Species diversity and speciation mechanisms in *Crenicichla* (Neotropical cichlids)

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Ph.D. Thesis

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Annotation

This thesis contributes to the knowledge of the species diversity of the *Crenicichla lacustris* sp. group in the La Plata River basin with description of three new species. Speciation mechanisms within two different species flocks from the middle Paraná/Iguazu and Uruguay Rivers were studied with a phylogenomic approach applying a novel genotyping method based on a Double-Digest Restriction site Adjacent DNA (ddRAD) sequencing. Our results support a repeated origin of morphological species being evolved several times sympatrically and independently in different drainages. A considerable role of hybridization/introgression as an evolutionary force was also proposed. The thesis further uncovers biogeographic aspects of the southern part of Brazilian shield and adjacent coastal rivers.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 9. září 2013 .....

Lubomír Piálek

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Piálek, L.**, Říčan, O., Almirón, A., Casciotta, J., 2010. *Crenicichla hu*, a new species of cichlid fish (Teleostei: Cichlidae) from the Paraná basin in Misiones, Argentina. Zootaxa 2537, 33–46 (IF = 0.974).
Lubomír Piálek participated in the field work, performed all the molecular methods and phylogenetic analyses, and wrote a substantial part of the manuscript.

II. Casciotta, J., Almirón, A., **Piálek, L.**, Gómez, S., Říčan, O., 2010. *Crenicichla ypo* (Teleostei: Cichlidae), a new species from the middle Paraná basin in Misiones, Argentina. Neotropical Ichthyology 8, 643–648 (IF = 1.048).
Lubomír Piálek participated in the field work and wrote a substantial part of the manuscript.

III. Říčan, O., **Piálek, L.**, Almirón, A., Casciotta, J., 2011. Two new species of *Australoheros* (Teleostei: Cichlidae), with notes on diversity of the genus and biogeography of the Río de la Plata basin. Zootaxa 2982, 1–26 (IF = 0.974).
Lubomír Piálek participated in the field work, phylogenetic analyses (molecular part) and preparation of the manuscript.

IV. **Piálek, L.**, Říčan, O., Casciotta, J., Almirón, A., Zrzavý, J., 2012. Multilocus phylogeny of *Crenicichla* (Teleostei: Cichlidae), with biogeography of the *C. lacustris* group: species flocks as a model for sympatric speciation in rivers. Molecular Phylogenetics and Evolution 62, 46–61 (IF = 4.066).
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V. Casciotta, J., Almirón, A., Aichino, D., Gómez, S., **Piálek, L.**, Říčan, O., in review. *Crenicichla taikyra* (Teleostei: Cichlidae), a new species of pike cichlid from the middle río Paraná, Argentina. Submitted to Zootaxa.
Lubomír Piálek performed several analyses approving the taxon statute and helped with preparation of the manuscript.

VI. **Piálek, L.**, Doubnerová, K., Petrusek, A., Casciotta, J., Almirón, A., Říčan, O., in preparation. Parallel evolution: Repeated origin of morphological species in Neotropical cichlids (*Crenicichla*) revealed by phylogenomics.
Lubomír Piálek participated in the field work, designed and performed the ddRADseq experiment, all the bioinformatic methods and phylogenetic analyses, and wrote a substantial part of the manuscript.

Disclaimer

This thesis contains one manuscript with a new name, but this new name is disclaimed for the purpose of Zoological nomenclature (International Code of Zoological Nomenclature). This means that the thesis may be cited in its own right, but it should not be cited as a source of nomenclatural statements.

Contents

Introduction

"With an estimated 3000 species, distributed from Central and South America, across Africa to Madagascar, the Middle East, and southern India, cichlid fishes (Cichlidae) represent the most species-rich family of vertebrates. In total they account for about 10% of extant teleost diversity. Throughout their distributional range, cichlids have repeatedly demonstrated their capacity for undergoing adaptive radiation, generating an outstanding variation of body shapes, color patterns, and behavior, and an enormous diversity of trophic and ecological specializations. This has made them an important model system for the field of evolutionary biology. With the completion of the first cichlid genome sequences, cichlid fishes are likely to receive even more attention in evolutionary research. Cichlids represent a unique model to study all aspects of evolution." (Koblmüller *et al.* 2011, Cichlid Evolution: Lessons in Diversification)

Cichlids and rapid diversification

Besides other interesting aspects of their biology, cichlids are a prime example of rapid adaptation to diverse habitats and trophic niches. Such syntopically living monophyletic assemblages of closely related species (forms) with a high level of endemicity are commonly indicated as species flocks (Salzburger and Meyer 2004) - a widely known evolutionary phenomenon in the Cichlidae. Most of the studies focused on such species complexes come from special lacustrine habitats where species have been demonstrated to evolve in sympatry, like East African Rift Valley, Cameroonian volcanic crater lakes, or more recently from Neotropical lakes (Salzburger and Meyer 2004; Kocher 2004; Schliewen 2005; Barluenga *et al.* 2006; Geiger *et al.* 2010). The latest works however suggest that cichlid species flocks can, under certain circumstances, evolve also in complex riverine habitats, e.g. the Lower Congo rapids (Schwarzer *et al.* 2011). In South America in the Amazon the candidates (since they have not yet been studied in an evolutionary context) for species flocks are the *Teleocichla* and *Crenicichla* complexes from the Xingu, Tapajós and Tocantins rivers (Kullander 1988; Stawikowski and Werner 2004) and the foremost example are the *Crenicichla* species complexes from the Uruguay and Paraná/Iguazu River drainages (Lucena and Kullander 1992; Piálek *et al.* 2012).

Crenicichla Heckel

The genus *Crenicichla* Heckel is the most speciose lineage of Neotropical cichlids, at present with 87 species (95 including *Teleocichla* Kullander, see further; http://www.fishbase.org; Kullander 1986; Ploeg 1991; Stawikowski and Werner 2004; Kullander *et al.* 2010; Piálek *et al.* 2012) but at least as many species are known and remain to be formally described (Stawikowski and Werner 2004; Piálek *et al.* 2012). Thus the number of valid species is increasing almost every year (Casciotta *et al*. 2006; Kullander and Lucena 2006; Lucena 2007; Casciotta and Almirón 2008; Montaña *et al.* 2008; Kullander 2009; Piálek *et al.* 2010; Casciotta *et al.* 2010; Varella *et al.* 2012; Kullander and Lucena

2013; Varella and Moreira 2013; Casciotta *et al.* in review). *Crenicichla* is primarily a predatory fish group with a long and slender body inhabiting a wide range of biotopes, from minute brooks to large rivers (pers. obs.). The genus has a widespread distribution in cis-Andean South America, ranging from Trinidad and the Orinoco basin to the Negro River in Patagonia, Argentina (Kullander 1986; Casciotta 1987), with a comparatively high diversity in the subtropical regions of South America (the *Crenicichla lacustris* group; Kullander *et al.* 2010). Kullander (1988) described several rheophilic species inhabiting the Brazilian and Guiana shield tributaries of the lower Amazon as a new genus, *Teleocichla* (with at present 8 valid species), but other authors (Ploeg 1991; López-Fernández *et al.* 2010) considered *Teleocichla* an ingroup of *Crenicichla*.

Phylogenetic relationships within *Crenicichla* were almost unknown (when work on this thesis started) and the genus was traditionally divided into several species groups (Kullander 1981, 1982, 1986; Ploeg 1991; Stawikowski and Werner, 2004; Kullander *et al.* 2010): the *C. lacustris* group (with 29 valid species), the *Crenicichla lugubris* group (15), the *Crenicichla reticulata* group (9), the *Crenicichla saxatilis* group (24), and the *Crenicichla wallacii group* (7); the classification of the type species *Crenicichla macrophthalma* as well as of *Crenicichla hemera* and *Crenicichla chica* in respect to these groups remains unclear (Kullander 1990, 1997; Varella *et al.* 2012). The species groups are mostly defined by the color pattern, several meristic characters, and geographic distribution.

Crenicichla lacustris species group

Most of the species groups of *Crenicichla* are largely sympatric, with distribution being centered in the Amazon and Orinoco drainages. The *C. lacustris* species group (a lineage of interest in this thesis), is, however, allopatric with respect to rest of the genus, distributed in the La Plata River basin (the Paraná and Uruguay Rivers) and in the Atlantic coastal rivers. When work on this thesis started, only seven endemic species were known from the Paraná River drainage, from which only three occur in the middle Paraná/lower Iguazu Rivers, an ichthyological province that as will be shown in this thesis is one of the most important diversity centers of the genus in the South America. Furthermore, one of these three species, *C. niederleinii*, is a taxon of unclear status best treated as a 'nomen nudum' (type specimen missing, type locality uncertain; Kullander 1981; Graça and Pavanelli 2007; Varella 2011; Piálek *et al.* 2012); the other two species (*C. tesay*, *C. yaha*) had previously been described by J. Casciotta and A. Almirón (our Argentinean collaborators in this project; Casciotta *et al.* 2006; Casciotta and Almirón 2008).

Considerably more had been published about the endemic association of *Crenicichla* from the Uruguay River drainage. In addition to the already known *C. celidochilus* (Casciotta 1987), Lucena and Kullander (1992) described (besides other two species; see Piálek 2012 *et al.* for details) five new taxa endemic to the Uruguay River drainage: a putatively monophyletic triplet of species (*C. missioneira*, *C. minuano*, *C. tendybaguassu*) with nearly identical color patterns (united also "by the particular coloration of males, not known from any other *Crenicichla* species"; Lucena and Kullander

1992), and, also, a uniquely colored (extensive spotting all over the body and fins) and possibly monophyletic species pair from the uppermost Uruguay River drainage (*C. igara*, *C. jurubi*). Species included within these two assemblages differ substantially in the their mouth and jaw characteristics (*C. missioneira* and *C. igara*, long piscivorous mouth with lower jaw prognathous; *C. minuano*, small terminal mouth with isognathous jaws; *C. tendybaguassu*, isognathous jaws and uniquely hypertrophied lips with long median lobes, *C. jurubi*, isognathous jaws and a massive lower pharyngeal jaw with molariform teeth). No other morphometric or meristic characters distinguishing between the species of a given assemblage were described or are known. All the above mentioned species were included by the authors in the newly proposed *C. missioneira* species group and characterized as a species flock.

Lucena and Kullander (1992) also mentioned several specimens collected "at the same time and at the same place" in the Forquila River, a left-hand tributary of the upper Uruguay, that resemble four different species (*C. celidochilus, C. jurubi, C. minuano, C. missioneira, C. tendybaguassu*) but "depart in the same way in color pattern from potential conspecifics collected elsewhere"; the authors therefore were reluctant to include them in the type material of the species in question. This little note to us suggested a parallel diversification process (according to the authors the putatively related species with different mouths share the same coloration, which is true also for the Forquilha River) which now gains a completely new dimension as will be shown in our study dedicated to possibly repeated origin of morphological species in the Uruguay River drainage (**PAPER VI**).

Fifteen years later, Lucena (2007) diagnosed two other species from the Upper Uruguay: *C. empheres* living above a high waterfall on the Chapeco River, and *C. hadrostigma* from below, occurring also down in the Uruguay River. Both new taxa were included by the author into the *C. missioneira* species group sensu Lucena and Kullander (1992), pointing out (besides other) the shared particular male coloration (a number of dark spots on the caudal peduncle and a series of narrow vertical single or double-bars along the middle portion of the body flank) between *C. hadrostigma*, *C. missioneira*, *C. minuano*, and *C. tendybaguassu*.

Even before the rapid increase in the species diversity of *Crenicichla* in the middle Paraná/Iguazu and Uruguay Rivers that coincided and partly is the result of this thesis, the diversity of *Crenicichla* already then seemed to be disproportionately high given the southern latitude of the area. This is in contrast to the situation in most Neotropical fish groups, which have the highest species diversity in the Amazon basin. It seems to be reasonable to look for the cause of this imbalance (if we do not consider an eventually disproportional interest of ichthyologists which can be true as well) also outside the biological forces of the diversification process.

Abiotic factors and biodiversity

As already suggested, cichlid species flocks can evolve in different types of macrohabitats (lakes, rivers) but the key factor is complexity of the habitats. No matter if a lake or a river, only a complex

set of niches from which fishes can choose and adapt to, can initiate the driving forces behind rapid diversification. The geological diversity (geodiversity) of a given river basin (or lake) thus seems to be the main abiotic factor responsible for rapid speciation (besides the inner potential of a group to diversify). A common geological feature of the Xingu (and Tapajós and Tocantins) and the Paraná/Iguazu/Uruguay River basins (where *Crenicichla* species flocks occur) is that both belong to the same geological formation known as Brazilian shield (which together with the Guiana shield form the geological core of the South American continent).

The southern part of the Brazilian shield centered on the Iguazu/middle Paraná/Uruguay Rivers is unique in South America by being composed of, and having exposed at its surface, volcanic flood basalts of the Paraná group, which are a direct result of the rifting between South America and Africa (Bryan *et al.* 2010). The Paraná flood basalts are part of the Paraná–Etendeka traps, which comprise a large igneous province shared between South America and Africa across the Atlantic Ocean, originating ca. 128 to 138 million years ago (Fodor *et al.* 1989). These flood basalts are the reason why the southern region of the Brazilian shield has the largest number and highest concentration of waterfalls in South America; as opposed to the Andes and Guiana shield's Tepuis (table-top mountains), these waterfalls have high biodiversity including freshwater fishes both below and above the falls. The region has hundreds of waterfalls and rapids ranging from those on the smallest tributaries to huge falls on mighty rivers and the falls are the products of crustal discontinuities within the deforming basalts. The most famous of these are the Iguazu Falls on the border between Argentina and Brazil. All rivers and tributaries in the Paraná group additionally flow in deeply incised canyons. The complex geomorphology of the rivers provides a bewildering diversity of habitats below the water surface and thus seems to promote diversification, which reached its peak in the cichlids, and not surprisingly in *Crenicichla*.

Aim of this thesis

The common aim of all papers included in this thesis is to contribute to the knowledge of the diversity of the *Crenicichla lacustris* species group and the speciation mechanisms behind it, to uncover also the biogeographic aspects of the southern part of Brazilian shield and adjacent coastal rivers. On account of the latter aim I have included also **Paper III**, which deals primarily with another genus of cichlids, *Australoheros*.

Results

I. **Piálek, L.**, Říčan, O., Almirón, A., Casciotta, J., 2010. *Crenicichla hu*, a new species of cichlid fish (Teleostei: Cichlidae) from the Paraná basin in Misiones, Argentina. Zootaxa 2537, 33–46.

II. Casciotta, J., Almirón, A., **Piálek, L.**, Gómez, S., Říčan, O., 2010. *Crenicichla ypo* (Teleostei: Cichlidae), a new species from the middle Paraná basin in Misiones, Argentina. Neotropical Ichthyology 8, 643–648.

III. Říčan, O., **Piálek, L.**, Almirón, A., Casciotta, J., 2011. Two new species of *Australoheros* (Teleostei: Cichlidae), with notes on diversity of the genus and biogeography of the Río de la Plata basin. Zootaxa 2982, 1–26.

IV. **Piálek, L.**, Říčan, O., Casciotta, J., Almirón, A., Zrzavý, J., 2012. Multilocus phylogeny of *Crenicichla* (Teleostei: Cichlidae), with biogeography of the *C. lacustris* group: species flocks as a model for sympatric speciation in rivers. Molecular Phylogenetics and Evolution 62, 46–61.

V. Casciotta, J., Almirón, A., Aichino, D., Gómez, S., **Piálek, L.**, Říčan, O., in review. *Crenicichla taikyra* (Teleostei: Cichlidae), a new species of pike cichlid from the middle río Paraná, Argentina. Submitted to Zootaxa.

VI. **Piálek, L.**, Doubnerová, K., Petrusek, A., Casciotta, J., Almirón, A., Říčan, O., in preparation. Parallel evolution: Repeated origin of morphological species in Neotropical cichlids (*Crenicichla*) revealed by phylogenomics.

Paper I

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Article

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Crenicichla hu, a new species of cichlid fish (Teleostei: Cichlidae) from the Paraná basin in Misiones, Argentina

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Abstract

A new species of *Crenicichla* Heckel, *C. hu*, is described from the arroyo Piray–Miní, a left-hand tributary of the río Paraná, Misiones province, Argentina. This new species is easily distinguished from its congeners in the La Plata basin and adjacent coastal rivers by the dark coloration (dark grey or dark brown to black), a color pattern consisting of 7 to 9 black irregular blotches on the flank, and 47–54 scales in the E1 row. Adult females have dorsal fin with an irregular color pattern formed by wide black and white longitudinal stripes and blotches. In addition to standard morphological comparisons, a brief molecular phylogenetic analysis of *Crenicichla* species from the province of Misiones is also introduced.

Key words: molecular phylogeny, systematics, taxonomy, Piray–Miní, ND2, NADH dehydrogenase

Resumen

Una nueva especie de *Crenicichla* Heckel, *C. hu*, es descripta del arroyo Piray-Miní, un afluente de la margen izquierda del río Paraná, provincia de Misiones, Argentina. Esta especie es facilmente reconocida entre las especies del género en la Cuenca del Plata y ríos costeros adyacentes por su coloración oscura (gris oscuro o castaño oscuro-negro), un patrón de coloración del flanco con 7 a 9 manchas irregulares negras, 47-54 escamas en la serie E1. Las hembras adultas poseen una aleta dorsal con un patrón de coloración irregular formado por bandas longitudinales y manchas, negras y blancas. Además de una comparación morfológica se presenta un breve análisis filogenético molecular de las especies del género presentes en la provincia de Misiones.

Introduction

The genus *Crenicichla* Heckel is the most speciose lineage of Neotropical cichlids. In the present conception the genus includes about 80 valid species (Casciotta *et al*. 2006; Kullander & Lucena 2006; Kullander *et al.* in press) and this number is rapidly increasing (*e.g.*, Casciotta *et al*. 2006; Kullander & Lucena 2006; Lucena 2007; Casciotta & Almirón 2008; Montaña *et al.* 2008; Kullander 2009). Stawikowski & Werner (2004) listed more than 120 known species including those yet undescribed. *Crenicichla* has a widespread distribution, ranging from northern South America to the río Negro in Patagonia, Argentina (Casciotta 1987).

Phylogenetic relationships within *Crenicichla* are almost unknown and the genus is traditionally divided into several species groups: the *C. lugubris* group, *C. reticulata* group, *C. saxatilis* group, *C. wallacii* group, and *C. lacustris* group s.l. (see below); according to some authors, *Teleocichla* Kullander, is a ingroup of *Crenicichla* (Kullander 1981, 1982, 1986; Ploeg 1991; Lucena & Kullander 1992; Kutty 2000; Stawikowski & Werner 2004; Kullander *et al.* in press). These species groups are mostly defined by coloration characters, as well as by biogeography as they basically correspond to major river drainages.

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Most Neotropical fish groups have the highest species diversity in the Amazon basin. On the contrary, the diversity of *Crenicichla* seems to be disproportionately high in the southern part of its distribution (Kullander 2009). At present there are 27 described species and several known but still undescribed from the La Plata basin and adjacent coastal drainages; with a few exceptions all these taxons fall into the above-mentioned *C. lacustris* group s.l. (Stawikowski & Werner 2004).

A small part (nine described species) of the *C. lacustris* group s.l. was recently studied by Kullander *et al.* (in press). With respect to this work, we can divide the group into the following subgroups:

The coastal drainages of Brazil have six endemic species: *C. iguapina* Kullander & Lucena, *C. lacustris* (Castelnau), *C. maculata* Kullander and Lucena, *C. mucuryna* von Ihering, *C. punctata* Hensel, and *C. tingui* Kullander & Lucena.

