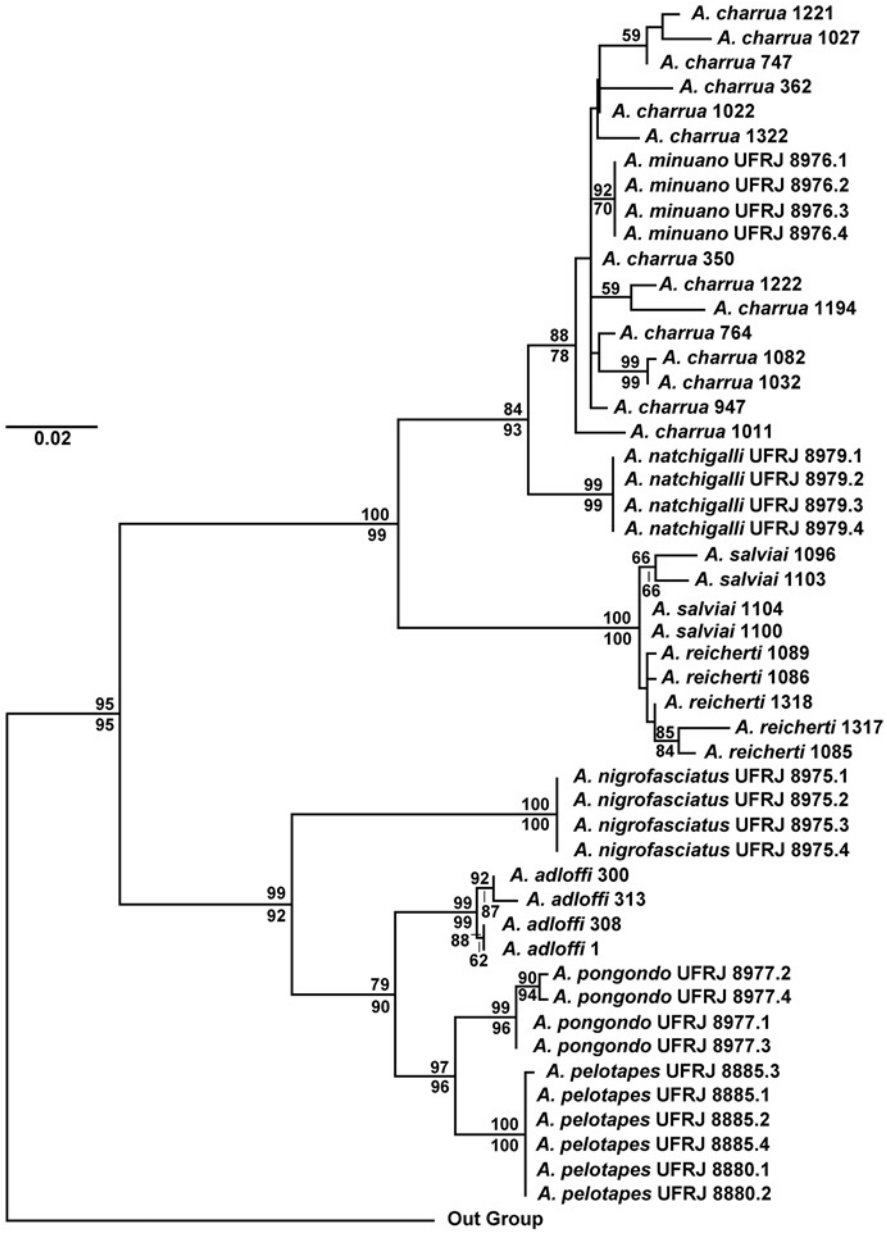

Fig. 1. Maximum likelihood tree of relationships among species of the *Austrolebias adloffi* group. Outgroups not represented. Single numbers after terminal species names are voucher numbers as in Genbank for material used in García *et al.* (2004); numbers preceded by UFRJ abbreviation after terminal species names indicate catalog numbers of specimens sequenced in the present paper. Numbers above and below branches are bootstrap values above 50 % for the Maximum Likelihood and Maximum Parsimony analyses, respectively.

from snout to end of dorsal and anal-fin bases, nearly straight on caudal peduncle. Body deep and compressed, greatest body depth at vertical through pelvic-fin base. Jaws short, snout blunt. Urogenital papilla cylindrical and short in males, its base attached to anal fin, tip free; urogenital papilla pocket-shaped in females, overlapping anal-fin origin.

Extremity of dorsal and anal fins rounded in both sexes. In females, anal fin sub-triangular with antero-median rays lengthened, distal portion thickened. Caudal fin rounded. Pectoral fin elliptical, posterior margin reaching vertical between base of 4th and 7th anal-fin rays in males, between urogenital papilla and anal-fin origin in females. Pelvic fin small, tip reaching base of 3rd anal-fin ray; medial membrane about 30–60 % coalesced. Dorsal-fin origin on vertical between base of 3rd and 5th anal-fin rays in males, between base of 1st and 2nd rays in females; second proximal radial of dorsal fin between neural spines of 7th and 9th vertebrae in males, between neural spines of 8th and 10th vertebrae in females; first proximal radial of anal fin between pleural ribs of 7th and 9th vertebrae in males, between pleural ribs of 8th and 10th vertebrae in females. Dorsal-fin rays 21–23 in males, 17–20 in females; anal-fin rays 23–26 in males, 20–23 in females; caudal-fin rays 20–22; pectoral-fin rays 11–12; pelvic-fin rays 5–6.

Scales large, cycloid. Trunk and head entirely scaled, except on infraorbital region and ventral surface of head. Body squamation extending over anterior 20 % of cau-

Fig. 2. *Austrolebias pelotapes*: UFRJ 8601, holotype, male, 31.0 mm SL; Brazil: Rio Grande do Sul: Pelotas.

Fig. 3. *Austrolebias pelotapes*, topotype, male, not preserved (photograph by Matheus V. Volcan).

dal-fin base; one irregular row of scale on middle portion of anal-fin base, no scales on dorsal-fin base. Frontal scales irregularly arranged; E-scales slightly overlapping medially. One supra-orbital scale. Longitudinal series of scales 27–29; transverse series of scales 12; scale rows around caudal peduncle 16. One to three minute contact organs on each scale of antero-ventral part of flank in males; minute contact organs on internal surface of three uppermost pectoral-fin rays in males; no contact organs on unpaired and pelvic fins.

Cephalic neuromasts: supraorbital 14–19, parietal 3, anterior rostral 1, posterior rostral 1, infraorbital 2 + 22–23, preorbital 2–3, otic 3, post-otic 3–4, supratemporal 1, median opercular 1, ventral opercular 2–3, preopercular plus mandibular 36–39, lateral mandibular 6–8, paramandibular 1. One neuromast per scale of lateral line. Two neuromasts on caudal-fin base.

Basihyal sub-triangular, basihyal cartilage about 40% of total length of basihyal. Six branchiostegal rays. Second pharyngobranchial teeth 3–6. Gill-rakers on first branchial arch 3+10. Vomerine teeth absent. Dermosphenotic absent. Ventral process of posttemporal well-developed. Total vertebrae 27–29.

Colouration. ***Males***. Flank usually with 7–9 grey bars, separated by light blue interspace; anterior bars darker and narrower, slightly wider than adjacent light blue interspaces, posterior bars 2–4 times wider than adjacent light blue bars (Fig. 3); in some specimens above 29 mm SL, flank bars increasing to 9–12 bars as result of ontogenetically appearing narrow light blue bars on middle of posterior wider grey bars (Fig. 2). Urogenital papilla grey. Side of head intense blue on opercular and infra-orbital region; black infra-orbital bar, wider close

Table 1. Morphometric data of *Austrolebias pelotapes*.

	holotype	paratypes	
	male	males (5)	females (6)
Standard length (mm)	31.0	26.3–31.4	22.8–29.4
Percent of standard length			
Body depth	44.5	39.4–45.3	38.7–44.4
Caudal peduncle depth	17.3	15.1–16.3	13.3–15.2
Pre-dorsal length	53.0	53.3–55.6	57.3–63.0
Pre-pelvic length	49.2	45.6–53.1	53.9–60.7
Length of dorsal-fin base	42.3	36.2–41.8	26.4–34.7
Length of anal-fin base	47.5	43.5–46.9	30.4–36.1
Caudal-fin length	32.1	30.4–35.1	33.8–42.1
Pectoral-fin length	23.9	22.6–30.3	23.9–32.2
Pelvic-fin length	10.3	8.2–11.8	10.9–15.1
Head length	31.8	30.2–33.4	30.9–34.2
Percent of head length			
Head depth	117	101–119	102–109
Head width	65	60–64	68–72
Snout length	14	14–16	13–17
Lower jaw length	21	17–22	17–20
Eye diameter	28	26–30	28–32