The río Uruguay has 11 endemic or nearly endemic species (the exception is *C. scottii* that enters the lower Paraná) in two species groups or complexes: 1. The *C. missioneira* group/complex which includes *C. celidochilus* Casciotta, *C. empheres* Lucena, *C. hadrostigma* Lucena, *C. igara* Lucena & Kullander, *C. jurubi* Lucena & Kullander, *C. minuano* Lucena & Kullander, *C. missioneira* Lucena & Kullander, *C. tendybaguassu* Lucena & Kullander; 2. The *C. scottii* group/complex with *C. gaucho* Lucena & Kullander, *C. prenda* Lucena & Kullander, *C. scottii* (Eigenmann).

The río Paraná has eight described endemic species: *C. haroldoi* Luengo & Britski, *C. iguassuensis* Haseman, *C. jaguarensis* Haseman, *C. jupiaensis* Britski & Luengo, *C. mandelburgeri* Kullander, *C. niederleinii* (Holmberg), *C. tesay* Casciotta & Almirón, and *C. yaha* Casciotta *et al*. Another species, *C. vittata* Heckel, occurs both in the Paraná and in the Uruguay drainage basins.

Furthermore, at least three species from other groups of *Crenicichla* enter the río Paraná drainage. These are *C. britskii* Kullander and *C. lepidota* Heckel from the *C. saxatilis* group, and *C. semifasciata* Heckel from the *C. reticulata* group.

Despite its small size, the province of Misiones is one of the regions with the highest biodiversity in Argentina (Bertonatti & Corcuera 2000). According to López *et al.* (2002) Misiones displays the highest rate of endemism of all Argentinean ichthyoregions. The borders of Misiones are defined by three major river drainages of the La Plata system, namely the río Paraná, the río Uruguay and the río Iguazú, each with a different set of species. The location of this small province is thus ideal for the study of faunal evolution in the larger context of the La Plata system.

Recently there have been numerous discoveries of new fish species from Misiones (Miquelarena *et al.* 2002; Rodríguez & Miquelarena 2005; Casciotta *et al*. 2006; Casciotta & Almirón 2008), including so far two new species of *Crenicichla, C. yaha* and *C. tesay.* The aim of this paper is to describe another new species of *Crenicichla* from the arroyo Piray–Miní belonging to the Paraná basin. We also provide a phylogenetic placement of the new species among Misioneran crenicichlas using molecular markers.

Material and methods

Morphology. Specimens were cleared and counterstained (C&S) following the method of Taylor & Van Dyke (1985). Measurements and counts were taken as described by Kullander (1986). Descriptions of pharyngeal teeth and counts of frashed zone concavities follow Casciotta & Arratia (1993). Holotype values are indicated by an asterisk. Body length is expressed as standard length (SL). E1 scale counts refer to the scales in the row immediately above that containing the lower lateral line (Lucena & Kullander 1992).

Institutional abbreviations are as listed in Leviton *et al.* (1985), except for AI (Asociación Ictiológica, La Plata, Argentina).

Molecular phylogeny. Twenty specimens from eleven localities representing nine prospective species were obtained during a field expedition to Misiones; two additional samples of *C. lacustris* and *C. punctata* were received commercially from the aquarium trade (Table 1). The mitochondrial gene ND2 (coding the NADH dehydrogenase subunit 2) including adjacent sequences of tRNA (in the order: part of tRNA-Gln, tRNA-Met, ND2, tRNA-Trp, part of tRNA-Ala) was sequenced in order to determine phylogenetic

relationships. Genomic DNA was extracted from ethanol-preserved gill tissue using a JETQUICK Tissue DNA Spin Kit (Genomed) following the standard protocol. The ND2 gene of ca. 1300 bp was amplified using PCR with the following primers: ILE-5' (CCG GAT CAC TTT GAT AGA GT) and ASN-3' (CGC GTT TAG CTG TTA ACT AA) (Wimberger *et al.* 1998). Each PCR reaction volume of 25 µl contained 12.5 µl of Combi PPP Master Mix (Top-Bio, http://www.top-bio.cz), 1.5 µl of each primer (10 pmol/µl), and 1 µl of DNA extract. Reaction mixtures were subjected to the following cycling protocol: 10 min. 95 °C, 36 x (10 s 96 °C, 20 s 50 °C, 90 s 68 °C), 10 min. 72 °C. PCR reactions were performed in a PTC-150 thermocycler (MJ Research) and PCR products were purified using the JETQUICK PCR Purification Spin Kit (Genomed). Sequencing reactions were performed following the standard protocol with the use of the same primers, and the products were analyzed in an ABI 3730XL automated sequencer (Applied Biosystems; both steps done by Macrogen Inc., Korea). Contiguous sequences of the gene segments were created by assembling DNA strands (forward and reverse) using BioLign 4.0.6.2 (Hall 2001) and aligned manually in BioEdit 7.0.9 (Hall 1999). All sequences were submitted to GenBank under Accession Nos. HM048873, HM048874, GQ328030 to GQ328048 (Table 1).

Separate maximum parsimony (MP) and Bayesian (BA) analyses were performed. Phylogenetic tree construction utilized the software PAUP* 4.0b10 (MP; Swofford 2001) and MrBayes 3.1.2 (BA; Huelsenback & Ronquist 2001; Ronquist & Huelsenbeck 2003). For MP analyses heuristic searches were performed to find the most parsimonious tree(s) using tree bisection-reconnection (TBR) branch-swapping and 1000 random sequence addition replicates with equal weight for all sites. Nonparametric bootstrapping (Felsenstein 1985) was used to measure the support of clades with 10000 total pseudoreplicates and TBR branch-swapping with 10 random sequence addition replicates per pseudoreplicate. The phylogenetic tree was rooted with two sequences of *Crenicichla lepidota* (C7, C34) from the *C. saxatilis* group (see Kullander *et al.* in press); the outgroup position of *C. lepidota* was further augmented with GenBank sequences of *Satanoperca jurupari* Heckel (Accession No. AB018971) and *Astronotus ocellatus* (Agassiz) (AB018972) in the role of an additional outgroup.

Akaike (AIC) criterion was used to select a model for BA analyses in MrModelTest 2.2 (Nylander 2004), a simplified version of ModelTest 3.06 (Posada & Crandall 1998) for use with MrBayes, and PAUP*. A Bayesian analysis using a Markov chain Monte Carlo simulation was run for 5 million generations, with trees sampled and saved every 100 generations (50000 trees saved per run). Two simultaneous analyses, each with ten chains, were performed using the computational facilities of the Computational Biology Service Unit of Cornell University (http://cbsuapps.tc.cornell.edu). The first trees from each run before reaching equilibrium were discarded as burn-in; convergence between the two runs was estimated using diagnostics criteria produced by the ‘sump’ command in MrBayes. The remaining trees were used for reconstruction of a 50% majority-rule consensus tree with the posterior probability (PP) values of the relevant branches displayed by the 'sumt' command.

Uncorrected pairwise divergences were counted in PAUP* with the use of the command 'showdist'.

Results

Molecular phylogenetic analyses

Alignment of the 22 sequences of the 1296 bp ND2 region contained 296 parsimony informative characters. MP analysis resulted in one parsimonious tree (Fig. 1; length, 636; CI, 0.74; RI, 0.79) that differs in one node from the consensus tree obtained from BA analysis (model, GTR+I+G; burn-in, 100). Testing the influence of the burn-in value on the consensus BA tree revealed absolute stability both of the tree topology and of the PP values within the whole investigated range (burn-in 100 to 42000).

The topology and node support of the recovered trees as well as the uncorrected pairwise divergences between relevant clades (Table 2) fully support the taxonomic distinctivness of the new *Crenicichla* species from the arroyo Piray–Miní.

TABLE 1. List of specimens used in the molecular phylogenetic analyses. Most of the samples collected during the field expedition to Misiones, Nov–Dec 2007

Specimen	Tissue No.	Catalogue No.	GenBank Access No.	Drainage	Locality	Coordinates
C. gaucho	C24		GQ328042	Uruguay	arroyo Fortalezza	26°45'57"S 54°10'57"W
C. hu	C13	MACN-ict 9430	GQ328038	Paraná	arroyo Piray–Miní	26°20'00"S 53°52'30"W
C. hu	C14	MACN-ict 9430	GQ328039	Paraná	arroyo Piray–Miní	26°20'00"S 53°52'30"W
C. lacustris	II74		HM048873	coastal	aquarium specimen	
C. lepidota	C7		GQ328030	Paraná	forest pool drained to río Iguazú	25°41'24"S 54°07'27"W
C. lepidota	C34		GQ328031	Uruguay	arroyo Patria	27°45'57"S 55°09'34"W
C. cf. mandelburgeri	C15		GQ328040	Paraná	arroyo Piray–Guazú	26°26'34"S 54°08'29"W
C. cf. mandelburgeri	C17		GQ328041	Paraná	arroyo Piray–Guazú	26°26'34"S 54°08'29"W
C.. mandelburgeri	C44	MACN-ict 9441	GQ328044	Paraná	arroyo Cuñapirú	27°05'19"S 54°57'08"W
C.. mandelburgeri	C49	MACN-ict 9440	GQ328045	Paraná	arroyo Cuñapirú	27°02'19"S 55°02'14"W
C. mandelburgeri	C51	MACN-ict 9439	GQ328046	Paraná	arroyo Guaruhape	26°53'02"S 54°53'33"W
C. mandelburgeri	C52	MACN-ict 9449	GQ328047	Paraná	arroyo Guaruhape	26°53'02"S 54°53'33"W
C. minuano	C80		GQ328048	Uruguay	small affluent of río Yaboti	27°14'15"S 54°02'39"W
C. missioneira	C36		GQ328043	Uruguay	arroyo Patria	27°45'57"S 55°09'34"W
C. punctata	DNA9		HM048874	coastal	aquarium specimen	
C. tesay	C1		GQ328032	Paraná	arroyo Deseado	25°40'16"S 53°55'59"W
C. tesay	C2		GQ328033	Paraná	arroyo Deseado	25°40'16"S 53°55'59"W
C. tesay	C11		GQ328036	Paraná	small stream, affluent to río Iguazú	25°46'59"S 54°02'16"W
C. tesay	C12		GQ328037	Paraná	small stream, affluent to río Iguazú	25°46'59"S 54°02'16"W
C. vittata	C62		HM048875	Paraná	laguna Iberá	28°32'47"S 57°11'45"W
C. yaha	C5		GQ328034	Paraná	arroyo Deseado	25°40'16"S 53°55'59"W
C. yaha	C6		GQ328035	Paraná	arroyo Deseado	25°40'16"S 53°55'59"W

TABLE 2. Uncorrected pairwise divergences of sequences of the ND2 gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 *C. gaucho* C24	-																				
2 *C. hu* C13	0.082	-																			
3 *C. hu* C14	0.082	0.001	-																		
4 *C. lacustris* II74	0.122	0.110	0.110	-																	
5 *C. lepidota* C7	0.162	0.160	0.161	0.172	-																
6 *C. lepidota* C34	0.162	0.162	0.162	0.173	0.012	-															
7 *C. cf. mandelburgeri* C15	0.075	0.036	0.036	0.114	0.160	0.160	-														
8 *C. cf. mandelburgeri* C17	0.075	0.036	0.036	0.114	0.160	0.160	0.000	-													
9 *C. mandelburgeri* C44	0.074	0.033	0.033	0.113	0.161	0.160	0.008	0.008	-												
10 *C. mandelburgeri* C49	0.073	0.034	0.034	0.112	0.159	0.158	0.009	0.009	0.002	-											
11 *C. mandelburgeri* C51	0.072	0.036	0.036	0.110	0.159	0.159	0.007	0.007	0.005	0.006	-										
12 *C. mandelburgeri* C52	0.072	0.036	0.036	0.110	0.159	0.159	0.007	0.007	0.005	0.006	0.000	-									
13 *C. minuano* C80	0.122	0.114	0.114	0.127	0.151	0.152	0.112	0.112	0.113	0.110	0.109	0.109	-								
14 *C. missioneira* C36	0.124	0.114	0.114	0.127	0.152	0.154	0.111	0.111	0.113	0.112	0.109	0.109	0.006	-							
15 *C. punctata* DNA9	0.057	0.085	0.085	0.121	0.170	0.170	0.077	0.077	0.083	0.082	0.080	0.080	0.124	0.124	-						
16 *C. tesay* C1	0.073	0.035	0.035	0.110	0.159	0.159	0.011	0.011	0.011	0.012	0.010	0.010	0.109	0.109	0.081	-					
17 *C. tesay* C2	0.073	0.035	0.035	0.110	0.159	0.159	0.011	0.011	0.011	0.012	0.010	0.010	0.109	0.109	0.081	0.000	-				
18 *C. tesay* C11	0.072	0.035	0.035	0.111	0.159	0.159	0.011	0.011	0.011	0.012	0.010	0.010	0.109	0.109	0.080	0.003	0.003	-			
19 *C. tesay* C12	0.071	0.036	0.036	0.110	0.158	0.158	0.012	0.012	0.012	0.012	0.011	0.011	0.110	0.110	0.079	0.004	0.004	0.001	-		
20 *C. vittata* C62	0.080	0.078	0.078	0.127	0.169	0.169	0.067	0.067	0.068	0.068	0.066	0.066	0.121	0.121	0.090	0.069	0.069	0.068	0.069	-	
21 *C. yaha* C5	0.074	0.037	0.037	0.115	0.161	0.161	0.008	0.008	0.010	0.010	0.011	0.011	0.110	0.109	0.077	0.013	0.013	0.013	0.014	0.067	-
22 *C. yaha* C6	0.074	0.037	0.037	0.115	0.161	0.161	0.008	0.008	0.010	0.010	0.011	0.011	0.111	0.109	0.077	0.013	0.013	0.013	0.014	0.067	0.000

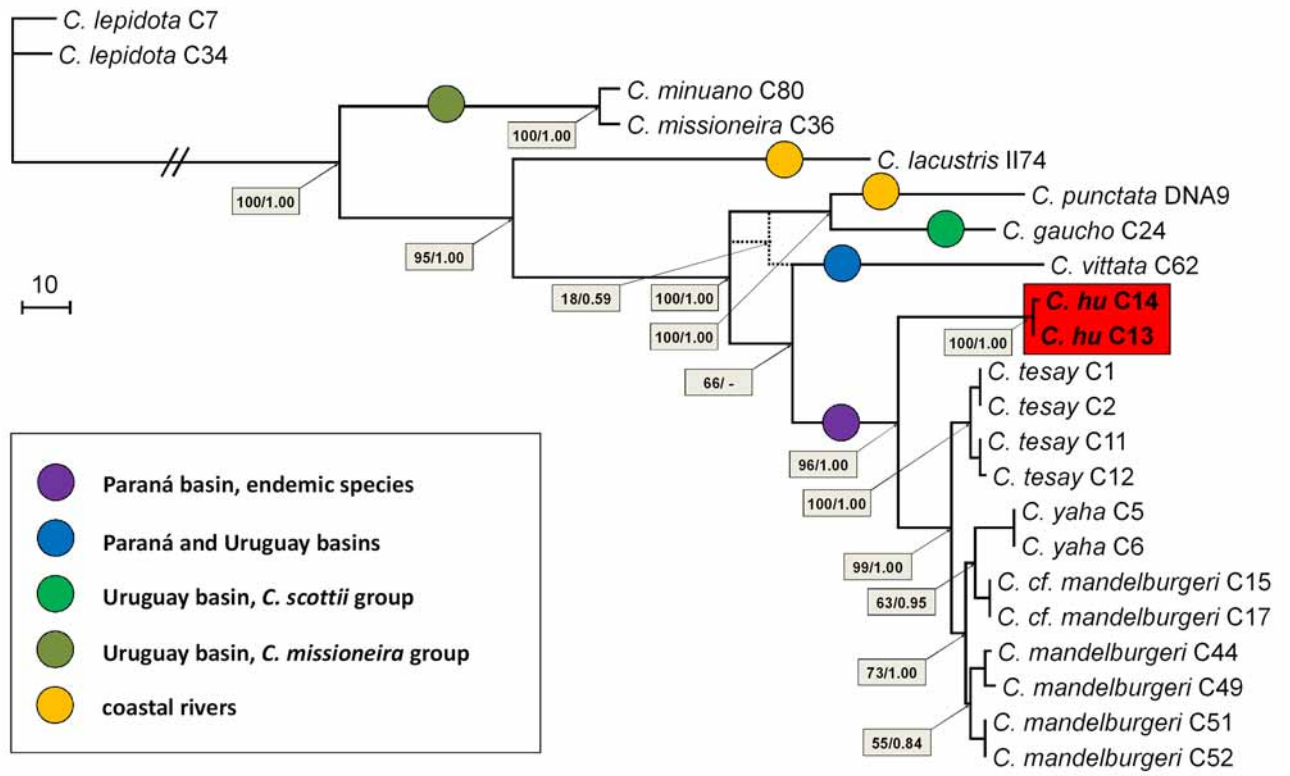

FIGURE 1. Maximum parsimony tree topology based on ND2 sequences; the dotted line displays the different topology of the alternative Bayesian inference. Numbers for each recovered node represent nonparametric bootstrap support (left) and Bayesian posterior probability (right), respectively.

Crenicichla hu, new species

(Figs. 2–5)

Holotype. MACN-ict 9429, 118.0 mm, Argentina, Misiones, río Paraná basin, arroyo Piray–Miní, 26°20'00.3"S 53°52'30.0"W, Nov 2007, O. Říčan *et al.* (Fig. 2).

Paratypes. All from Argentina, same data as the holotype. MACN-ict 9430, 17 ex., 76.9–153.0 mm. AI 261, 4 ex., 96.3–110.0 mm. AI 262, 1 ex. (C&S) 93.9 mm, same data as holotype (Figs. 3–4).

Diagnosis. *Crenicichla hu* is distinguished from all known species of the La Plata basin and adjacent coastal rivers by the following combination of characters: 1. dark grey or dark brown to black color of body and fins, 2. 7 to 9 black irregular blotches on the flank, 3. 47–54 scales in row E1, 4. the dorsal fin of adult females with a color pattern formed of black and white longitudinal stripes and/or blotches.

Since the molecular analysis confirmed close relations between *C. hu* and its biogeographic congeners from the Paraná basin (Fig. 1), a detailed comparative analysis was performed on all 13 known species inhabiting the Paraná drainage basin, either exclusively or partly:

Crenicichla hu is distinguished from *C. britskii* and *C. lepidota* (both *C. saxatilis* group) by the absence of the distinctive humeral spot vs. a humeral spot present (synapomorphy of the group). *Crenicichla hu* is distinguished from *C. haroldoi* by the absence of dots on lateral line scales vs. brown dots present on each lateral line scale. It differs from *C. iguassensis* and *C. tesay* in the absence of small dots on the flank vs. numerous scattered small dots present. *Crenicichla hu* is distinguished from *C. jaguarensis*, *C. vittata*, and adults of *C. mandelburgeri* by the absence of a lateral band vs. a lateral band present. It differs from *C. jaguarensis* in the absence vs. presence of the caudal spot. Further, *Crenicichla hu* differs from *C. mandelburgeri* and *C. niederleinii* in the absence vs. presence of the narrow vertical double-bars on the flank. It is also distinguished by a low number of scales in a lateral row, 47–54 vs. 56–65 in *C. niederleinii* and 78–85 in *C. vittata*. *Crenicichla hu* differs from *C. jupiaensis* in the absence vs. presence of numerous narrow

vertical bars on the flank, a well developed (but composed of spots) suborbital stripe vs. reduced to a few spots posteriorly to the orbit, a cheek bearing 4 to 6 scale rows vs. a naked cheek, and the absence vs. presence of a thin black line on the posterior margin of the preopercle. *Crenicichla hu* lacks several regular parallel rows of small dark spots vs. present in *C. scottii*. The new species is distinguished from *C. semifasciata* (*C. reticulata* group) by having about half of the caudal fin scaled vs. this fin scaled over most of its surface. *C. hu* further has the ascending arm of the premaxilla longer than the dentigerous one vs. shorter in *C. semifasciata*. Finally, *C. hu* is distinguished from *C. yaha* by the head depth 17.9–20.8% vs. 15.1–18.1% of SL, and lower jaw slightly prognathous vs. jaws isognathous or upper jaw slightly prognathous.