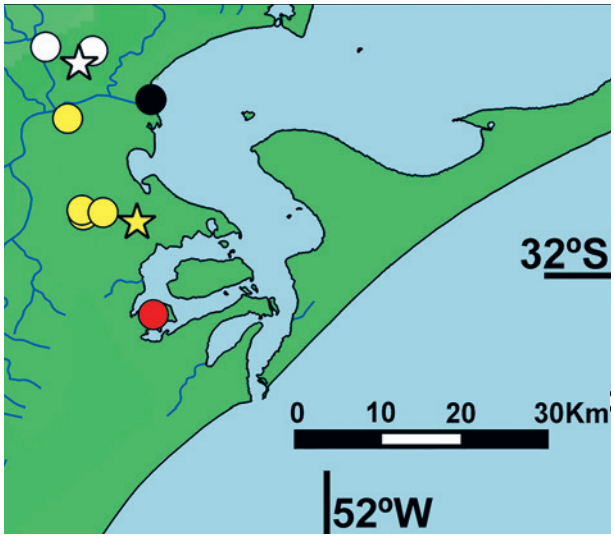

Fig. 4. Geographical distribution of species of the *Austrolebias adloffi* group in the region of confluence of the Canal de São Gonçalo with Lagoa dos Patos. White dots, *A. pelotapes*; yellow, *A. pongondo*; black, *A. nigrofasciatus*; red, *A. minuano*.

eye, gradually narrowing ventrally; elongate black supraorbital bar, with distinctive narrow extension over neuromast parietal series. Iris dark yellow, with dark black bar through centre of eye. Dorsal fin dark bluish grey, darker near fin base; few light blue dots and short bars on basal half of fin; transverse sub-basal row of light blue dots. Anal fin bluish grey, basal half with light blue dots and narrow diffusing bars, distal half intense blue; black blotch, more visible in preserved specimens, on posterior portion of dorsal and anal fins, anteriorly limited by narrow oblique light blue bar. Caudal fin intense blue to dark bluish grey on dorsal portion; sometimes light blue dot on middle of fin base. Pelvic fin intense blue. Pectoral fin hyaline with black ventral margin.

***Females*.** Flank light brownish grey; grey marks variable in form, ranging from spots vertically elongated to bars, marks sometimes absent; venter silver; no black spot on anterocentral portion of flank; two black spots vertically arranged on posterior portion of caudal peduncle, often coalesced to form 8-shaped spot, sometimes absent. Opercular region pale blue to pale golden. Iris light yellow, with grey bar through centre of eye. Infraorbital and supraorbital bars grey. Unpaired fins hyaline, with faint grey bars on basal portion of dorsal and anal fins; paired fins hyaline.

Genetic distance. To *A. pongondo*: 2.7–3.6%; to *A. nigrofasciatus*: 8.2–8.4%; to *A. adloffi*: 4.5–5.3 %; to other species of the *A. adloffi* species group: 13.5–16.7%; intraspecific: 0–0.2.

Distribution and habitat. *Austrolebias pelotapes* is known from temporary pools and swamps associated to small streams tributaries to the northern margin of the Canal de São Gonçalo, Patos-Mirim lagoon system, Rio Grande do Sul, Brazil (Fig. 3). In the type locality, water samples revealed the parameters: pH 6.4–6.8, dissolved oxygen 5.7–8.7 mg/l, and water temperature 16.7–25.9°C. Intensive field studies in the last 10 years have shown that all populations of this species are situated within or adjacent to urban areas of the town of Pelotas, in aquatic habitats being drastically reduced, making the species severely threatened with extinction. Given the small area of occurrence and the strong decline of habitats, *A. pelotapes* should be considered as a critically endangered species.

Etymology. The name *pelotapes* is the old usage for the town of Pelotas, from the Spanish *pelota* (ball), referring to a little boat made in cow hide, and *tapes*, referring to the indigenous tribe inhabiting the area occupied in the past by the new species.

Austrolebias pongondo Costa & Morevy sp. nov.

Fig 5, Table 2

Holotype: UFRJ 9583, male, 37.0 mm SL; Brazil: Estado do Rio Grande do Sul: Município de Pelotas: swamp at the road to Torotama island, Arraial, Povo Novo district, 31°55′54″S 52°14′42″W; M. Cheffe *et al.*, 21 Aug. 2001.

Paratypes: All from Município de Pelotas, Estado do Rio Grande do Sul, southern Brazil: UFRJ 9584, 2 males, 30.5–34.4 mm SL, 6 females, 31.0–37.0 mm SL; collected with holotype. – UFRJ 9585, 3 males, 33.9–40.9 mm SL, 2 females, 33.1–37.1 mm SL; same locality as holotype; M. Cheffe *et al.*, 15 Aug. 2001. – CIMC 4540, 8 males, 27.8–35.9 mm SL, 7 females, 22.5–31.8 mm SL; swamp close to the road BR-392, localidade de Capão Seco, Povo Novo district, 31°55′21″S 52°18′47″W; M. Cheffe *et al.*, 30 Aug. 2000. – CIMC 42098, 12 males, 26.7–36.1 mm SL, 21 females, 26.3–36.4 mm SL; road BR-392, 31°54′59″S 52°18′53″W; M. Burns & E. Silveira, 18 Aug. 2011. – CIMC 42099, 20 males, 18.8–32.3 mm SL, 25 females, 14.5–30.7 mm SL; road BR-392, 31°55′23″S 52°18′45″W; M. Cheffe *et al.*, 17 Aug. 2011. – CIMC 42100, 2

Fig. 5. *Austrolebias pongondo*, UFRJ 9583, holotype, male, 37.0 mm SL; Brazil: Rio Grande do Sul: Pelotas.

males, 30.8–33.2 mm SL, 16 females, 19.1–35.3 mm SL; road BR-392, 31°55′20″S 52°18′49″W; M. Burns, 5 Oct. 2011. – CIMC 42103, 3 males, 30.5–34.5 mm SL, 3 females, 29.7–34.2 mm SL; road BR-392, 31°54′59″S 52°18′53″W; M. Burns, 5 Oct. 2011. – CIMC 42108, 5 males, 29.4–36.3 mm SL, 1 female, 31.2 mm SL; road BR-392, 31°54′58″S 52°18′55″W; M. Burns, 15 Sep. 2011. – CIMC 42114, 2 males, 31.9–38.2 mm SL, 2 females, 29.0–32.7 mm SL; road BR-392, 31°54′48″S 52°18′55″W; M. Burns, 5 Oct. 2011. – CIMC 42116, 1 male, 30.6 mm SL, 8 females, 26.2–32.5 mm; Banhado do Cemitério, 31°55′23″S 52°18′46″W, Povo Novo district; M. Burns, 5 Oct. 2011. – UFRJ 8977, 3 males, 25.8–31.8 mm SL, 3 females, 28.6–31.3 mm SL (DNA); same locality as CIMC 42116; M. Cheffe & M. Burns, 22 Sep. 2011. – CIMC 5666, 1 male, 42.9 mm SL; swamp near Banhado da Mulata, 31°55′10″S 52°17′16″W; M. Cheffe *et al.*, 21 Aug. 2001. – UFRJ 9581, 12 males, 24.1–30.9 mm SL, 35 females, 23.7–29.6 mm SL; UFRJ 9582, 2 males, 29.4–31.5 mm SL, 3 females, 27.6–28.5 mm SL (C&S); swamp close to the road BR-392, Capão Seco, 31°47′59″S 52°19′59″W; M. Cheffe & M. Burns, 30 Oct. 2000. – CIMC 5671, 5 males, 30.5–37.8 mm SL, 7 females, 30.1–42.4 mm SL; same locality as UFRJ 9581; M. Cheffe & F. Silveira, 23 Aug. 2001.