FIGURE 2. *Crenicichla hu*, female, holotype, MACN-ict 9429, 118.0 mm, arroyo Piray-Miní, 26°20'00"S 53°52'30"W.

FIGURE 3. *Crenicichla hu* male, live specimen, paratype, MACN-ict 9430, 153.0 mm.

Description. Morphometric data of the holotype and paratypes is given in Table 3. Body elongate, depth 21.5 to 25.6% of SL (Fig. 2). Head slightly deeper than wide. Snout short, bluntly pointed in lateral view, 2.5 to 3.0 times in HL. Lower jaw slightly prognathous. Tip of maxilla not reaching anterior margin of orbit in most specimens (reaching in four specimens, MACN-ict 9429). Lower lip widely interrupted medially. Nostrils dorsolateral, nearer anterior margin of orbit than snout tip. Posterior margin of preopercle weakly serrated (21 ex.*) or smooth (3 ex., MACN-ict 9430). Scales on flank strongly ctenoid. Head scales cycloid. Predorsal scales small, superficially embedded in skin. Prepelvic scales smaller than predorsal ones.

Interopercle naked. Cheek scaled, 4 to 6 scales below eye embedded in skin. Scales in E1 row 47(1), 51(2), 52(4), 53(5), 54(9*). Scales in transverse row 10/14(1), 11/13(1), 11/14(7), 11/15(2), 11/16(3*), 11/17(3), 12/13(1), 12/14(2), 12/15(1). Three scale rows between lateral lines. Upper lateral line scales 18(1), 19(1), 21(3), 22(8), 23(4*), 24(2), 25(2). Lower lateral line scales 10(1), 11(7), 12(5), 13(1), 14(5), 15(2*). Dorsal, anal, pectoral and pelvic fins naked. Dorsal fin XVIII,10(1); XX,12(2); XX,13(1); XXI,10(3*); XXI,11(8); XXI,12(4); XXII,11(1). Anal fin II,10(1); III,8(2); III,9(14*); III,10(3). Pectoral fin 15(10*), 16(11). Caudal-fin squamation extending almost to middle of fin in larger specimens, no more than the basal third of caudal fin in smaller ones. Soft-dorsal fin rounded or pointed tip, surpassing caudal-fin base. Tip of anal fin reaching caudal-fin base (not reaching in three specimens, AI 261 and MACN-ict 9429). Caudal fin rounded. Pectoral fin rounded, almost reaching the tip of pelvic fin. Microbranchiospines present on second through fourth gill arches. Gill rakers externally on first gill arch: 1 on epibranchial, 1 on angle, and 8 on ceratobranchial. Three to five patches of unicuspid teeth on fourth ceratobranchial. Lower pharyngeal tooth plate with unicuspid recurved and curved crenulated bicuspid teeth, those of posterior and medial row larger than remaining ones (Fig. 5). Upper pharyngeal tooth plate with unicuspid and bicuspid teeth. Frashed zone bearing one concavity with small unicuspid teeth. Premaxillary ascending process longer than dentigerous one. Premaxilla with 24(1) unicuspid teeth on outer row, larger than inner ones. Five teeth rows near symphysis. Dentary with 25(1) unicuspid teeth on outer row, 4 rows near symphysis. Total vertebrae 35 (1 C&S ex.). Premaxillary and dentary outer row teeth slightly movable, inner ones fully depressible.

TABLE 3. Proportional measurements in percents of Standard length of the holotype and 21 paratypes of the new species *Crenicichla hu*. SD=standard deviation.

	Holotype	Range	Mean	SD
Standard length (mm)	118.0	76.9–153.0		
Head length	33.7	31.0–35.4	33.4	1.18
Snout length	12.5	10.7–13.4	12.0	0.75
Head depth	16.9	15.1–18.1	16.2	0.74
Body depth	25.3	21.5–25.6	23.2	1.24
Orbital diameter	5.8	5.0–7.3	6.2	0.54
Interorbital width	8.6	6.7–9.0	7.9	0.69
Pectoral fin length	19.1	19.1–23.1	20.5	0.92
Caudal peduncle depth	14.5	11.7–14.5	12.9	0.60
Caudal peduncle length	16.8	13.8–16.8	15.1	0.99

Coloration in alcohol. Background of body deeply dark, almost black in large specimens; smaller ones (75–95 mm) dark brown. Deep grey preorbital stripe between anterior margin of orbit to snout tip, only visible in smaller specimens. Postorbital stripe between posterior margin of orbit to preopercle distal margin, deep grey; only visible in smaller specimens. Suborbital stripe black almost reaching ventral margin of cheek; wide (up to six dots) and fragmented. Flank with 7 to 9 black irregular blotches just below upper lateral line and reaching faintly dorsal-fin base. Posteriormost blotch extending or not onto caudal peduncle. Dorsal, anal, and caudal fins dark grey or black, dorsal and anal fins with numerous dark scattered dots on their surface, also present in caudal fin in smaller specimens. Dorsal fin (females) with an irregular color pattern formed by black and white longitudinal stripes and blotches (3 ex., AI 261 and MACN-ict 9430; Fig. 4) or a black longitudinal stripe (sometimes reduced to a single blotch) with white margin (2 ex.*, MACN-ict 9429; Fig. 2). Caudal fin with a black subcircular spot well separated from base of fin, just above midline of caudal fin. Pectoral and pelvic fins smoky.

Coloration in live specimens. Same as color in alcohol (Figs. 3–4). Live specimens lack almost all carotenoid or physical reflective colors, the overall color is dark grey or dark brown to black. Some female specimens show a faint orange area behind the pectoral fin. Outline of the black areas in the dorsal fin of females milk-colored (Fig. 4).

FIGURE 4. *Crenicichla hu* female, live specimen, paratype, MACN-ict 9430, 122.0 mm.

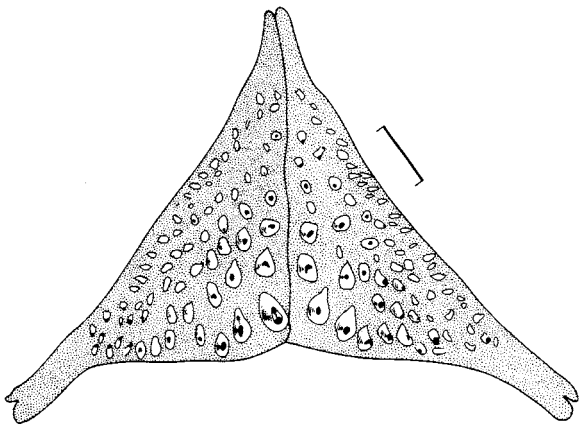

FIGURE 5. *Crenicichla hu*, lower pharyngeal tooth plate in occlusal view, AI 262, 93.9 mm SL. Scale bar: 1 mm.

Ecological notes. The arroyo Piray–Miní (the type and only-known locality) has clear and rapidly flowing water. The depth of the arroyo Piray–Miní is variable, 0.20 to 1.40 m. The bottom consists of mud, sand, and mostly stones. Some areas have scarce submerged vegetation (Figs. 6–7).

Etymology. The specific epithet hu is a Guaraní word *hû* that means black in allusion to the ground color of the body and fins.

Discussion. Molecular phylogeny divides the Misioneran crenicichlas into several clades (Fig.1). The basal-most species is *C. lepidota* (*C. saxatilis* group; Fig. 1; tree additionaly rooted with *Satanoperca jurupari* and *Astronotus ocellatus*; see Methods). The two philosophically distinct computing methods (MP, BA) inferred a robust phylogenetic hypothesis of nearly identical topologies that supports the biogeographic foundation of the recognized species groups. All species from the Misioneran part of the Paraná drainage basin (*C. hu*, *C. tesay*, *C. yaha*, and *C. mandelburgeri*) were grouped together in one clade with conclusively high support (Fig. 1; Paraná endemic species; bootstrap, 96; PP, 1.00). The newly described *Crenicichla hu* is recovered in a basal position of this clade. *Crenicichla mandelburgeri* appears non-monophyletic (see below).

The position of *C. vittata* inhabiting both the Paraná and the Uruguay basins differs between the BA and MP hypotheses, in the latter forming a monophyly together with the endemic Paraná species (Fig. 1; bootstrap, 66). The morphologically distinct *Crenicichla* species from the Uruguay basin formed two independent clades (Fig. 1; *C. missioneira* and *C. scottii* groups). Two species from the coastal rivers (*C. lacustris, C. punctata*) do not form a monophyletic lineage in either of the two phylogenies.

FIGURE 6. Arroyo Piray–Miní, the type locality of *Crenicichla hu*.

The phylogeny of a few species of *Crenicichla* from southern South America was recently studied by Kullander *et al.* (in press). That study supports virtually the same relationships between the above-mentioned clades. The endemic Paraná species group was represented in their analysis by only one taxon (*C. iguassuensis*) as were the coastal-river drainages (*C. punctata*). The Uruguay basin was represented by the *C. scottii* group (*C. scottii*) and the *C. missioneira* group (*C. missioneira, C. minuano, C. celidochilus, C. empheres, C. tendybaguassu*). There is thus no overlap with our taxon sampling of the endemic Paraná clade.

Evaluating the uncorrected pairwise divergences between the gene sequences of *Crenicichla hu* and the other species, the supposed higher evolutionary rate in the Geophagini tribe of cichlids must be taken in account (Farias *et al.* 1999, 2000; Pereyra & García 2008). Referring to ND2 sequences, the lowest divergence between a haplotype of *C. hu* and a haplotype of the nearest species (*C. mandelburgeri* C44) is 3.3% (Table 2). On the other hand, substantially lower values of divergences between formerly described species can be found (*e.g., C. missioneira* C36 vs. *C. minuano* C80, 0.6%; *C. tesay* C1 vs. *C. mandelburgeri* C51, 1.0%; *C. yaha* C5 vs. *C. mandelburgeri* C44, 1.0%). Despite the little-known divergence rate in Geophagini (López-Fernández *et al.* 2005), molecular divergences between the newly described species and its phylogenetic neighbours are substantial.

With the newly described *C. hu*, fourteen species of *Crenicichla* have now been recorded from the río Paraná basin. Some of them (i.e. *C. britskii, C. haroldoi*, and *C. jaguarensis*) are restricted to the Upper Paraná basin (Resende 2003; Reis *et al.* 2003). *Crenicichla jupiaensis* and *C. niederleinii* are found both in the Upper and Middle Paraná basin. Several species (i.e. *C. lepidota, C. mandelburgeri, C. scottii, C. semifasciata* and *C. vittata*) inhabit only the lower–middle part of the river; *C. vittata* occurs also in the Uruguay basin. *Crenicichla iguassuensis* and *C. tesay* are only present in the Iguazú basin. *Crenicichla yaha* is registered from the arroyo Urugua–í (Paraná basin) and Iguazú basin above the Cataratas del Iguazú.

The high diversity of ichthyofauna in the Argentinean province of Misiones has already been stressed. Three new *Crenicichla* species have recently been described from the río Paraná tributaries in Misiones (*C. yaha, C. tesay*, and *C. hu*), and several additional putatively new species are known to us from these tributaries (pers. obs).

We also confirm the presence of *Crenicichla mandelburgeri* in Misiones. Our material was compared with material from the type localities of *C. mandelburgeri* (both morphology and the ND2 gene sequences; not

shown). This taxon, however, demonstrates geographical variation as well as phylogeographic structure (Fig. 1), and we thus cannot rule out the presence of a species complex; the specimens from Piray-Guazu (haplotypes C15, C17) that infringe on the monophyly of this species are therefore referred to as *C. cf. mandelburgeri*.

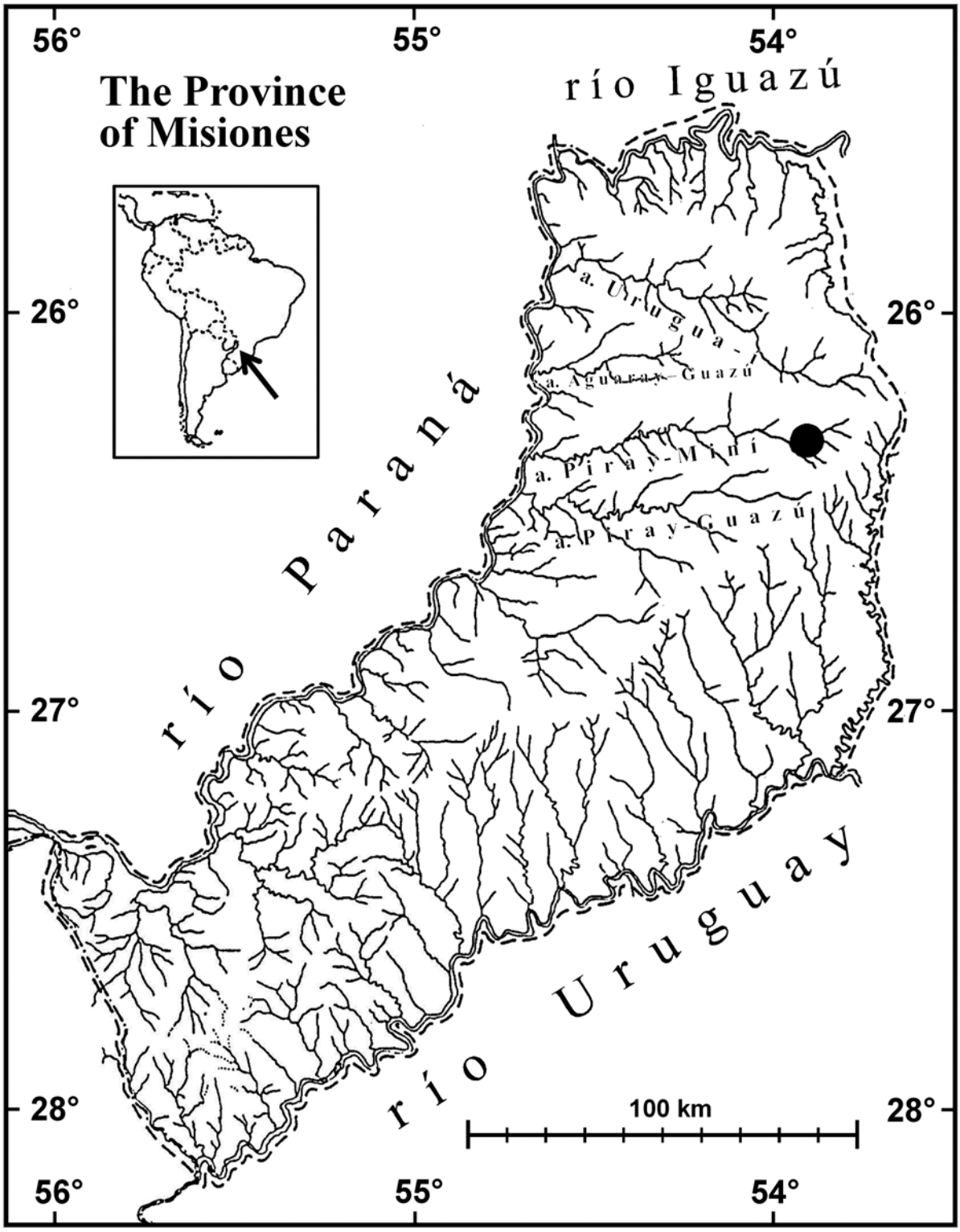

FIGURE 7. Hydrological map of the Province of Misiones. The type locality is marked with a solid circle.

Based on our field observations, *Crenicichla missioneira*, *C. minuano* and *C. gaucho*, described from the Middle río Uruguay in Rio Grande do Sul, Brazil and also cited by Lucena & Kullander (1992) from

Misiones, are quite common in the río Uruguay tributaries in Misiones. *Crenicichla hadrostigma*, described from the Upper río Uruguay in Santa Catarina, Brazil is known so far from one locality in Misiones (Lucena 2007), confirming the possibility of finding additional Upper Uruguay *Crenicichla* species in Misiones. On this note, Lucena & Kullander (1992) also cite *C. tendybaguassu* from Misiones.

So far there are no records in Misiones of four *Crenicichla* species which are known from the Upper río Uruguay basin in Brazil (*C. prenda*, *C. empheres*, *C. igara* and *C. jurubi*), whose boundary with the Middle río Uruguay is recognised as being at the Salto Moconá (Yucumã), just downstream from the río Pepirí–Guazú which forms the eastern border between Misiones, Argentina and Santa Catarina, Brazil (Zaniboni Filho & Schulz 2003). The ranges of these species could thus primarily be outside of Misiones as they are also not known from the Middle río Uruguay in Rio Grande do Sul, Brazil. However, the presence of these species in Misiones cannot be ruled out (see *C. hadrostigma* and *C. tendybaguassu* above).

Despite its small size, the province of Misiones shows biogeographic structuring which cannot be explained merely by diversity on a broader scale. Both the río Paraná and río Uruguay tributaries in Misiones are divided from the main rivers by waterfalls close to their mouths, but the northern tributaries of the Paraná in particular and the Iguazú itself in Misiones have a significant number of endemics which are so far not known outside of Misiones (i.e. Paraguay or Brazil). Among *Crenicichla* these are *C. tesay* from río Iguazú and *C. yaha* from río Iguazú and arroyo Urugua–í and *C. hu* from arroyo Piray–Miní (Fig. 7). Two putative new species are further known from the arroyo Urugua–í (pers. obs.), which is located between the río Iguazú and arroyo Piray–Miní. These three drainages together with arroyo Aguaray–Guazú form the northern part of Misiones. From the southeastern-most point of this part of Misiones starts the watershed between the río Paraná and río Uruguay. Tributaries of the río Paraná from here to the southeast (starting with arroyo Piray–Guazú, Fig. 7) have a diferent fauna of *Crenicichla* (dominated by *C. mandelburgeri*). A very similar pattern is also observed among *Australoheros* Říčan & Kullander, but with the exception that south from arroyo Piray–Miní there are so far no known species of *Australoheros* in the río Paraná tributaries in Misiones (*A. kaaygua* Casciotta *et al.*, *A. tembe* (Casciotta *et al.*) [and likely *A. guaraní* Říčan & Kullander] are known again only from the northern tributaries).