Diagnosis. *Austrolebias pongondo* is distinguished from all other species of the *A. adloffi* group, except *A. pelotapes*, by the following combination of character states: a transverse row of small spots on the middle portion of the dorsal fin in males (*vs.* transverse row absent in all other species), unpaired fins with a single row of light blue spots on their basal portion in males, and with a distinctive dark grey zone on the posterior portion of the dorsal and anal fins (*vs.* multiple rows of blue dots and no distinctive dark grey zone in *A. charrua* and *A. minuano*), and caudal peduncle in males predominantly dark brownish grey or dark grey to black, with narrow vertical light blue zones (*vs*. alternating dark grey and light blue bars, dark grey bars slightly wider, equal or narrower than light blue bars, in *A. adloffi*, *A. nachtigalli*, *A. nigrofasciatus*, *A. reicherti*). *Austrolebias pongondo* is distinguished from *A. pelotapes* by the former having the urogenital papilla free from the anal fin in males (*vs*. basal portion of the urogenital papilla attached to the anterior margin of the anal fin); 20–21 neuromasts in the supraorbital series (*vs*. 14–19) and 24–27 neuromasts around orbit (*vs*. 22–23); the presence of dark grey to black bars on the flank strongly contrasting with the light blue interspace (*vs.* grey bars slightly contrasting with the light blue background in *A. pelotapes*) and a row of blue dots on the basal portion of the caudal fin (*vs*. absence, sometimes a single dot).

Austrolebias pongondo is distinguished from all other species of the *A. adloffi* group by six unique nucleotide substitutions: cytb.568(G > T), cytb.581(T > G), cytb.641(A > G), cytb.643(G > A), cytb.648(T > C), cytb.750(G > C); it is similar to *A. pelotapes* and distinguished from all other congeners of the *A. adloffi* group by four unique nucleotide substitutions: cytb.357(T > C), cytb.393(C > T), cytb.580(A > G), cytb.660(C > T); it is also distinguished from *A. pelotapes* by the latter having five unique nucleotide substitutions: cytb.219(A > G), cytb.243(T > C), cytb.480(T > C), cytb.501(G > A), cytb.663(C > T). Other nucleotide loci useful to distinguish *A. pongondo* from *A. pelotapes* are: cytb.165(T), cytb.258(G), cytb.345(A), cytb.408(G), cytb.569(T) (*vs*. cytb.165(C), cytb.258(A), cytb.345(G), cytb.408(A), cytb.569(C) in *A. pelotapes*).

Description. Morphometric data appear in Table 2. Largest male examined 42.9 mm SL; largest female examined 42.4 mm SL. Dorsal and ventral profiles convex from snout to end of dorsal and anal-fin bases, nearly straight on caudal peduncle. Body deep and compressed, greatest body depth at vertical through pelvic-fin base. Jaws short, snout blunt. Urogenital papilla cylindrical and short in males, free from anal fin; urogenital papilla pocket-shaped in females, overlapping anal-fin origin.

Extremity of dorsal and anal fins rounded in both sexes. In females, anal fin sub-triangular with antero-median rays lengthened, distal portion thickened. Caudal fin rounded. Pectoral fin elliptical, posterior margin reaching vertical between base of 6th and 7th anal-fin rays in males, between base of 1st and 3rd anal-fin rays in females. Pelvic fin small, tip reaching base of 4th anal-fin ray; medial

membrane about 10–50 % coalesced. Dorsal-fin origin on vertical between base of 3rd and 6th anal-fin rays in males, between base of 1st and 3rd rays in females; second proximal radial of dorsal fin between neural spines of 8th and 10th vertebrae in males, between neural spines of 9th and 12th vertebrae in females; first proximal radial of anal fin between pleural ribs of 7th and 9th vertebrae in males, between pleural ribs of 8th and 10th vertebrae in females. Dorsal-fin rays 19–22 in males, 15–20 in females; anal-fin rays 22–25 in males, 19–23 in females; caudal-fin rays 22–23; pectoral-fin rays 11; pelvic-fin rays 4–5.

Scales large, cycloid. Trunk and head entirely scaled, except on infraorbital region and ventral surface of head. Body squamation extending over anterior 20 % of caudal-fin base; one irregular row of scale on middle portion of anal-fin base, no scales on dorsal-fin base. Frontal scales irregularly arranged; E-scales slightly overlapping medially. One or two supra-orbital scales. Longitudinal series of scales 27–28; transverse series of scales 11; scale rows around caudal peduncle 16. One to five minute contact organs on scales of antero-ventral part of flank in males; minute contact organs on internal surface of three uppermost pectoral-fin rays in males; no contact organs on unpaired and pelvic fins.