Comparative material. A list of comparative material of *C. scottii* and *C. vittata* is available in Casciotta (1987). In addition, the following material was studied: *Crenicichla hadrostigma*: Argentina. AI 220, 1 ex., 72.8 mm, Misiones, Itacaruare, río Uruguay basin. *Crenicichla iguassuensis*: Brasil. FMNH 54159 (holotype), 137 mm, Porto Uniao da Victoria, Rio Iguassu. *Crenicichla jupiaensis*: Argentina. AI 226, 2 ex., 87.7–93.0 mm, Corrientes, río Paraná at Yahapé. AI 227, 1 ex., 60.7 mm, Corrientes, río Paraná at Yahapé. *Crenicichla lepidota*: Argentina. MACN-ict 5067, 4 ex., 67.7–113.4 mm, Misiones, Represa Estación Experimental Cerro Azul. FML 00528, 1 ex., 111.5 mm, Salta, Luna Muerta, Hickman. MACN-ict 3656, 2 ex., 116.0–165.7 mm, Formosa, Riacho de Oro. MACN-ict 7275, 1 ex., 151.6 mm, Chaco, Esteros del Palmar. FML 00312, 1 ex., 138.0 mm, Corrientes, Isla Apipé Grande, Ituzaingó. MACN-ict 4091, 1 ex., 98.4 mm, Entre Ríos, río Uruguay, Concepción del Uruguay. MACN-ict 2314, 6 ex., 59.9–104.2 mm, Buenos Aires, Isla Martín García. Uruguay. MNHNM 2087, 1 ex., 72.9 mm, Departamento Colonia, arroyo Limetas. *Crenicichla cf. mandelburgeri*: MACN-ict 9439, 2 ex., 83.7–93.0 mm, Misiones, arroyo Guaruhape en ruta 220, río Paraná basin. MACN-ict 9440, 2 ex., 72.6–82.3 mm, Misiones, arroyo Cuñapirú, in route 223 near Ruiz de Montoya, río Paraná basin. MACN-ict 9441, 7 ex., 56.0–93.0 mm, Misiones, arroyo Cuñapirú (arroyo Tucangua), río Paraná basin. MACN-ict 9442, 2 ex., 102.2–208 mm, Misiones, arroyo Chapa, ruta 6, río Paraná basin. *Boggiana ocellata*: Paraguay. MSNG 33700 (holotype), 257.5 mm, Puerto 14 de Mayo, Bahía Negra, Chaco Boreal. *Crenicichla semifasciata*: Argentina. MACN-ict 3683, 1 ex., 68.8 mm, Formosa, Riacho de Oro. MACN-ict 6239, 1 ex., 176,6 mm, Entre Ríos, arroyo Curupí. *Crenicichla tesay*: MACN-ict 9016 (holotype), 115.1 mm, Argentina, Misiones, río Iguazú basin, arroyo Verde. *Crenicichla yaha*: Argentina, Misiones. MACN-ict 8924 (holotype), 103.7 mm, arroyo Urugua–í in Isla Palacios. AI 199, 1 ex., 116.6 mm, río Iguazú basin, arroyo Benavente. MTD-F 30606 (paratype), 1 ex., 105.9 mm, arroyo Urugua–í in ruta provincial 19, Parque Provincial Islas Malvinas. AI 200 (paratype), 1 ex., 135.8 mm SL, arroyo Uruzú (affluent of A. Urugua–í) in ruta provincial 19, Parque Provincial Islas Malvinas. AI 202 (paratypes), 4 ex., 1 (C&S) 37.4–48.5 mm, arroyo Urugua–í in Isla Palacios.

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Paper II

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Crenicichla ypo (Teleostei: Cichlidae), a new species from the middle Paraná basin in Misiones, Argentina

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A new species of *Crenicichla*, *C. ypo*, is described from the Arroyo Urugua-í, a left-hand tributary of the middle Paraná River, Misiones province, Argentina. The new species is recognized by 6 to 8 irregular blotches along the upper lateral line, absence of scattered dark spots on flanks, low number (47-55) of E1 scales, and a slightly prognathous lower jaw. Females have a distinctive coloration of the dorsal fin, with a wide black longitudinal stripe on the distal portion with an equally wide red stripe below it.

Una nueva especie de *Crenicichla*, *C. ypo*, es descripta de la cuenca del arroyo Urugua-í, tributaria de la margen izquierda del río Paraná medio, provincia de Misiones, Argentina. La nueva especie es reconocida por tener 6 a 8 manchas irregulares sobre la línea lateral superior, ausencia de pequeñas manchas oscuras dispersas sobre el flanco, bajo número (47-55) de escamas en la serie E1 y la quijada inferior levemente prognata. Las hembras tienen una coloración distintiva en la aleta dorsal con una banda ancha negra en la porción distal y otra roja del mismo ancho por debajo de esta.

Key words: Water dweller, Urugua-í basin, Taxonomy.

Introduction

The genus *Crenicichla* Heckel includes at present about 80 valid species and is the most speciose genus within the family Cichlidae (Kullander, 2003, 2009; Casciotta *et al*., 2006). Most *Crenicichla* species are found in tropical and subtropical cis-Andean drainages (Kullander & Lucena, 2006), although few of them, such as *Crenicichla lepidota* Heckel, *C. vittata* Heckel, and *C. scottii* (Eigenmann) also inhabit temperate waters in the La Plata River basin in Buenos Aires province and northern Patagonia in Argentina (Casciotta, 1987).

The Paraná River basin with 3,100,000 km^2 is the second largest basin of South America, and fourteen species of *Crenicichla* are known from that basin (Kullander, 2003, 2009; Casciotta *et al*., 2006; Casciotta & Almirón, 2008). Some of them, such as *C. haroldoi* Luengo & Britski, *C. jaguarensis* Haseman, and *C. britskii* Kullander are restricted to the upper Paraná basin. *Crenicichla jupiaensis* Britski & Luengo and *C. niederleinii* (Holmberg) also occur in the middle Paraná basin (Kullander, 2003; Casciotta *et al*., 2007), and *C. mandelburgeri* Kullander is endemic to the middle Paraná basin (Kullander, 1981, 2009; pers. obs.). *Crenicichla iguassuensis* Haseman and *C. tesay* Casciotta & Almirón are restricted to the Iguazú River above the Cataratas del Iguazú (Casciotta & Almirón, 2008). *Crenicicla yaha* Casciotta, Almirón & Gómez has an interesting distribution ocurring both in the Iguazú above the Cataratas del Iguazú and in the adjacent arroyo Urugua-í (middle Paraná basin). *Crenicichla semifasciata* (Heckel), *C. lepidota*, *C. scottii*, and *C. vittata* are found both in the lower and middle Paraná basin (*C. scottii* in lower only), and the last three species also are present in the Uruguay River (Casciotta, 1987; Lucena & Kullander, 1992).

The aim of this paper is to describe a new species of *Crenicichla* restricted to the arroyo Urugua-í, middle Paraná basin, Argentina.

Material and Methods

We use the following nomenclature in naming of drainages. River is used to designate large international drainages (*e.g*. Uruguay River), while arroyo ("stream" in Spanish) is used for smaller, exclusively Argentinean drainages (*e.g*. arroyo Urugua-í). This nomenclature bypasses the confusion between similar names of distinct drainages (*e.g*. Portuguese spelling of Uruguai for the Uruguay River *vs*. arroyo Urugua-í).

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Division of the Paraná River into sections differs substantially between various authors (*e.g*. Carolsfield *et al*., 2004; Iriondo *et al*., 2007). In this text we refer to the middle Paraná River as to the section from its confluence with the Paraguay River upstream to the Saltos del Guairá. Today this natural upper barrier of the middle Paraná is replaced by the Itaipu hydroelectrical dam.

Specimens were cleared and counterstained (c&s) following the method of Taylor & van Dyke (1985). Measurements and counts were taken as described by Kullander (1986). Pharyngeal teeth description and counts of frashed zone concavities follow Casciotta & Arratia (1993). Holotype values are indicated by an asterisk. Body length is expressed as standard length (SL). E1 scale counts refer to the scales in the row immediately above that containing the lower lateral line (Lucena & Kullander, 1992).

Institutional abbreviations are as listed in Ferraris (2007), except for AI (Asociación Ictiológica, La Plata, Argentina).

Crenicichla ypo, new species
Figs. 1-4

Holotype. MACN-ict 9431, 105.5 mm SL, Argentina, Misiones, Paraná basin, arroyo Urugua-í, at Establecimiento "Alto Paraná", approx. 25º57.9'S 54º06.5'W, Feb 1986, Gómez *et al*.

Paratypes. All from Argentina, Misiones, Paraná River basin. AI 212, 4, 102.0-130.0 mm SL, arroyo Falso Urugua-í, 25º58'26.2"S 54º15'28.5"W, Nov 2007, Casciotta *et al*. AI 263, 1 c&s, 95.3 mm SL, arroyo Urugua-í basin, arroyo Grapia, 6 km north from Colonia Gobernador J. J. Lanusse, approx. 25º52.2'S 54º10.4'W, Nov 1986, Gómez *et al*. MACN-ict 9432, 3, 101.0-116.0 mm SL, arroyo Urugua-í basin, arroyo Grapia, 6 km north from Colonia Gobernador J. J. Lanusse, approx. 25º52.2'S 54º10.4'W, Nov 1986, Gómez *et al.* MACN-ict 9433, 1, 133.0 mm SL, arroyo Uruzú at route 19, Parque Provincial Islas Malvinas, approx. 25º56.3'S 54º13.0'W, Sep 1986, Gómez *et al*. MACN-ict 9434, 1, 111.0 mm SL, arroyo Urugua-í and route 19, Parque Provincial Islas Malvinas, approx. 25º56.3'S 54º13.0'W, Feb 1986, Gómez *et al*. MACN-ict 9435, 1, 137.0 mm SL, arroyo Urugua-í and route 19, Parque Provincial Islas Malvinas, approx. 25º56.3'S 54º13.0'W, Sep 1986, Gómez *et al*. MACN-ict 9436, 1, 123.0 mm SL, arroyo Urugua-í in Isla Palacio, approx. 25º52.8'S 54º24.0'W, Feb 1986, Gómez *et al*. MACN-ict 9437, 1, 123.0 mm SL, same data as holotype. MACN-ict 9438, 3, 89.8-109.0 mm SL, arroyo Falso Urugua-í, 25º58'26.2"S 54º15'28.5"W, Nov 2007, Casciotta *et al*.

Diagnosis. The new species is recognized in the Paraná River basin by the following combination of characters: 6 to 8 irregular blotches along the upper lateral line, absence of scattered dark spots on flanks, low number (47-55) of E1 scales, and a slightly prognathous lower jaw. Females have a distinctive coloration of the dorsal fin, with a wide black longitudinal stripe on the distal portion with an equally wide red stripe below it.

Crenicichla ypo lacks the humeral spot present in *C. britskii* and *C. lepidota*. Lateral line scales in *C. ypo* are without brown dots such as are present on each scale in *C. haroldoi*. Numerous scattered dark spots on flanks are absent in *C. ypo* that distinguishes this species from *C. iguassuensis* and *C. tesay*. *Crenicichla ypo* has a distinct caudal spot, inconspicuous or absent in *C. jaguarensis*. *Crenicichla ypo* lacks the lateral stripe displayed in *C. jaguarensis*, *C. mandelburgeri*, and *C. vittata*.

Crenicichla ypo differs from *C. jupiaensis* in having lower jaw slightly prognathous, having a well-developed suborbital stripe composed of spots, and the cheek bearing up to 8 scale rows *vs*. isognathous jaws, a suborbital stripe reduced to a few spots posterior to the orbit, and a naked cheek.

Crenicichla ypo does not bear well developed vertical bars which are reduced to irregular blotches. This distinguishes this species from those with complete vertical bars: *C. jupiaensis*, juveniles of *C. mandelburgeri*, and *C. niederleinii*.

Crenicichla ypo differs from *C. niederleinii* and *C. vittata* in having a low number of E1 scales (47-55 *vs*. 56-65 and 78-85, respectively).

Parallel and thin longitudinal stripes are absent in *C. ypo vs*. present in *C. scottii*.

Crenicichla ypo is easily distinguished from *C. semifasciata* in having the ascending arm of the premaxilla longer than the dentigerous one, the blotches on flanks including the upper lateral line and extending 3 to 4 scale rows above and below it, and having about half of the caudal fin scaled *vs*. ascending arm of the premaxilla shorter than the dentigerous one, the flanks bearing quadrangular blotches placed below the upper lateral line or lateral band, and caudal fin scaled in most of its surface.

The new species differs from *C. yaha* in having the lower jaw slightly prognathous and head depth 14.5-17.6% of SL *vs*. isognathous or upper jaw slightly prognathous and head depth 17.9-20.8% of SL. Females of *C. ypo* are distinguished from females of *C. yaha* by having dorsal fin with a wide black stripe above a red stripe *vs*. dorsal fin with a wide black irregular stripe.

Description. Body elongate, depth 4.2 to 4.8 times in SL. Head as deep as wide or slightly deeper. Snout short, bluntly pointed in lateral view, 2.5 to 3.0 times in head length. Lower jaw slightly prognathous. Tip of maxilla reaching anterior margin of orbit in most specimens. Lower lip widely interrupted medially. Nostrils dorsolateral, close to anterior margin of orbit (12; MACN- ict 9431, 9432, 9434, 9435, 9437, 9438, AI 212, AI 263) or close to snout tip (5; MACN-ict 9432, 9433, 9436, AI 212). Posterior margin of preopercle serrated (12; MACN-ict 9431, 9432, 9433, 9434, 9435, 9437, 9438, AI 212, 263), or smooth on one or both sides (6; MACN-ict 9432 right side, MACN-ict 9436 both sides, MACN-ict 9438 left side, AI 212 right side); variation of the last two characters does not display any biogeographical pattern. Scales on flank strongly ctenoid. Head scales cycloid. Predorsal scales small, superficially embedded in skin. Prepelvic scales smaller than predorsal ones. Interopercle naked. Cheek scaled, 5 to 8 scales below eye embedded in skin. Scales in E1 row 47(2*), 48(1), 51(3), 53(5), 54(3), 55(3). Scales in transverse row 9/15(1), 10/14(1), 10/15(3), 10/16(3), 11/14(1), 11/15(6*), 11/16(2). Three scale rows between lateral lines. Upper lateral line scales 20(1), 21(2), 22(1), 23(4*), 24(1), 25(7), 27(1). Lower lateral line scales 1(1), 5(1), 9(1), 10(2), 11(6), 12(4*), 13(1), 14(1). Dorsal, anal, pectoral and pelvic fins naked. Dorsal fin XX,10(1), XXI,10(2), XXI,11(3),

Fig. 1. *Crenicichla ypo*, holotype, MACN-ict 9431, 105.5 mm SL. Argentina, Misiones, arroyo Urugua-í.

XXI,12(1), XXII,10(3), XXII,11(5*), XXII,12(1). Anal fin III,7(1), III,8(13*), III,9(3). Pectoral fin 15(1), 16(16*). Caudal-fin squamation not reaching the middle of fin. Soft-dorsal fin rounded or pointed, extending beyond caudal-fin base. Tip of anal fin usually not reaching caudal-fin base (reaching in 4; MACN-ict 9431, 9432, 9433, AI 212). Caudal fin rounded. Pectoral fin rounded, reaching the tip of pelvic fin. Microbranchiospines present on second through fourth gill arches. Gill rakers externally on first gill arch: 3 on epibranchial, 1 on angle, and 8 on ceratobranchial. Two to five patches of unicuspid teeth on fourth ceratobranchial. Lower pharyngeal tooth plate with unicuspid recurved and curved crenulated bicuspid teeth, those of posterior and medial row larger than remaining ones (Fig. 4). Upper pharyngeal tooth plate with unicuspid and bicuspid teeth. Frashed zone bearing one concavity with small unicuspid teeth. Premaxillary ascending process longer than dentigerous process. Premaxilla with 20(1) unicuspid teeth on outer row, larger than inner ones. Five tooth rows near symphysis. Dentary with 25(1) unicuspid teeth on outer row, four rows near symphysis. Total vertebrae: 37 (1 c&s). Premaxillary and dentary outer row teeth slightly movable, inner ones fully depressible.

Colour upon capture. Background colour of body grey. Deep grey preorbital stripe between anterior margin of orbit and snout tip, visible only in small specimens. Postorbital stripe between posterior margin of orbit and preopercle or opercle

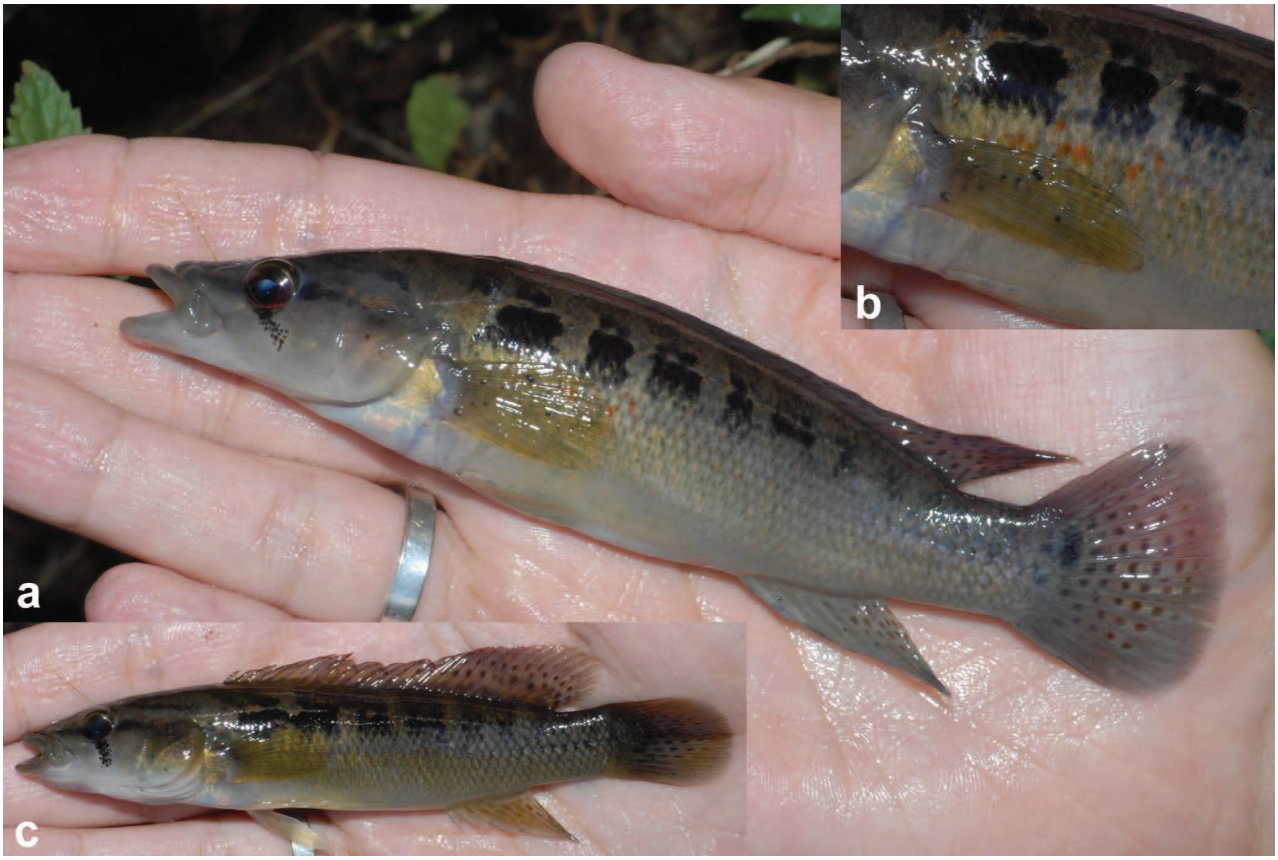

Fig. 2. *Crenicichla ypo*, AI 212, live male paratypes: **a**) 113.1 mm SL; **b**) detail of the orange dots on flank; and **c**) 102.7 mm SL, showing a spotted dorsal fin.

Fig. 3. *Crenicichla ypo*, MACN-ict 9438, 104.0 mm SL, female, paratype: **a**) a freshly collected specimen damaged from gillnets; **b**) detail of the dorsal fin showing the diagnostic black-red stripe pattern of females.

distal margin deep grey. Suborbital stripe black almost reaching ventral margin of cheek; wide and fragmented (up to eight dots wide). Flanks with 6 to 8 irregular black blotches below (up to four scales) and above (up to three scales) upper lateral line, marginally reaching dorsal-fin base. Posteriormost blotch not extending onto caudal peduncle. Dorsal, anal, and caudal fins pale grey, males with numerous dark scattered dots on dorsal, anal, and caudal fins, (Fig. 2) which are absent or rarely seen in females. Caudal fin with a black subcircular spot, in some specimens bearing an irregular white ring, just above of midline of caudal fin. Pectoral and pelvic fins pale grey. Some male specimens with several irregular orange dots on flank at level and behind pectoral fin (Fig. 2b).

Females with head, upper half of flank, and caudal fin deep grey. Yellow and orange pigment on flank at level and behind pectoral fin. Dorsal and anal fins of females lacking small dark dots, few of them present on caudal fin. Females with a distinctive coloration of the dorsal fin, with a wide black longitudinal stripe on the distal region of dorsal fin and an equally wide red stripe below it (Fig. 3).

Colour in alcohol. Similar to that of live specimens with a tendency to become pale. Conserved specimens lack the carotenoid pigments, such as orange dots on flank of males, yellow or orange area on flank of females, and wide red stripe in dorsal fin of females.

Distribution. *Crenicichla ypo* is found in the arroyo Urugua-í basin, middle Paraná River basin, Misiones province, Argentina (Figs. 5-6).

Etymology. The specific epithet *ypo*, is a Mbya Guaraní word *y po* that means water dweller.

Habitat. *Crenichla ypo* was collected both before and after the Urugua-í hydroelectrical dam was built in 1989 (see material);

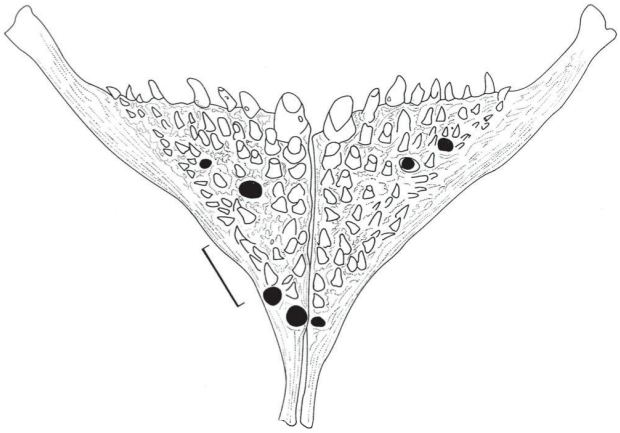

Fig. 4. *Crenicichla ypo*, AI 263, 95.3 mm SL, lower pharyngeal tooth plate in occlusal view. Scale bar = 1 mm.

Table 1. Proportional measurements in percents of standard length of holotype and 16 paratypes of *Crenicichla ypo*. SD = Standard deviation.

	Holotype	Range	Mean	SD
Standard length (mm)	105.5	89.8-137.0	-	-
Head length	34.6	32.0-34.6	33.2	0.76
Snout length	11.5	10.8-12.8	11.8	0.64
Head depth	15.6	14.5-17.6	16.0	0.97
Body depth	21.1	20.5-23.9	22.3	1.05
Orbital diameter	6.6	5.7-7.1	6.4	0.37
Interorbital width	6.8	6.2-7.9	7.1	0.50
Pectoral-fin length	19.7	18.7-22.0	20.1	0.84
Caudal-peduncle depth	12.3	10.9-12.9	12.1	0.60
Caudal-peduncle length	15.4	14.3-16.7	15.5	0.55

the species presently occurs also directly in the reservoir (pers. obs.). The arroyo Urugua-í is a moderately fast flowing river with tributaries of an average depth of 1 m outside of the dam influence. Macrophytes such as *Echinodorus uruguayensis* Arechavaleta and *Potamogeton pseudopolygonus* Hagström are present. The bottom consists of mud, sand with gravel and/or bedrock. After dam construction some parts of impoundment lake are up to 6 m deep and some previous localities like Isla Palacio are below the water surface. *Crenicichla ypo* is sympatric with *C. yaha* and one additional undetermined *Crenicichla* species (pers. obs.).

Discussion

The new species, *Crenicichla ypo*, is in its morphology more similar to other species of *Crenicichla* from the Paraná River basin, than to species from the Uruguay River (*C. celidochilus*, *C. empheres*, *C. gaucho*, *C. hadrostigma*, *C. igara*, *C. jurubi*, *C. minuano*, *C. missioneira*, *C. prenda*, *C. scottii*, *C. tendybaguassu*). These Uruguayan species are traditionally included in the *C. missioneira* and *C. scottii* species groups (Lucena & Kullander, 1992; extended by Kullander *et al*., 2010), and differ from the herein discussed taxa in a combination of color-pattern and meristic characters (Lucena & Kullander, 1992). The relationships of the new species with the Paraná River *Crenicichla* is also confirmed with analysis of molecular data (mitochondrial genes ND2 and cytochrome b) which included all at-present-known species from the Misiones province (Piálek *et al*., in prep.). Therefore a detailed morphological comparative analysis was directed towards the *Crenicichla* species from the Paraná River basin.

Crenicichla ypo inhabits the arroyo Urugua-í basin, a left-hand tributary of the middle Paraná River that drains roughly 3,000 km^2 of the northern part of Misiones. This river as well as most of the other tributaries of Paraná, Uruguay, and Iguazú basin in the central and northern portions of the province are divided from their main streams by a significant number of waterfalls, highest of which are invariably found closest to their mouths (pers. obs.). The isolation by high-level riverbed drops lasted apparently long enough so that many endemic taxa have evolved within these streams.

High level of endemism of the Misioneran ichthyological ecoregion (López *et al*., 2002; López *et al*., 2005) has been recently accentuated by a description of a number of endemic fish species, especially from the central and northern parts of the province, where endemism seems to be the highest (*e.g. Australoheros kaaygua* Casciotta *et al*., Iguazú; *Cnesterodon pirai* Aguilera *et al*., Cuñá-Pirú; *Crenicichla tesay*, Iguazú basin; *Crenicichla yaha*, arroyo Urugua-í and Iguazú; *Hisonotus hungy* Azpelicueta *et al*., Tirica, Paraná; *Rhamdella cainguae* Bockmann & Miquelarena, Cuñá-Pirú).

Crenicichla ypo is yet another faunal element of the unique hydrography of Misiones, an hyperdiverse area lying at the intersection of three major drainages (Paraná, Uruguay, and Iguazú).

Fig. 5. Arroyo Falso Urugua-í, one of the paratype localities of *Crenicichla ypo*.

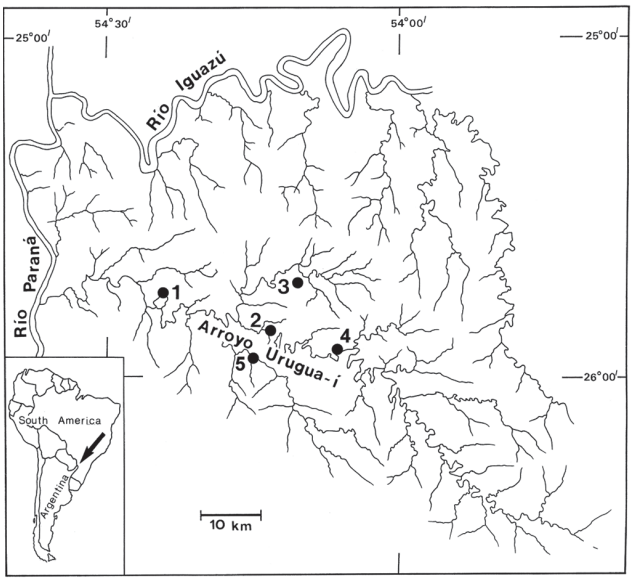

Fig. 6. Distribution of *Crenicichla ypo* in the Province of Misiones, Argentina. 1- Isla Palacio, 2- Parque provincial Isla Malvinas, 3- Arroyo Grapia and Arroyo Uruzú, 4- Establecimiento "Alto Paraná" (type-locality), and 5- Arroyo Falso Urugua-í.

Comparative material. A list of comparative material of *C. scottii* and *C. vittata* is available in Casciotta (1987). In addition, the following material was studied: *Crenicichla hadrostigma*, AI 220, 1, 72.8 mm SL, Argentina, Misiones, Uruguay River basin, Itacaruare. *Crenicichla iguassuensis*, FMNH 54159, holotype, 137 mm SL, Brazil, rio Iguaçu, Porto União da Victoria. *Crenicichla jupiaensis*: Argentina, Corrientes, Paraná River at Yahapé: AI 226, 2, 87.7-93.0 mm SL; AI 227, 1, 60.7 mm SL. *Crenicichla lepidota*: Argentina: Buenos Aires, Isla Martín García: MACN-ict 2314, 6, 59.9-104.2 mm SL. Chaco, Esteros del Palmar: MACN-ict 7275, 1, 151.6 mm SL. Corrientes, Isla Apipé Grande, Ituzaingó: FML 312, 1, 138.0 mm SL. Entre Ríos, Uruguay River, Concepción del Uruguay: MACN-ict 4091, 1, 98.4 mm SL. Formosa, Riacho de Oro: MACN-ict 3656, 2, 116.0-165.7 mm SL. Misiones, Represa Estación Experimental Cerro Azul: MACN-ict 5067, 4, 67.7-113.4 mm SL. Salta, Luna Muerta, Hickman: FML 528, 1, 111.5 mm SL. Uruguay: Departamento Colonia, arroyo Limetas: MNHNM 2087, 1, 72.9 mm SL. *Crenicichla* cf. *mandelburgeri*: Argentina: Misiones, Paraná River basin, arroyo Chapa at route 6: MACN-ict 9442, 2, 102.2-208 mm SL. Misiones, Paraná River basin, arroyo Cuñapirú, at route 223 near Ruiz de Montoya: MACN-ict 9440, 2, 72.6-82.3 mm SL. Misiones, Paraná River basin, arroyo Cuñapirú (arroyo Tucangua): MACN-ict 9441, 7, 56.0-93.0 mm SL. Misiones, Paraná River basin, arroyo Guaruhape at route 220: MACN-ict 9439, 2, 83.7-93.0 mm SL. *Crenicichla ocellata*, MSNG 33700, holotype, 257.5 mm SL, Paraguay, Puerto 14 de Mayo, Bahía Negra, Chaco Boreal. *Crenicichla semifasciata*: Argentina: Entre Ríos, arroyo Curupí: MACN-ict 6239, 1, 176,6 mm SL. Formosa, Riacho de Oro: MACN-ict 3683, 1, 68.8 mm SL. *Crenicichla tesay*, MACN-ict 9016, holotype, 115.1 mm SL, Argentina, Misiones, Iguazú River basin, arroyo Verde. *Crenicichla yaha*: Argentina: Misiones, Iguazú River basin, arroyo Benavente: AI 199, 1, 116.6 mm SL. Misiones, Paraná River basin, arroyo Urugua-í at Isla Palacio: MACN-Ict 8924, holotype, 103.7 mm SL. Misiones, Paraná River basin, arroyo Urugua-í at provincial route 19, Parque Provincial Islas Malvinas: MTD-F 30606, paratype, 105.9 mm SL. Misiones, Paraná River basin, arroyo Urugua-í at provincial route 19, arroyo Uruzú, Parque Provincial Islas Malvinas: AI 200, paratype, 135.8 mm SL. Misiones, Paraná River basin, arroyo Urugua-í at Isla Palacio: AI 202, paratypes, 4 (1 c&s), 37.4-48.5 mm SL.

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Article

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Two new species of *Australoheros* (Teleostei: Cichlidae), with notes on diversity of the genus and biogeography of the Río de la Plata basin

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Abstract

Two new species of *Australoheros* Říčan and Kullander are described. *Australoheros ykeregua* **sp. nov.** is described from the tributaries of the río Uruguay in Misiones province, Argentina. *Australoheros angiru* **sp. nov.** is described from the tributaries of the upper rio Uruguai and middle rio Iguaçu in Brazil. The two new species are not closely related, *A. ykeregua* is the sister species of *A. forquilha* Říčan and Kullander, while *A. angiru* is the sister species of *A. minuano* Říčan and Kullander. The diversity of the genus *Australoheros* is reviewed using morphological and molecular phylogenetic analyses. These analyses suggest that the described species diversity of the genus in the coastal drainages of SE Brazil is overestimated and that many described species are best undestood as representing cases of intraspecific variation. The distribution patterns of *Australoheros* species in the Uruguay and Iguazú river drainages point to historical connections between today isolated river drainages (the lower río Iguazú with the arroyo Urugua–í, and the middle rio Iguaçu with the upper rio Uruguai). Molecular clocks are used to date these and other biogeographic patterns.

Key words: *Australoheros*, new species, Cichlidae, phylogeny, South America, biogeography, Brazilian shield

Resumen

Dos nuevas especies de *Australoheros* Říčan y Kullander son descriptas. *Australoheros ykeregua* **sp. nov.** es descripta de tributarios del río Uruguay en la provincia de Misiones, Argentina. *Australoheros angiru* **sp. nov.** es descripta de tributarios del rio Uruguai superior y rio Iguaçu medio en Brasil. Las dos especies nuevas no se encuentran estrechamente relacionadas, *A. ykeregua* is la especie hermana de *A. forquilha* Říčan y Kullander, mientras que *A. angiru* es la especie hermana de *A. minuano* Říčan y Kullander. La diversidad del género *Australoheros* es revisada usando análisis filogenéticos morfológicos y moleculares. Estos análisis sugieren que la diversidad específica del género en las cuencas costeras del sudeste del Brasil se encuentra sobreestimada. Los patrones de distribución de las especies de *Australoheros* en las cuencas de los ríos Uruguay e Iguazú señalan una conexión histórica de cuencas que no se mantiene en la actualidad (río Iguazú inferior con el arroyo Urugua-í y rio Iguaçu medio con el rio Uruguai superior). Relojes moleculares son usados para datar estos y otros patrones biogeográficos.

Introduction

The genus *Australoheros* Říčan & Kullander with at present 20 valid species is rapidly becoming one of the most speciose genera of heroine cichlids. Twelve new species from the Atlantic coastal drainages of Brazil (Ottoni & Costa 2008; Ottoni *et al.* 2008; Ottoni & Cheffe 2009; Ottoni 2010), and seven new species from the Río de la Plata basin (Uruguay, Iguazú and Paraná river drainages) (Casciotta *et al.* 1995; Casciotta *et al.* 2006; Říčan & Kullander 2003, 2008) were described recently.

Říčan and Kullander (2006, 2008) have reviewed the species diversity of the genus *Australoheros* in the Río de la Plata basin. The authors reported a considerable diversity of this cichlid fish genus in this river drainage. Based

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on personal observation and also according to Ottoni and Costa (2008), Ottoni *et al.* (2008), Ottoni and Cheffe (2009) and Ottoni (2010), the *Australoheros* species from the rivers of the Atlantic coast of Brazil are rather similar to each other, with exception of *A. taura* Ottoni and Cheffe. The species from the Río de la Plata river drainages, on the other hand, show a wider spectrum of morphological and color pattern variation.

The highest diversity of *Australoheros* in the Río de la Plata basin is so far known from the río Uruguay drainage, which has four endemic species; *A. scitulus* (Říčan and Kullander), *A. charrua* Říčan and Kullander, *A. forquilha* Říčan and Kullander, *A. minuano* Říčan and Kullander. The río Paraná drainage has two endemic species (*A. guarani* Říčan and Kullander, *A. tembe* [Casciotta *et al.*]). Only two species are (in the Río de la Plata basin) presently known to occur in two separate river drainages (*A. facetus* [Jenyns], *A. kaaygua* Casciotta *et al.*).

New data have recently become available and demonstrate that the diversity described above is still underestimated, since *A. kaaygua* and *A. forquilha* as presently understood hide considerable variation, which better corresponds to four rather than two species. The aim of this paper is to describe this variation and to demonstrate that the species of *Australoheros* from the Río de la Plata basin reveal some interesting biogeographic patterns.

Material and methods

River names terminology. Rivers flowing through both Spanish and Portuguese speaking countries (*e.g.* Argentina *vs.* Brazil) usually vary in their names. Typical examples in our case are the río Iguazú (in Argentina), but rio Iguaçu (in Brazil), or the río Uruguay (in Argentina and Uruguay), but rio Uruguai in Brazil. We keep this difference in names throughout the text because it helps in pointing out which part of the river in which country we mean without the necessity to repeat the name of the country. If the river drainage is meant in general, the Spanish version is used. The rio Uruguai (Brazil) is not to be confused with the arroyo Urugua–í, which is a tributary of the río Paraná in Misiones, Argentina.

Morphological methods. In this work, we use character-based and tree-based approaches to analyze morphological characters as two tests of species delimitation.

Character-based delimitation. Character-based species delimitation involves finding diagnostic character states that represent seemingly fixed differences between the putative species, or differences that are at least non overlapping (*e.g.* Říčan & Kullander 2006). This approach is useful but lacks the clear relationship to estimated patterns of gene flow that the phylogenetic component of the tree-based approach offers.

Tree-based delimitation. Tree-based delimitation with morphology, although advocated by some authors (*e.g.* Baum & Donoghue 1995), has rarely been used by empirical systematists (*e.g.* Hollingsworth 1998; Wiens & Penkrot 2002; Říčan & Kullander 2006, 2008). The tree-based approach provides the parsimonious solution of character distribution, a homology hypothesis, and presents monophyletic groups, which are compared with results of the character-based approach. This two-step system, combining character- and tree-based approaches, has multiple advantages over a single step system (see Říčan and Kullander, 2006, 2008).
We complement our tree-based morphological delimitation with molecular data.

Characters. Measurements and counts were taken as described by Kullander (1986). Measurements were taken with digital calipers to 0.1 mm and are made point to point except for head length and snout length, which are projections from the anterior tip of the premaxilla to the orbital margin and the posterior margin of the gill cover, respectively. Scale rows are numbered as described by Kullander (1990), *i.e.* the horizontal row including the lower lateral line is designated as row E0, and the rows are counted as E1, E2 *etc.* dorsally, and H1, H2 *etc.* ventrally. Dorsal and anal fin rays, pterygiophores and vertebrae were counted on X-radiographs. Vertebral counts include the last halfcentrum. Color marking terminology follows Kullander (1983, 1986) and Říčan *et al.* (2005). Bars are counted and numbered in postero-anterior succession (Kullander 1983; Kullander & Silfvergrip 1991; Říčan *et al.* 2005). In the Description sections the number of specimens is indicated in parentheses, values of the holotype are indicated by an asterisk. Body length is expressed as standard length (SL).

Institutional abbreviations are as listed in Leviton *et al.* (1985) and Leviton and Gibbs (1988), except for AI (Asociación Ictiológica, La Plata, Argentina) and MACN-ict (Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Argentina).