Cephalic neuromasts: supraorbital 20–21, parietal 3, anterior rostral 1, posterior rostral 1, infraorbital 2–3 + 24–27, preorbital 3, otic 3–4, post-otic 3–4, supratemporal 1, median opercular 1, ventral opercular 2–3, pre-opercular 22–28; mandibular 13–15 (pre-opercular plus mandibular 27–33), lateral mandibular 5–6, paramandibular 1. One neuromast per scale of lateral line. Two neuromasts on caudal-fin base.

Basihyal sub-triangular, basihyal cartilage about 40 % of total length of basihyal. Six branchiostegal rays. Second pharyngobranchial teeth 3. Gill-rakers on first branchial arch 3 + 10. Vomerine teeth absent. Dermosphenotic absent. Ventral process of posttemporal well-developed. Total vertebrae 27–29.

Colouration. ***Males.*** Flank usually with 7–10 dark grey to black bars separated by light blue interspace; anterior bars narrower, about equal in width or slightly wider than adjacent light blue interspaces, posterior bars 1.5–2 times wider than adjacent light blue bars. Urogenital papilla grey. Side of head light blue on opercular and infra-orbital region; dark grey infra-orbital bar, wider close eye, gradually narrowing ventrally; elongate black supra-orbital bar, with distinctive narrow extension over neuromast parietal series. Iris dark yellow, with dark black bar through centre of eye. Dorsal and anal fins dark bluish grey, darker near fin base, with few light blue dots and narrow bars parallel to fin rays on basal half of fin; transverse sub-basal row of light blue dots; black blotch, more visible in preserved specimens, on posterior portion of dorsal and anal fins, anteriorly limited by narrow oblique light blue bar. Caudal fin bluish grey, lighter on basal portion; transverse row light blue dots on fin base. Pelvic fin bluish grey. Pectoral fin hyaline with black ventral margin.

Table 2. Morphometric data of *Austrolebias pongondo*.

	holotype	paratypes	
	male	males (5)	females (6)
Standard length (mm)	37.0	33.9–40.9	31.4–37.1
Percent of standard length			
Body depth	41.1	37.9–43.7	36.8–40.0
Caudal peduncle depth	15.7	14.5–15.7	13.4–15.6
Pre-dorsal length	52.1	51.7–56.6	56.4–61.0
Pre-pelvic length	45.6	44.6–50.7	52.2–56.4
Length of dorsal-fin base	44.6	37.1–42.1	27.7–32.3
Length of anal-fin base	45.8	43.9–48.0	24.5–30.8
Caudal-fin length	33.1	32.3–35.9	32.6–35.6
Pectoral-fin length	26.9	24.6–32.1	23.9–29.8
Pelvic-fin length	11.8	9.9–12.6	13.0–14.5
Head length	29.5	29.5–32.4	27.5–30.8
Percent of head length			
Head depth	122	111–117	104–113
Head width	66	60–68	73–81
Snout length	18	15–18	16–18
Lower jaw length	20	18–21	17–20
Eye diameter	28	28–31	31–33

Females. Flank light brownish grey; grey marks variable in form, ranging from spots vertically elongated to bars, marks sometimes absent; venter silver; no black spot on anterocentral portion of flank; two black spots vertically arranged on posterior portion of caudal peduncle, often coalesced to form 8-shaped spot, sometimes absent. Opercular region pale blue. Iris light yellow, with grey bar through centre of eye. Infraorbital and supraorbital bars grey. Unpaired fins hyaline, with faint grey bars on basal portion of dorsal and anal fins; paired fins hyaline.

Genetic distance. To *A. pelotapes*: 2.7–3.6 %; to *A. nigrofasciatus*: 9.0–9.7 %; to *A. adloffi*: 4.2–5.3 %; to other species of the *A. adloffi* species group: 12.7–17.4 %; intraspecific: 0–0.7.