Characters used in the present study include the following (plus color pattern) characters (HL: head length; SL: standard length): HL/SL, snout L/HL, body depth/SL, orbital diameter/HL, head width/HL, interorbital dist./HL,

preorbital dist./HL, caudal peduncle L/caudal peduncle depth, pectoral fin L/SL, ventral fin L/SL, last dorsal fin spine L/SL, and the following counts: scale counts (E0, L1, L2, scales between anterior insertion of the dorsal fin and the upper lateral line, scales between the posterior end of the upper lateral line and the dorsal fin, cheek scale rows), first ceratobranchial gill-rakers, caudal vertebrae, caudal peduncle vertebrae, anal pterygiophores anteriorly from the first haemal spine, anal-fin spines, anal-fin rays, anal-fin total, dorsal-fin spines, dorsal-fin rays, dorsal-fin total, pectoral-fin rays.

Molecular characters include the mitochondrial cytochrome *b* gene.

Molecular methods. Sequences of the mitochondrial cytochrome *b* gene from 38 specimens representing eight *Australoheros* species (and three outgroup taxa) make up our molecular data set (Table 1). New sequences have been deposited in GenBank under the following accession numbers: HQ197686–HQ197712.

DNA was extracted from small pieces of muscle or gill (10 to 25 mg) using the DNeasy™ Tissue Kit (Qiagen). The entire cytochrome *b* gene (1.3 kb) was PCR amplified with primers GLuDGL-TGA CTT GAA RAA CCA YCG TTG (Palumbi *et al.* 1991) and H15915-AAC TGC AGT CAT CTC CGG GTT ACA AGA C (Irwin *et al.* 1991). PCR reactions were carried out with initial denaturation at 94°C for 5 min, followed by 30 cycles with denaturation at 94°C for 1 min, primer annealing at 45 to 50°C for 40 s and primer extension at 72°C for 1 min. PCR was finished by final extension at 72°C for 5 min. PCR products were purified by ethanol precipitation or using Microcon PCR Filter Units (Millipore) and directly sequenced on an automated DNA sequencer using BigDye™ Terminator Cycle Sequencing Kit v.3.1 (PE Applied Biosystems). Sequencing reaction products were cleaned by ethanol precipitation or with DyeEx 2.0 Spin Kit (QIAGEN) and then resolved on ABI Prism 310 Genetic Analyser (Perkin Elmer). Except the amplification primers, the following additional primers were used for sequencing: modified L14952 of Lydeard *et al.* (1995; TCA TCC GTC GCC CAC AT), modified L15162 of Taberlet *et al.* (1992; CCA TGA GGA CAA ATA TC), and L15299 (Lydeard & Roe 1997). Chromatograms were assembled and checked by eye for potential mistakes using SEQMAN II of the DNASTAR software package (http://www.dnastar.com). Edited sequences were aligned using the default settings in ClustalX software (Thompson *et al.* 1997). The alignment was manually revised in BIOEDIT (Biological sequence alignment editor v5.0.9, http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The alignment includes no gaps.

Phylogenetic analyses. The morphological data set is coded with populations as terminal units (PTU) to enable tree-based species delimitation. The morphological matrix inludes 39 characters, of which 26 are multistate and 20 are ordered. See Appendices 1 and 2 for details. Morphological data for the Atlantic coast species of Brazil are taken from the respective species descriptions.

Qualitative characters were coded using the majority approach. Some characters, such as the number of abdominal bars have been coded using the scaled coding (Campbell & Frost 1993). The states are ordered under the assumption that traits pass through a polymorphic stage between absence and fixed presence. The scaled method is advantageous in that it allows polymorphisms to act as synapomorphies.

Quantitative characters have been coded using the gap weighting method (GW) of Thiele (1993). Thiele's implementation of gap weighting involves finding (for a given character) the mean value of the trait in each species in the analysis, the range of mean species values among taxa (*i.e.* the species with the greatest mean value and the species with the lowest), and then dividing this range into smaller ranges or segments equal to the maximum number of character states allowed by the phylogenetic software program (*e.g.* 32 for PAUP*; Swofford 2001). We have used a less fine grained spacing, thus having in most cases less than 32 states. Species are then assigned states based on these ranges, and the character is ordered. Evolving from low to high mean trait values (or vice versa) therefore requires passing through many intermediate states and requires many steps, whereas smaller changes in trait values involve fewer state changes and fewer steps. An important advantage of the gap-weighting method is that it incorporates information on the distance between states, weighting the changes according to the difference between mean species values.

We have used the between-state scaling (Wiens 2001) to weight quantitative characters against qualitative characters. This weighting scheme assigns transformations between species with fixed, adjacent values of meristic variables (*e.g.* 13 to 14 vertebrae) the same weight as changes in binary variables (0 to 1), and species with intermediate mean values (*e.g.* 13.5) receive proportionally intermediate weights. The consistency index is reported with uninformative characters excluded.

The phylogenetic analyses were performed using PAUP* 4b.10 (Swofford 2001) with maximum parsimony (MP). Analyses included 500 random sequence additions, 10 trees kept per addition, and a hs (heuristic) search on

TABLE 1. Locality data for DNA samples. Accession numbers highlighted in bold indicate new sequences generated for this study.

Species	DNA label	Locality	Drainage	GPS	GenBank
A. angiru		Brazil, Santa Catarina	rio Iguaçu		AY998658
A. facetus	A24	Paraguay, Itapua, P09-03	río Paraná	27°05'26.16"S, 55°53'13.02"W	**HQ197709**
A. facetus	A25	Paraguay, Itapua, P09-04	río Paraná	27°05'26.16"S, 55°53'13.02"W	**HQ197710**
A. facetus	A26	Argentina, Corrientes, Laguna Iberá	río Paraná	28°32'47.28"S, 57°11'44.70"W	**HQ197711**
A. facetus	A27	Argentina, Corrientes, Laguna Iberá	río Paraná	28°32'47.28"S, 57°11'44.70"W	**HQ197712**
A. facetus	H18	Argentina, Catamarca	río Paraná	28°28'08.37"S, 65°46'44.30"W	**HQ197703**
A. facetus	H19	Argentina, Catamarca	río Paraná	28°28'08.37"S, 65°46'44.30"W	**HQ197704**
A. facetus		Argentina, Entre Ríos	río Uruguay		AY843387
A. facetus		Argentina, Entre Ríos	río Uruguay		AY998665
A. facetus		Argentina, Entre Ríos	río Uruguay		AY998667
A. facetus		Uruguay, Maldonado	Río de la Plata		AY998666
A. forquilha	A22	Brazil, Rio Grande do Sul, B902, río forquilha	rio uruguai	27°37'26.34"S, 51°45'00.12"W	**HQ197707**
A. forquilha	A23	Brazil, Rio Grande do Sul, B902, río forquilha	rio uruguai	27°37'26.34"S, 51°45'00.12"W	**HQ197708**
A. kaaygua	H1	A07-02, arroyo Lobo	río Iguazú	25°42'34.79"S, 54°05'39.42"W	**HQ197686**
A. minuano		Uruguay, Salto	río Uruguay		AY998659
A. scitulus	A20	Argentina, Misiones, arroyo Itacaruare, A09-01	río Uruguay	27°52'33.80"S, 55°16'35.07"W	**HQ197705**
A. scitulus	A21	Argentina, Misiones, arroyo Itacaruare, A09-02	río Uruguay	27°52'33.80"S, 55°16'35.07"W	**HQ197706**
A. scitulus	H16	Argentina, Corrientes, A07-23	río Uruguay	29°36'49.60"S, 58°07'6.12"W	**HQ197701**
A. scitulus	H17	Argentina, Corrientes, A07-24	río Uruguay	29°36'49.60"S, 58°07'6.12"W	**HQ197702**
A. scitulus		Uruguay, Colonia	Río de la Plata	34°19'07" S, 59°20'13" W	AY998662

continued next page

TABLE 1. (continued)

Species	DNA label	Locality	Drainage	GPS	GenBank
A. scitulus		Uruguay, Colonia	Río de la Plata	34°19'07" S, 59°20'13" W	AY998661
A. scitulus		Argentina, Entre Ríos	Río Uruguay	31°53'55" S, 58°19'55" W	AY998663
A. tembe	H2	Argentina, Misiones, A07-04, arroyo Falso Urugua–í	arroyo Urugua–í, río Paraná	25°58'26.20"S, 54°15'28.78"W	**HQ197687**
A. tembe	H3	Argentina, Misiones, A07-04, arroyo Falso Urugua–í	arroyo Urugua–í, río Paraná	25°58'26.20"S, 54°15'28.78"W	**HQ197688**
A. tembe		Argentina, Misiones, arroyo Tirica	arroyo Urugua–í, río Paraná		AY998660
A. tembe		Argentina, Misiones	arroyo Urugua–í, río Paraná		AY843373
A. ykeregua	H4	Argentina, Misiones, A07-08, arroyo Fortaleza	río Uruguay	26°45'56.63"S, 54°10'57.43"W	**HQ197689**
A. ykeregua	H5	Argentina, Misiones, A07-08, arroyo Fortaleza	río Uruguay	26°45'56.63"S, 54°10'57.43"W	**HQ197690**
A. ykeregua	H6	Argentina, Misiones, A07-10A, arroyo Paraiso	río Uruguay	27°14'15.07"S, 54°02'38.49"W	**HQ197691**
A. ykeregua	H7	Argentina, Misiones, A07-10A, arroyo Paraiso	río Uruguay	27°14'15.07"S, 54°02'38.49"W	**HQ197692**
A. ykeregua	H8	Argentina, Misiones, A07-10B, arroyo Paraiso	río Uruguay	27°14'15.07"S, 54°02'38.49"W	**HQ197693**
A. ykeregua	H9	Argentina, Misiones, A07-10B, arroyo Paraiso	río Uruguay	27°14'15.07"S, 54°02'38.49"W	**HQ197694**
A. ykeregua	H10	Argentina, Misiones, A07-11, arroyo Shangai	río Uruguay	27°28'13.83"S, 54°41'24.52"W	**HQ197695**
A. ykeregua	H11	Argentina, Misiones, A07-11, arroyo Shangai	río Uruguay	27°28'13.83"S, 54°41'24.52"W	**HQ197696**
A. ykeregua	H12	Argentina, Misiones, A07-12, arroyo Guerrero	río Uruguay	27°45'57.45"S, 55°09'33.75"W	**HQ197697**
A. ykeregua	H13	Argentina, Misiones, A07-12, arroyo Guerrero	río Uruguay	27°45'57.45"S, 55°09'33.75"W	**HQ197698**
A. ykeregua	H14	Argentina, Misiones, A07-13, arroyo Tamandua	río Uruguay	27°05'56.53"S, 54°45'48.89"W	**HQ197699**
A. ykeregua	H15	Argentina, Misiones, A07-13, arroyo Tamandua	río Uruguay	27°05'56.53"S, 54°45'48.89"W	**HQ197700**

the saved trees to find all the shortest trees. Bootstrap analyses were done using the same approach, with 5 random sequence additions per one bootstrap. Bootstrap analyses were run with 1000 replications.

Characters have been mapped onto phylogeny using the software package Mesquite (Maddison and Maddison 2004).

Since the sister group of *Australoheros* is not established (Říčan *et al.* 2008), we have used a composite outgroup based on a reconstructed ancestor of the CAM heroine cichlids (Říčan *et al.* 2008).

Molecular data set. The molecular cytochrome *b* matrix was analyzed using MP in PAUP* 4b.10 with the same settings as the morphological data set and with Bayesian inference (BI) using MrBayes version 3.01 (Huelsenbeck & Ronquist 2001). The evolutionary model that best fits the analyzed sequence data set was selected using Modeltest and the Akaike information criterium (Posada & Crandall 1998). The Bayesian tree was inferred using the selected GTR+I+G model with partitioning by codon, with two MCMC chains for 5 million generations, sampling each hundredth tree, and discarding first 25% trees as burn-in. Statistical support for recovered clades was assessed using posterior probabilities (BI) and bootstrap (MP).

All molecular divergences mentioned in this text are uncorrected pairwise divergences reported by PAUP* with the use of the command 'showdist'.

Results

Tree-based delimitation. The phylogenetic analysis of the morphological matrix of 39 characters (Appendices 1 and 2) resulted into two MP trees (L=693; CI=0.51; RI=0.66) (Fig. 1). The two trees differ only in the internal topology of *A. angiru*. *Australoheros ykeregua* is found as the sister group of *A. forquilha*. *Australoheros kaaygua* and *A. angiru* are not conspecific, and not even sister groups. The validity of all species, including *A. ykeregua* and *A. angiru*, are supported by this morphological tree-based delimitation.

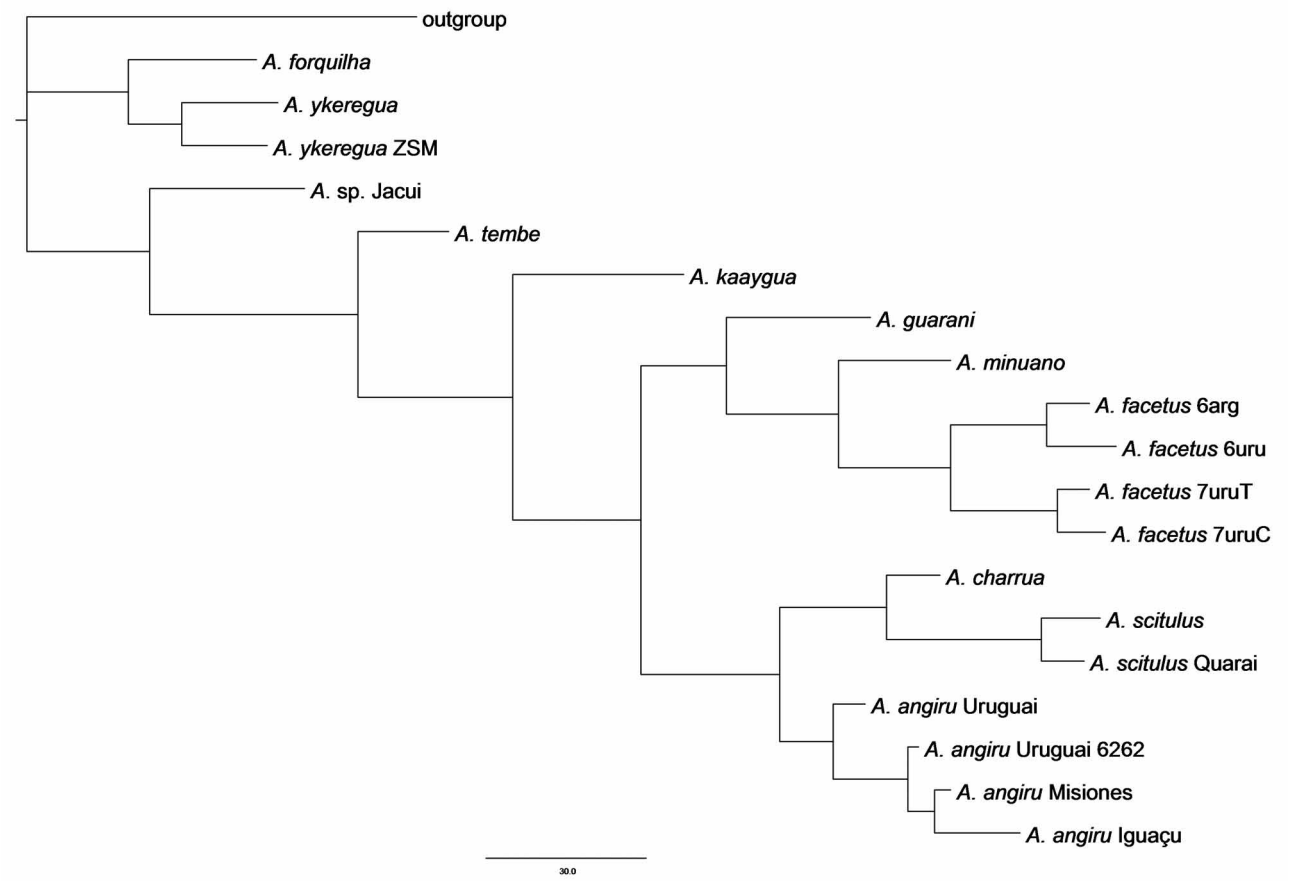

FIGURE 1. Tree-based delimitation using MP phylogenetic analysis of morphological data. The tree shown is one of two MP trees (L= 693; N=2; CI=0.51; RI=0.66), which differ only in the internal topology of *A. angiru*. Branch lengths represent morphological divergences.

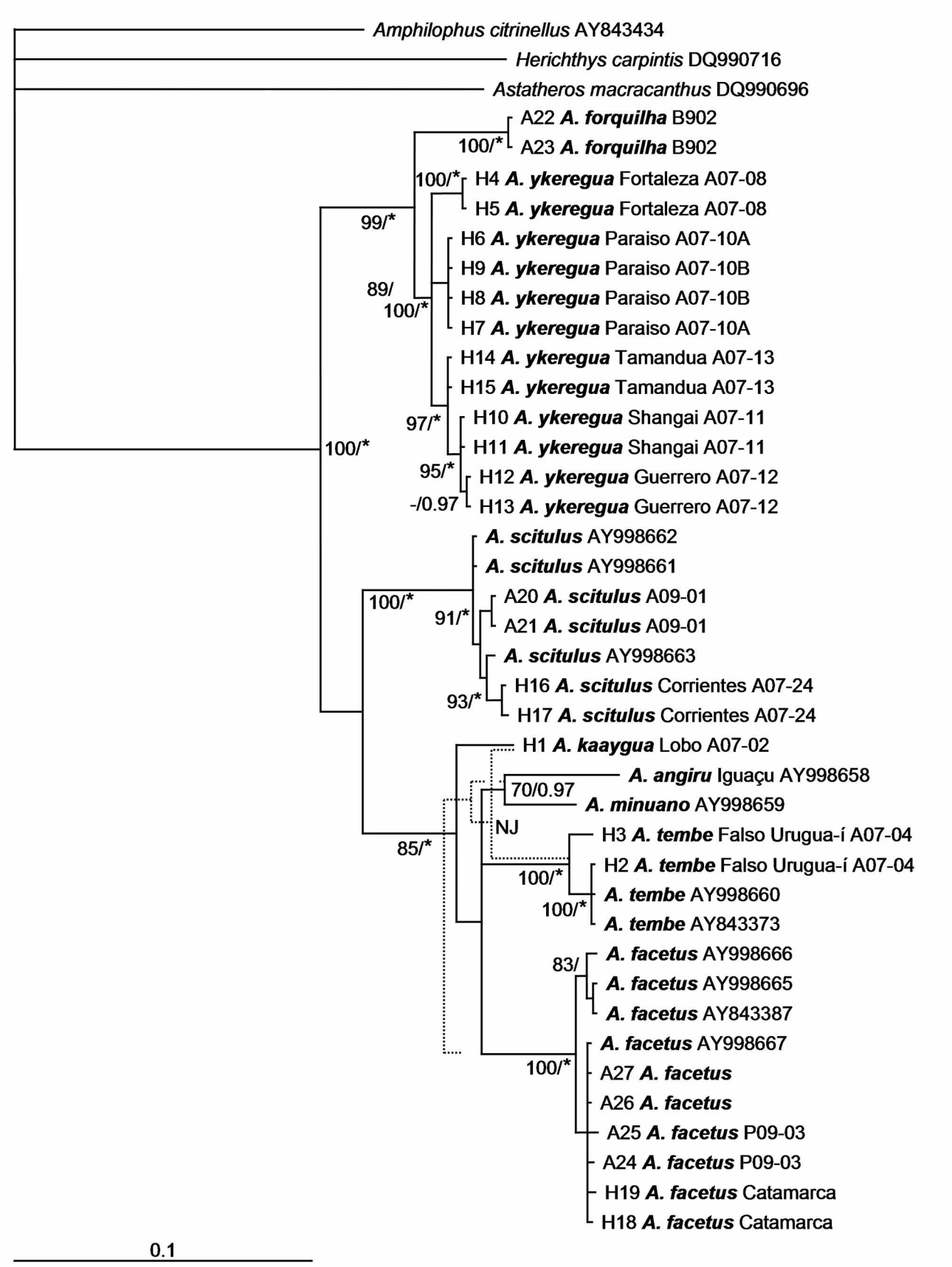

FIGURE 2 . Molecular phylogeny of the Río de la Plata basin *Australoheros* species using BI. Node support values shown for MP/BI analyses. The alternative dotted topology represents neighbor-joining (NJ) analysis. Asterisk denotes posterior probability of 1.00.