Distribution and habitat. *Austrolebias pongondo* is known only from temporary pools and swamps in the floodplains south of the São Gonçalo channel, Povo Novo, Rio Grande, Rio Grande do Sul state, southern Brazil. The several pools and swamps of this area were significantly reduced after duplication of the road BR-392 between 2011 and 2012. Consequently, at least material CIMC 4595, CIMC 5671, CIMC 42098, CIMC 42099, CIMC 42100, CIMC 42103, CIMC 42108 and CIMC 42114 refer to pools today extinct. Considering the small area of distribution and the intense recent decline of the resting populations, *A. pongondo* should be indicated to be listed among the critically endangered species of southern Brazil.

Etymology. The name pongondó is the local designation for the people living in the village of Povo Novo, Rio Grande municipality, to where the new species is endemic.

Fig. 6. *Austrolebias nigrofasciatus*, topotype, male, not preserved (photograph by Matheus V. Volcan).

Discussion

The analyses support two new exclusive lineages, herein identified as *A. pelotapes* and *A. pongondo* (Fig. 1). Whereas all known localities of *A. pelotapes* are situated near the left bank of the Canal de São Gonçalo, all localities recorded for *A. pongondo* occur between the right bank of the Canal de São Gonçalo and the Lagoa dos Patos (Fig. 4). These ranges indicate that the Canal de São Gonçalo has been a historical barrier for gene flow among these two lineages.

Austrolebias pelotapes and *A. nigrofasciatus* are found in close adjacent areas, with their type localities separated by only about 10 km. Interestingly, colour patterns exhibited by males of both species are very distinctive, with the pattern of dark and light bars nearly opposite in width (*i.e.*, dark bars wider than light bars in *A. pelotapes vs.* narrower in *A. nigrofasciatus* (Figs. 3 and 5). *Austrolebias pelotapes* also differs from *A. nigrofasciatus*, by the latter species having the dorsal-fin origin slightly anterior or slightly posterior to the anal-fin origin in males and anterior to the anal-fin origin in females (*vs*. dorsal-fin origin always posterior to the anal-fin origin, between base of the 3rd and 5th anal-fin rays in males, between base of the 1st and 3rd anal-fin rays in females). In addition, the phylogenetic analysis consistently indicates that these two species are not close relatives, with *A. pelotapes* being more closely related to *A. pongondo* and *A. adloffi*. The last species is endemic to an area close to the city of Porto Alegre (Costa, 2006), in the opposed extremity of the Lagoa dos Patos, about 240 km from the type locality of *A. pelotapes*, suggesting a complex historical biogeographical pattern for the group.

Costa (2006) provided a monographic revision of *Austrolebias*, clarifying old taxonomic problems after examining data taken from type material of nominal species described between the late 19th century and first half of the 20th century, as well as describing six new species. Among the new species were two taxa belonging to the *A. adloffi* species group, endemic to river basins connected to the Lagoa Mirim lagoon: *A. nachtigalli* from the floodplains of the Arroio Grande and adjacent areas in southern Brazil, and *A. salviai* from the Río Tacuarí in Uruguay. The type locality of the latter species was within the geographical range of another nominal species of the *A. adloffi* group, *A. reicherti*, which was described while Costa's (2006) revision was in progress (Loureiro & García, 2004). The type material of *A. reicherti* included material from both the Cebollatí and Tacuarí river drainages, but the type locality was designated as 1 km north of the Vergara town, in the Río Cebollatí drainage (Loureiro & García, 2004). Costa (2006) examined material from the Río Cebollatí drainage, identifying it as *A. charrua*, thus concluding that *A. reicherti* would be a synonym of *A. charrua*. However, both *A. reicherti* and *A. charrua* occur in neighbouring areas of the Río Cebollatí drainage (García, 2006), later proving Costa's (2006) synonymy to be equivocal (Loureiro & García, 2008).

Besides considering *A. reicherti* as a valid species, Loureiro & García (2008) considered *A. salviai* as a synonym of *A. reicherti*. However, just comparing the type material of *A. salvia* from the Río Tacuarí drainage to the data presented in the original description of *A. reicherti* from its type locality, it is possible to realise some differences in colour pattern and general morphology. For example, male specimens of *A. reicherti* have black bars on the caudal peduncle about thrice wider than interspace (Loureiro & García, 2004: fig. 2B), whereas in *A. salvia* all flank black bars are very narrow, with the bars on the caudal peduncle being about one third width

of interspace (Costa, 2006: fig. 51). In addition, southern populations from the Río Cebollatí drainage, including the type locality of *A. reicherti*, and northern populations from the Río Tacuarí drainage, including the type locality of *A. salvia*, appear as recently diverging exclusive lineages in Loureiro & García (2008: fig. 3), suggesting that they may represent closely related, distinct species, when adopting exclusiveness as a criterion to delimitate species. The present tree topology (Fig. 1), using sequences provided by García (2006) for *A. reicherti* and *A. salviai*, also indicates close relationships but without resolution within the lineage. However, the high genetic distances found among individuals of *A. reicherti* and *A. salviai* populations may not correspond to real genetic variability, but to some error in DNA sequencing process, since so high variability in cytb sequences has neither been found in other congeners (our sequences provided in the present paper), nor in other genera of the Cynolebiini (Costa *et al.*, 2012, 2014a, b; Costa & Amorim, 2014). Furthermore, different lineages of the geographically widespread *A. charrua* are clustered with *A. minuano*, suggesting the existence of distinct species hidden under the name *A. charrua*, which is consistent with the great morphological variation recorded by Costa (2006) and the broad genetic distance between populations (up to 3.7 %). Thus we recommend deeper studies with species of the *A. adloffi* before synonymies being formally made.

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Appendix 1

List of specimens, and respective catalogue numbers, localities, coordinates and GenBank accession numbers

Species · Catalog number · Locality · GenBank

Austrolebias pelotapes
- UFRJ 8800.1 · Pelotas · KJ475078
  31º 42′ 29″S 52º 21′ 39″W
- UFRJ 8800.2 · Pelotas · KJ475079
  31º 42′ 29″S 52º 21′ 39″W
- UFRJ 8885.1 · Pelotas · KJ475080
  31º 42′ 46″S 52º 18′ 02″W
- UFRJ 8885.2 · Pelotas · KJ475081
  31º 42′ 46″S 52º 18′ 02″W
- UFRJ 8885.3 · Pelotas · KJ475082
  31º 42′ 46″S 52º 18′ 02″W
- UFRJ 8885.4 · Pelotas · KJ475083
  31º 42′ 46″S 52º 18′ 02″W

Austrolebias nigrofasciatus
- UFRJ 8975.1 · Laranjal · KJ475084
  31º 46′ 32″S 52º 12′ 36″W
- UFRJ 8975.2 · Laranjal · KJ475085
  31º 46′ 32″S 52º 12′ 36″W
- UFRJ 8975.3 · Laranjal · KJ475086
  31º 46′ 32″S 52º 12′ 36″W
- UFRJ 8975.4 · Laranjal · KJ475087
  31º 46′ 32″S 52º 12′ 36″W

Austrolebias minuano
- UFRJ 8976.1 · Quinta · KJ475088
  32º 03′ 01″S 52º 13′ 25″W
- UFRJ 8976.2 · Quinta · KJ475089
  32º 03′ 01″S 52º 13′ 25″W
- UFRJ 8976.3 · Quinta · KJ475090
  32º 03′ 01″S 52º 13′ 25″W
- UFRJ 8976.4 · Quinta · KJ475091
  32º 03′ 01″S 52º 13′ 25″W

Austrolebias pongondo
- UFRJ 8977.1 · Povo Novo · KJ475092
  31º 55′ 23″S 52º 18′ 45″W
- UFRJ 8977.2 · Povo Novo · KJ475093
  31º 55′ 23″S 52º 18′ 45″W
- UFRJ 8977.3 · Povo Novo · KJ475094
  31º 55′ 23″S 52º 18′ 45″W
- UFRJ 8977.4 · Povo Novo · KJ475095
  31º 55′ 23″S 52º 18′ 45″W

Austrolebias nachtigalli
- UFRJ 8979.1 · Arroio Grande · KJ475096
  32º 14′ 32″S 53º 04′ 07″W
- UFRJ 8979.2 · Arroio Grande · KJ475097
  32º 14′ 32″S 53º 04′ 07″W
- UFRJ 8979.3 · Arroio Grande · KJ475098
  32º 14′ 32″S 53º 04′ 07″W