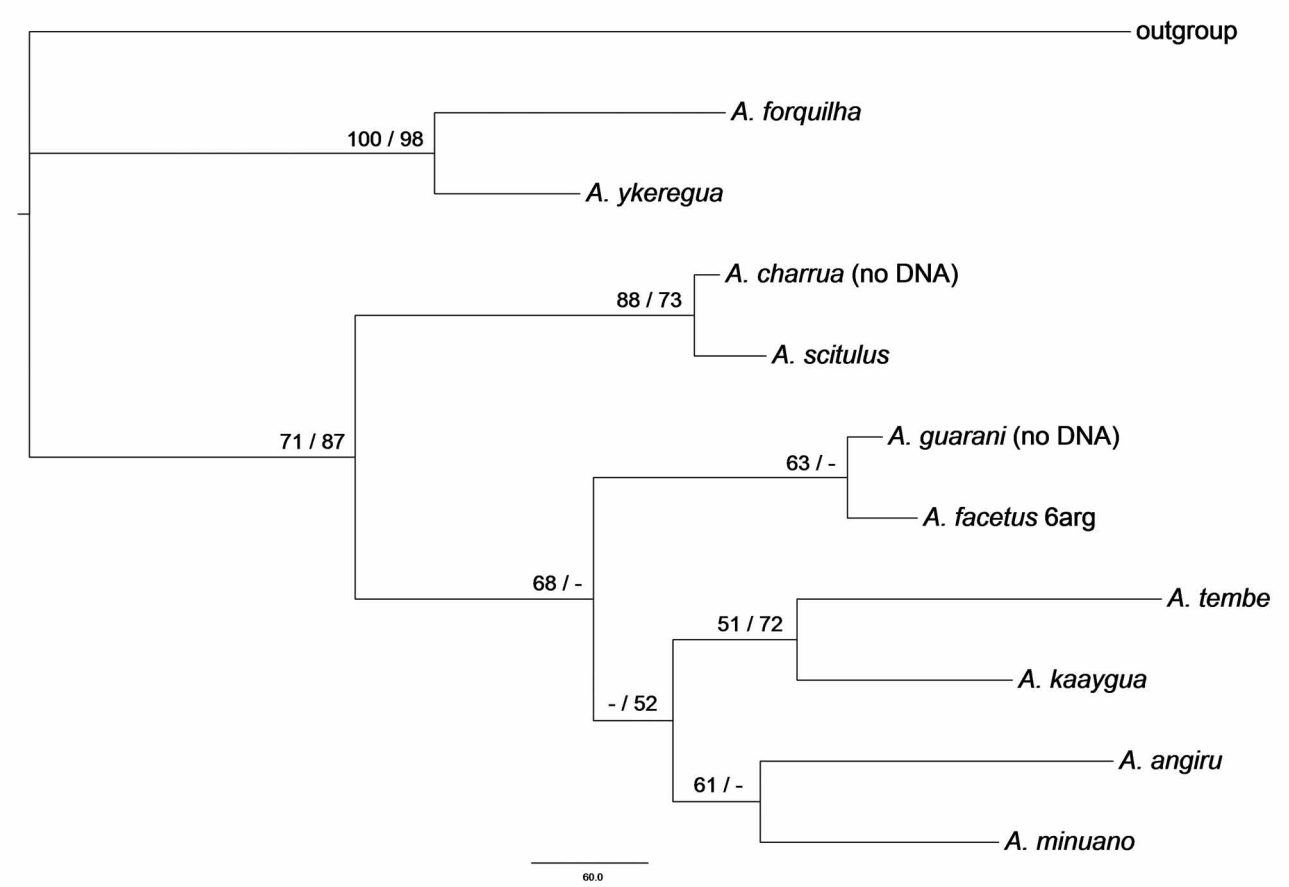

FIGURE 3. Combined MP morphological-molecular phylogeny with between-state scaling internal weighting between morphological and molecular data (L=2457; N=1; CI=0.58; RI=0.49). Node support values show MP bootstrap for two types of analyses (left: between-state scaling internal weighting structure / right: all characters weighted equally).

The phylogenetic analysis of the molecular cyt*b* matrix is shown in Fig. 2. The results are similar to those from the morphological analysis, with *A. ykeregua* and *A. forquilha* as sister groups, and *A. kaaygua* and *A. angiru* as not conspecific and not immediately related. The validity of all species is again supported.

The combined morphological-molecular phylogenetic analysis (Fig. 3) supports the results of the independent analyses (Figs 1 and 2). *Australoheros ykeregua* and *A. forquilha* are sister groups, with a mean divergence of 2.3% in the cyt*b* gene. *Australoheros kaaygua* and *A. angiru* are not conspecific, separated by a divergence of 4.8% in the cyt*b* gene. *Australoheros angiru* is the sister species of *A. minuano* (cyt*b* divergence of 4.2%). *Australoheros kaaygua* is the sister group of *A. tembe* (mean cyt*b* divergence of 3.8%). *Australoheros guarani* and *A. facetus*, and *A. scitulus* and *A. charrua* are additional sister groups (DNA data not available for *A. guarani* and *A. charrua*).

Our tree-based delimitation analyses thus support the distinctivness of *A. ykeregua* from *A. forquilha*, and of *A. angiru* from *A. kaaygua*.

Character-based delimitation. Character based delimitation, in agreement with tree-based delimitation, supports the distinctivness of *A. ykeregua* from *A. forquilha*, and of *A. angiru* from *A. kaaygua* (Tables 2 and 3). For separating characters see the taxonomy section below.

TABLE 2. Meristics of the two new species (*A. ykeregua*, *A. angiru*) and the two species with which they have been previously associated (*A. forquilha*, *A. kaaygua*).

Dorsal fin count frequences		XIV 12	XV 8	XV 9	XV 10	XV 11	XVI 7	XVI 8	XVI 9	XVI 10	XVI 11	XVII 7	XVII 8	XVII 9	XVII 10
A. ykeregua					3	3			3	16	22			1	2
A. forquilha						1				5	5				
A. angiru	upper rio Uruguai								16	13			2		
	rio Iguaçu								3	1					
A. kaaygua			1	2			1	8	1					1	

Anal fin count frequences		V 7	V 8	V 9	VI 6	VI 7	VI 8	VI 9	VII 7	VII 8	VII 9	VIII 6	VIII 7	VIII 8
A. ykeregua			3	1		14	29	3						
A. forquilha						1	7	1		2				
A. angiru	upper rio Uruguai					2	3		17	8		1		
	rio Iguaçu								1	3				
A. kaaygua		2			7	5								

		vertebrae 13 12	13 13	13 14	13 15	14 12	14 13	14 14	pectoral fin rays 12	13	14	15	C1 gill rakers 5	6	7	8	9
A. ykeregua			3	19			3			14	18	15			3	13	4
A. forquilha				5	6					6	3				4	5	
A. angiru	upper rio Uruguai		29	2					11	5			4	11	1		
	rio Iguaçu	1	3							4			1	2	1		
A. kaaygua			1						9	5				1			

		caudal peduncle vertebrae -2	-1.5	-1	-0.5	0	0.5	1	1.5	2	2.5	3	3.5
A. ykeregua								1	5	11	6	1	
A. forquilha										3	3	5	
A. angiru	upper rio Uruguai			3	1	4	3	10	1				
	rio Iguaçu		2	2									

		anal pterygiophores 11 1	11 2	12 1	12 2	13 1	13 2	13 3	14 1	14 2	14 3	15 2	dorsal pterygiophores 9	10	11	12
A. ykeregua		10	5	3	4									6	16	
A. forquilha			1	6	2	2									5	6
A. angiru	upper rio Uruguai	1	1	7	8	3	3						1	13	9	
	rio Iguaçu			1		1	2							3	1	
A. kaaygua				1												

		E0 scale counts 23	24	25	26	L1 scale counts 13	14	15	16	17	18	19	L2 scale counts 6	7	8	9	10	11
A. ykeregua			1	32	13		1	1	5	15	19	5		1	4	26	10	5
A. forquilha					9					2	5	2			4	3	2	
A. angiru	upper rio Uruguai	3	16	4					1	6	8			4	7	4		
	rio Iguaçu																	

Taxonomy

Australoheros ykeregua sp. nov.

(Figs. 4, 5, 6, 7).

"Cichlasoma" cf. *tembe* (arroyo Fortaleza)—Casciotta et *al.* 2003: 68, 70
"Cichlasoma" cf. *tembe*—Stawikowski and Werner 2004: 455
Australoheros sp. Forquilha—Říčan and Kullander 2006: 6
Australoheros forquilha (non-type material from ZSM)—Říčan and Kullander 2008: 16

TABLE 3. Proportional measurements in percents of standard length (SL) of the two new species (*A. ykeregua, A. angiru*) and the two species with which they have been previously associated (*A. forquilha, A. kaaygua*). SD=standard deviation.

		A. forquilha			*A. ykeregua*	
	N	Min-Max	Mean ± SD	N	Min-Max	Mean ± SD
Head length	10	31.5 – 34.6	33.2 ± 1.2	49	33.2 – 39.1	36.2 ± 1.2
Snout length	10	7.6 – 12.6	10.5 ± 1.6	49	8.8. – 18.4	14.9 ± 2.3
Body depth	10	40.9 – 46.6	43.9 ± 1.9	49	41.7 – 47.8	44.9 ± 1.5
Orbital diameter	10	9.3 – 12.6	11.3 ± 0.8	49	8.1 – 13.8	10.5 ± 1.4
Head width	10	15.6 – 18.0	16.5 ± 0.7	49	16.0 – 19.1	17.6 ± 0.6
Interorbital width	10	8.7 – 11.5	10.1 ± 0.9	49	8.7 – 14.3	10.9 ± 1.6
Preorbital distance	10	6.4 – 10.8	9.1 ± 1.4	49	6.4 – 12.3	9.3 ± 1.3
Caudal peduncle depth	10	16.6 – 18.3	17.4 ± 0.5	49	15.6 – 18.8	17.2 ± 0.7
Caudal peduncle length	10	8.9 – 11.1	10.2 ± 0.7	49	8.4 – 13.9	10.9 ± 1.3
Pectoral fin length	10	25.6 – 29.5	26.9 ± 1.2	49	25.9 – 32.5	29.4 ± 1.6
Ventral fin length	10	22.1 – 29.6	26.1 ± 2.2	49	23.3 – 34.7	29.6 ± 2.0
continued.						
		A. angiru			*A. kaaygua*	
	N	Min-Max	Mean ± SD	N	Min-Max	Mean ± SD
Head length	16	31.7 – 36.2	33.3 ± 1.5	13	35.2 – 38.4	37.0 ± 1.02
Snout length	16	7.8 – 11.4	9.5 ± 0.9	13	8.9 – 13.0	10.9 ± 1.16
Body depth	16	46.2 – 51.5	49.6 ± 1.2	13	40.7 – 46.7	43.8 ± 1.71
Orbital diameter	16	10.8 – 13.5	11.8 ± 0.8	13	9.8 – 12.9	11.2 ± 1.19
Head width	16	16.4 – 20.5	17.7 ± 1.2	13	17.9 – 23.4	19.6 ± 1.4
Interorbital width	16	10.3 – 12.7	11.8 ± 0.6	13	10.1 – 15.1	11.7 ± 1.42
Preorbital distance	16	6.3 – 8.3	7.3 ± 0.6	13	7.3 – 11.0	8.9 ± 1.25
Caudal peduncle depth	16	17.8 – 19.4	18.5 ± 0.5	13	13.9 – 17.6	16.2 ± 1.0
Caudal peduncle length	16	5.5 – 9.2	7.4 ± 0.9	13	8.9 – 11.0	10.4 ± 0.79
Pectoral fin length	16	28.1 – 32.4	30.3 ± 1.4	13	27.3 – 31.7	29.0 ± 1.38
Ventral fin length	16	28.3 – 37.6	32.4 ± 3.1	13	26.4 – 35.3	28.8 ± 2.81

Holotype. MACN-ict 9467, 102.0 mm SL, Argentina, río Uruguay basin, arroyo Paraiso (or Canal Muerto), 27°14'15.1" S, 54°02'38.5" W, col: Říčan *et al.*, December 2007.

Paratypes. 30 specimens, 39.5–136.8 mm SL, all from Argentina, Misiones province, río Uruguay basin. MACN-ict 9468, 4 ex., 39.5–108.7 mm SL, same data as holotype. MACN-ict 9469, 3 ex., 101.1–136.8 mm SL, arroyo Fortaleza, 26°45'56.6" S, 54°10'57.4" W, col: Říčan *et al.*, December 2007. AI 270, 3 ex. (C&S), 57.0–64.0 mm SL, arroyo Fortaleza, 26°45'56.6" S, 54°10'57.4" W, col: Casciotta *et al.*, April 2000. MACN-ict 9470, 3 ex., 90.5–112.0 mm SL, arroyo Guerrero, 27°45'57.4" S, 55°09'33.7" W, col: Říčan *et al.*, December 2007. MACN-ict 9471, 4 ex., 86.5–102.1 mm SL, arroyo Shangai or arroyo Pindaiti, 27°28'13.8" S, 54°41'24.5" W, col: Říčan *et al.*, December 2007. MACN-ict 9472, 13 ex., 47.0–86.3 mm SL, arroyo Tamandua, 27°05'56.5" S, 54°45'48.9" W, col: Říčan *et al.*, December 2007.

Additional non-type material. ZSM 23060b, 6 ex., río Soberbio, El Soberbio, col: J. Foerster, 1966. ZSM 23482b, 13 ex., río Soberbio, El Soberbio, col: J. Foerster, 1966. ZSM 23482c, 2 ex. (C&S), río Soberbio, El Soberbio, col: J. Foerster, 1966.

Diagnosis. *Australoheros ykeregua* is distinguished from all *Australoheros* species except *A. forquilha* (with which it was previously associated) in having a series of opalescent pale blue dots along the postero-lateral border

of the suborbital series (dark markings in preserved specimens), in having checkerboard-spotted dorsal, anal and caudal fins (red spots in live animals and dark grey in preserved specimens), a red to orange branchiostegal membrane, mouth and lower head area and base of pectoral fin, by having comparativelly thick lips (shared also with *A. tembe*), the lower jaw shorter than the upper, by having 25–26 E0 scales (vs. less than 25), by having the longest dorsal fin scale cover (shared also with *A. tembe*), and by the narrowest head (head width less than 50% *vs.* more than 50% of HL), shortest interorbital (10.9% of SL) and longest preorbital (9.3% of SL) distances.

Australoheros ykeregua is distinguished from *A. forquilha* by not having opalescent pale blue dots on each body scale, by not having them widely distributed on the head, but limited to a single line below the suborbital series, and in having a red coloration limited to the head region and the base of the pectoral fin (*vs.* red coloration on the whole belly to the end of the anal fin). Further distinguished by lower counts of caudal vertebrae (13–14 *vs*. 14–15), less caudal peduncle vertebrae (modally 2 *vs.* modally 3), lower total dorsal fin counts (25–26 *vs.* 26–27) and 25 *vs.* 26 E0 scales.

Australoheros ykeregua is distinguished from the only other similar species, *Australoheros tembe*, by the above listed unique characters and by coloration (shared only with *A. forquilha*) and additionally by a shorter caudal peduncle (including 2 *vs.* 3 vertebrae) and more dorsal fin rays (10–11 *vs.* 9).

For distinguishing characters from all other *Australoheros* species see the Notes section.

FIGURE 4. *Australoheros ykeregua*, MACN-ict 9467, 102.0 mm SL, holotype, right side (reversed). This specimen does not show vertical bars after preservation, but see Fig. 7 of the same specimens photographed alive.

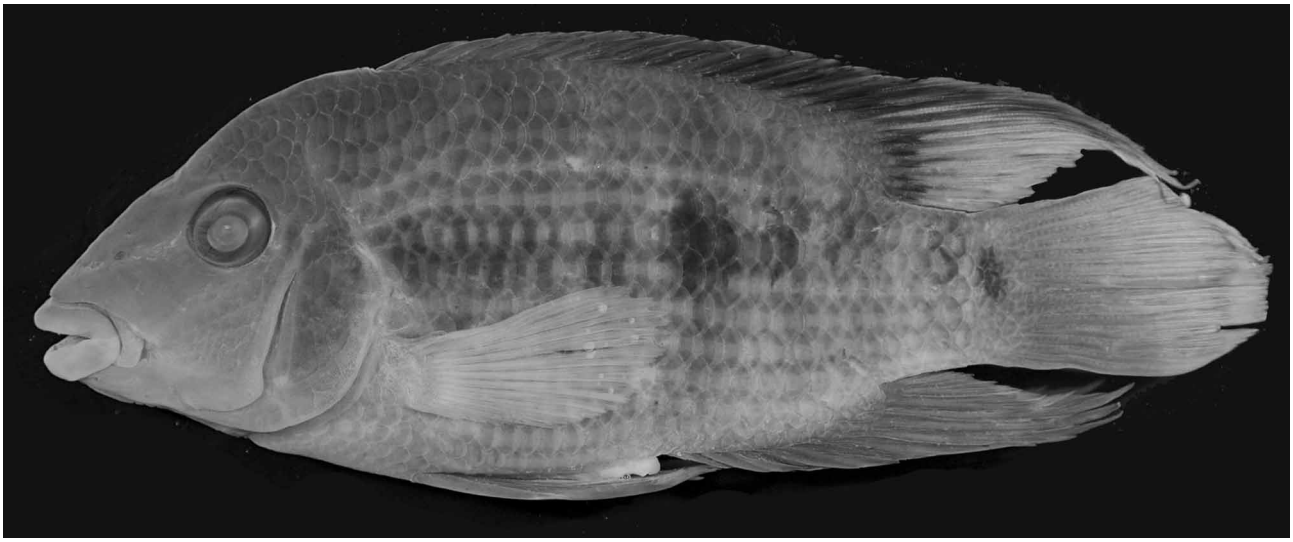

FIGURE 5. *Australoheros ykeregua*, MACN-ict 9470, 90.5 mm SL. This specimens shows the dark color of the dorsal fin and the midlateral blotch and vertical bars.

FIGURE 6. *Australoheros ykeregua*, MACN-ict 9472, 66.2 mm SL. This specimen shows a continuous lateral band extending beyond the midlateral spot and the checker-board spot pattern of unpaired fins (also evident in the holotype and the majority of specimens).

Description. Based on specimens over 60 mm SL. Meristic data are summarized in Table 2, morphometric data are summarized in Table 3.

Body rather slender (44.9% SL), head with a rounded profile, mouth subterminal with comparativelly thick lips, short interorbital (10.9% SL) and long preorbital (9.3% SL) distances. Lacrimal bone deeper than wide. A rather long caudal peduncle containing modally two vertebrae, 14 caudal vertebrae. Caudal peduncle considerably deeper than long (mean length 63% of depth).

Scales on chest smaller than half the size of the biggest scales in the E0 row above the pectoral fin. About 8 scale rows between the opercular flap and the anterior insertion of the pelvic fin in the holotype. Scales in E0 row 24(1), 25(32*), 26(13). Upper lateral line scales 14(1), 15(1), 16(5), 17(15), 18(19*), 19(5). Lower lateral line scales 7(1), 8(4), 9(26*), 10(10), 11(5). Scales between upper lateral line and dorsal-fin scale cover 3 posteriorly, 4 plus two small parallel scales anteriorly, forming a sheath of smaller scales arranged in pairs per scale row, along the insertion of the dorsal fin. Cheek scale rows 4(2), 5(16*), 6(1). Dorsal fin with interradial scales appearing from 13^{th}(1), 14^{th}(6), 15^{th}(8), 16^{th}(4*) spine membrane, in single rows. One (5), two (10*) or three (4) last interradial membranes without scales. Anal fin with one basal scale row; interradial scales in single rows, from the 4^{th}(1), 5^{th}(8), 6^{th}(10*) spine membrane lacking on one (17*) or two (2) last interradial membranes. Caudal fin densely scaled, scales ctenoid; interradial scales in one or two rows; posterior margin of scaly area concave, extending to between one-third and middle of caudal fin.

Soft dorsal fin pointed, extending to the middle or almost to the end of the caudal fin. D. XV,10(3), XV,11(3), XVI,9(2), XVI,10(16), XVI,11(22*), XVII,9(1), XVII,10(2). Soft anal fin pointed, of about the same length as dorsal fin. A. V,8(3), V,9(1), VI,7(14), VI,8(29*), VI,9(3). Anal fin pterygiophores 11(15), 12(7). Pelvic fin base slightly posterior of pectoral fin base; first branched ray longest. Pelvic fin not reaching (2), reaching (10) or surpassing (7*) anal fin origin. Pectoral fin shorter than pelvic fin, with a rounded tip. P. 13(14), 14(18), 15(15*). Caudal fin with rounded corners.

Oral jaw teeth caniniform, slightly curved. Outer row teeth increasing in size symphysiad, upper-jaw anterior teeth more robust, lower-jaw anterior teeth subequal.

Lower pharyngeal tooth plate in a dissected specimen about one quarter wider than long (length 59–62% of width). Dentigerous area wider than long. 7–9 teeth along midline, 22–26 teeth along posterior margin. Posterior teeth tend to be progressively more compressed, except for medial teeth. Larger teeth medially and posteriorly, gradually smaller anteriad and laterad. Posterior teeth with forwards curved posterior cusp and subapical anterior shelf. Large laterally compressed teeth with a second cusp projecting anteriorly from shelf.

Gill rakers externally on first gill arch: 1-2 epibranchial, 1 in angle, 7(3), 8(13), 9(4) ceratobranchial.

Vertebrae 13+13=26(3), 13+14=27(19), 14+13=27(3). Caudal peduncle contains 1(1), 1.5(5), 2(11), 2.5(6), 3(1) vertebrae.

Color pattern in alcohol. Six or seven vertical flank bars, a midlateral blotch in the fourth flank bar (sensu Říčan *et al.* 2005), a caudal fin spot, and the caudal peduncle bar make up the principal markings. Base of caudal spot at level of the lower lateral line. Lateral band 1, 1/2 or 2 scales deep posteriorly from the posterodorsal edge of opercular to the midlateral blotch (not clearly visible in the holotype). Lateral band extending behind the midlateral blotch, widening towards the end of dorsal-fin base level in five adult specimens and in eight juveniles (arroyo Tamandua, MACN-ict 9472).

Vertical bars are relatively wide, indistinct in their ventral parts. The fourth bar, bearing the midlateral blotch is centered above the anteriormost portion of the anal fin. Many thin parallel stripes on flanks, more evident on lower half of body.

Dorsal fin with a dark pigmentation from interradial membranes from 8th or 9th spine to 3rd to 4th branched ray. This pigmentation extended to the tip of the dorsal filament. Same dark pigmentation on basal third of the remaining branched rays. Soft dorsal and anal fins, and caudal fin with dark spots in a checker-board pattern on interradial membranes (missing in some specimens).

One (MACN-ict 9468, MACN-ict 9472), two (in the holotype MACN-ict 9467 and in MACN-ict 9468, MACN-ict 9472) or three (MACN-ict 9472) small and inconspicuous dark blotches below the orbit along the postero-lateral border of the suborbital series.

Life coloration. The most distinct color markings include the diagnostic 1) red/orange branchiostegal membrane, base of pectoral fin, mouth and lower head area, 2) the single interrupted line of blue dots along the suborbital series (dark blotches in preserved specimens), and 3) the checkerboard pattern of red dots on unpaired fins (Fig. 7). This character combination is unique among *Australoheros*. The most similar species, *A. forquilha*, is easily distinguished in that the blue dots are not limited to a single line below the orbit. Instead, they cover the whole head and are present in all body scales and are also present on all fins except the pectorals (see Fig. 7 and also *"Cichlasoma"* cf. *tembe* in Stawikowski & Werner 2004, p. 455).

Distribution. *Australoheros ykeregua* is so far known only from Argentinean territory in the tributaries of the río Uruguay below the Salto Moconá, province of Misiones.

Etymology. The Guaraní word *ykeregua* means neighbor (vecino in Spanish). The etymology is based on the fact that *A. ykeregua* and *A. forquilha* have been preliminarily treated as conspecific (Říčan & Kullander 2008). New data have however demonstrated that they are two sister group species living in the same river drainage (río Uruguay), though not sympatrically.

Notes. Říčan and Kullander (2006, 2008) treated part of the ZSM non-type material from Argentina as conspecific with *A. forquilha*. New fresh material collected in 2007 has revealed that the Argentinean and Brazilian material do not represent the same species. The ZSM lots 23060 and 23482 have been divided since they contained two different species and lots ZSM 23060b, 23482b and 23482c hold *A. ykeregua*.

We hypothesize that the barrier between the two species, *A. forquilha* and *A. ykeregua*, is formed by the Salto Moconá on the río Uruguay just below the confluence with the río Pepirí Guazú (which forms the international border between Argentina and Brazil). The two species are closely related, but important differences in morphology and DNA demonstrate that there is no gene flow between them and they are thus evolutionarily independent units.

Additional diagnostic characters that separate *Australoheros ykeregua* from all other species except *A. forquilha* and *A. tembe* are as follows. From *A. facetus*, by having more caudal vertebrae (14 *vs*. 13), more caudal peduncle vertebrae (2 *vs*. 0–1), more E0 scales (25–26 *vs*. 24), and by a longer snouth (14.9 *vs*. 9.4 % SL) and a longer preorbital distance (9.3 *vs*. 5.7 % SL).

Australoheros ykeregua is additionally distinguished from *A. kaaygua* by having more caudal vertebrae (14 *vs*. 13), more C1 gill rakers (8 *vs*. 6), more caudal peduncle vertebrae (2 *vs*. 0–1), more E1 scales (18 *vs*. 16) and by a slightly longer snouth (14.9 *vs*. 10.9 % SL). It is additionally distinguished from *A. minuano* by lacking a pinkish body coloration of live specimens, by having more caudal vertebrae (14 *vs*. 13), more pectoral fin rays (14 *vs*. 12), more C1 gill rakers (8 *vs*. 6), more caudal peduncle vertebrae (2 *vs*. 0–1), more E0 scales (25–26 *vs*. 24), and by a longer snouth (14.9 *vs*. 10.6 % SL) and a longer preorbital distance (9.3 *vs*. 6.0 % SL).

Australoheros ykeregua is distinguished from *A. guarani* by also having more caudal vertebrae (14 *vs*. 13), more pectoral fin rays (14 *vs*. 13), more C1 gill rakers (8 *vs*. 7), more E0 scales (25–26 *vs*. 24), more caudal peduncle vertebrae (2 *vs*. 0–1), and by a shorter head (36.2 *vs*. 32.4 % SL), longer snouth (14.9 *vs*. 8.5 % SL), and less deep body (44.9 *vs*. 48.1 % SL). It is additionally distinguished from *A. charrua* by lacking a pinkish body coloration of live specimens, by less anal fin spines (5–6 *vs*. 7–8), more C1 gill rakers (8 *vs*. 6), more caudal peduncle

vertebrae (2 *vs*. 0–1), by a slightly longer head (36.2 *vs*. 32.4 % SL), slightly longer preorbital distance (9.3 *vs*. 7.3 % SL) and by a longer snouth (14.9 *vs*. 8.5 % SL).

FIGURE 7. Color plate. Horizontaly from upper left to lower right. *Australoheros forquilha*, rio Forquilha, rio Uruguai drainage, Rio Grande do Sul, Brazil (not preserved). *Australoheros ykeregua* (MACN-ict 9467, holotype), río Uruguay drainage, arroyo Paraiso (or Canal Muerto), Misiones province, Argentina. *Australoheros kaaygua* (MACN-ict 9473), río Iguazú drainage, small stream 7 km SW from Andresito, Misiones province, Argentina. *Australoheros angiru*, male in neutral colors, rio Chopim, rio Iguaçu drainage, Paraná, Brazil (not preserved). *A. angiru*, male and female in breeding colors guarding fry, same locality (not preserved). All *A. angiru* photographs courtesy of Wolfgang Staeck.

Australoheros ykeregua is additionally distinguished from *A. scitulus* in lacking the dark spot-markings on the head and anterior part of body, less dorsal fin spines (16 *vs*. 17), more dorsal fin rays (10–11 *vs*. 9–10), less anal fin spines (5–6 *vs*. 8–9), more pectoral fin rays (14 *vs*. 13), by more C1 gill rakers (8 *vs*. 6), more caudal peduncle vertebrae (2 *vs*. 0) and less deep body (44.9 *vs*. 47.7 % SL). It is also distinguished from *A. angiru* by lacking the yellow background coloration, yellow iris and red dorsal and ventral margins and corners of the caudal fin in live specimens, by having more dorsal fin rays (10–11 *vs*. 9–10), less anal fin spines (6 *vs*. 7), more caudal vertebrae (14 *vs*. 13), more pectoral fin rays (14 *vs*. 12), more C1 gill rakers (8 *vs*. 6), more E0 scales (25–26 *vs*. 24), more caudal peduncle vertebrae (2 *vs*. 0–1), a longer head (36.2 *vs*. 33.3 % SL), a longer snouth (14.9 *vs*. 9.5 % SL), a less deep body (44.9 *vs*. 49.6 % SL) and a longer preorbital distance (9.3 *vs*. 7.3 % SL).

Australoheros ykeregua is distinguished from *A. acaroides* by also having more caudal vertebrae (14 *vs*. 13), more caudal peduncle vertebrae (2 *vs*. 0–1), less anal fin spines (6 *vs*. 7), more E0 scales (25 *vs*. 23–24), more C1 gill rakers (8 *vs*. 6), and a smaller interorbital distance (33 *vs*. 43 % HL). It is additionally distinguished from *A. taura* by lacking a pink to red body coloration of live specimens, more caudal vertebrae (14 *vs*. 13), more C1 gill rakers (8 *vs*. 7), and a deeper body (44.9 *vs*. 41.4 % SL) and a smaller interorbital distance (33 *vs*. 41% HL).

Australoheros ykeregua is additionally distinguished from all the Atlantic coast species north of *A. acaroides* and *A. taura* (*A. autrani*, *A. barbosae*, *A. capixaba*, *A. ipatinguensis*, *A. macacuensis*, *A. macaensis*, *A. muriae*, *A. paraibae*, *A. ribeirae*, *A. robustus*, *A. saquarema*) by having more caudal vertebrae (14 *vs*. 12 or 13), more caudal peduncle vertebrae (2 *vs*. 0), less anal fin spines (6 *vs*. 7), a smaller interorbital distance (33 *vs*. 41% HL), and a shorter pelvic fin (<30 *vs*. >30 % SL).

Australoheros angiru **sp. nov.**

(Figs 7, 8, 9).

"Cichlasoma" facetum—Staeck 1998a: 62–63; 1998b: 81–85
"Cichlasoma" sp. Iguaçu—Staeck 2003: 64–65
"Cichlasoma" sp. Iguaçu—Stawikowski and Werner 2004: 455
Australoheros sp. jacutinga—Říčan and Kullander 2006: 6
Australoheros kaaygua—Říčan and Kullander 2008: 28 (in part)

Holotype. MCP 13937, 73.2 mm SL, Brazil, Santa Catarina State, rio Uruguai drainage, rio Jacutinga, road BR 283 from Ceará to Concordia, col: Bergmann *et al.*, October 1988.

Paratypes. 13 specimens, 24.6–77.0 mm SL, all from Brazil. Santa Catarina State, rio Uruguai drainage: MCP 13383, 6 ex., 24.6–77.0 mm SL, rio Jacutinga, road BR 283 from Ceará to Concordia, col: Reis *et al.*, February 1989. MCP 12509, 1 ex., 75.0 mm SL, same data as holotype. MCP 13011, 6 ex., 44.2–61.4 mm SL, rio Jacutinga, road BR 283 from Ceará to Concordia, col: Reis *et al.*, December 1988.

Additional non-type material. Paraná State, rio Iguaçu drainage: NUP 3913, 2 ex., rio São Pedro, tributary to rio Iguaçu, Pinhão county, 26º05´S, 51º45'W, col: Nupélia staff, March 1993. NUP 3914, 1 ex, rio Iratim (Linígrafo), tributary to rio Iguaçu, Palmas county, boundary with Pìnhão-PR, 26º05´S, 51º45´W, col: Nupélia staff, April 1993. NUP 3915, 1 ex, rio São Pedro, tributary to rio Iguaçu, Pinhão county, 26º05´S, 51º45'W, col: Nupélia staff, March 1993. Rio Grande do Sul State, rio Uruguai drainage: MCP 46328, 13 ex., Sanga das Aguas Frias, Irai, col: Malabarba *et al.*, 1985. Argentina, Misiones province, río Uruguay drainage: ZSM 23482a, 1 ex., P, río Soberbio, El Soberbio, col: J. Foerster, 1966. ZSM 23060a, 4 ex., río Soberbio, El Soberbio, col: J. Foerster, 1966. ZSM 23060c, 2 ex. (C&S), río Soberbio, El Soberbio, col: J. Foerster, 1966.

Diagnosis. *Australoheros angiru* is one of the most deep-bodied species of *Australoheros* (body depth in SL >49%; shared with *A. guarani* and *A. facetus*). It has been previously associated with *A. kaaygua*, but it is the sister species of *A. minuano* based on DNA characters.

Australoheros angiru is distinguished from *A. kaaygua* by having less scale rows between anterior end of dorsal fin and upper lateral line (ch4 states 1–2 *vs.* 0), by a very narrow or missing caudal base spot, by a pure yellow ground color (*vs.* yellowish-green), by yellow eyes (*vs.* dark green), by more scales between anterior end of dorsal fin and upper lateral line (5 *vs.* 4), more anal fin spines (7 *vs.* 6), more anal fin rays (> 7 *vs.* < 7), more dorsal fin rays (9 *vs.* 8), less E0 scales (24 *vs.* > 25), more L1 scales (> 17–18 *vs.* 16), less L2 scales (8 *vs.* > 9), and by a being more deep-bodied (49.6% *vs.* 43.8% SL), and having a shorter caudal peduncle (7.4% *vs.* 10.4% SL).

Australoheros angiru is distinguished from *A. minuano* by a large and dominant midlateral blotch, very narrow or missing caudal base spot, by lacking a pinkish body coloration, by a small terminal or subterminal mouth (*vs.* large supraterminal), by more scales between the anterior end of the dorsal fin and the upper lateral line (5 *vs.* 4), less anal fin rays (7 *vs.* 8), less dorsal fin rays (9 *vs.* 10), and by slight differences in body depth (49.6% *vs.* 46.9% SL) and in preorbital distance (7.3% *vs.* 6.0% SL).

For distinguishing characters to all other *Australoheros* species see the Notes section.

FIGURE 8. *Australoheros angiru*. Holotype, MCP 13937, 73.2 mm SL, rio Jacutinga, rio Uruguai drainage, Brazil.

FIGURE 9. *Australoheros angiru*. Paratype, MCP 13011, 48.1 mm SL, rio Jacutinga, rio Uruguai drainage, Brazil.

Description. Based on specimens over 60 mm SL, with notes on smaller specimens. Meristic data are summarized in Table 2, morphometric data are summarized in Table 3.

Comparatively deep bodied (mean body depth 49.6% SL). Snout short, straight in lateral view. Jaws isognathous. Mouth small.

Scales on head and chest not distinctly smaller than on flanks. Scales in E0 row 23(3), 24(16*), 25(4). Upper lateral line scales 16(1), 17(6*), 18(8). Lower lateral line scales 7(4), 8(7*), 9(4). Scales between upper lateral line and dorsal fin 4 anteriorly, 1 large plus 1 small posteriorly. Cheek scale rows 3(14*), 4(2). About 8 scale rows between the opercular flap and the anterior insertion of the pelvic fin. Dorsal fin with one basal scale row, starting from the 7th or 8th spine and running posteriad; interradial scales appear from 14th or 15th spine membrane, in single rows. Anal fin with one basal scale row; interradial scales in single rows, from penultimate spine. Caudal fin densely scaled, scales ctenoid; interradial scales in single rows; hind margin of scaly area concave, extending to between one-third and middle of caudal fin.

Soft dorsal fin pointed, extending beyond middle of caudal fin. D. XVI,9(16*), XVI,10(13), XVII,8(2). Soft anal fin pointed, of about the same length as dorsal fin. A. VI,7(2), VI,8(3), VII,7(17*), VII,8(8), VIII,6(1). Anal fin pterygiophores 11(2), 12(22*), 13(7). First pelvic fin ray longest, extending up to the second anal fin spine. Pectoral fin with a rounded tip, third and fourth rays longest, extending just to the midlateral blotch. P. 12(11*), 13(5). Caudal fin rounded to subtruncate.

All teeth caniniform, slightly curved. Outer row teeth increasing in size symphysiad, upper jaw anterior teeth longest, lower jaw anterior teeth subequal. Number of lower jaw teeth up to 16 in one outer hemiseries, upper jaw tooth row much shorter, with about 7 or 8 teeth in one outer hemiseries. Lower pharyngeal tooth plate not studied. Gill rakers externally on first gill arch, 2 epibranchial, 1 in angle, 5(4), 6(11*), 7(1) ceratobranchial.
Vertebrae 13+13=26(29*), 13+14=27(2). Caudal peduncle with no vertebrae (10) or containing 0.5(4), 1(14*), 1.5(1) vertebrae.

Color pattern in alcohol. Six to seven vertical flank bars, a caudal peduncle bar confluent with the caudal-base bar, and a midlateral stripe bearing the midlateral blotch in the fourth flank bar (sensu Říčan *et al.* 2005) make up the principal markings. All fins and body are without conspicuous spots or blotches. The midlateral stripe is more distinct anteriorly from the midlateral blotch than posteriorly, and the midlateral blotch itself is a dominant coloration pattern element. Vertical bars are relatively wide, faint, indistinct in their ventral parts. The midlateral stripe posteriorly from the midlateral blotch does not align with the lower lateral line and aligns with the E1 scale row and does not continue in the E0 scale row. Posteriorly from the midlateral blotch, the stripe is slightly decomposed into two blotches in the respective vertical flank bars. The blotch posterior from the midlateral blotch is centered in the same scale row as the midlateral blotch (*i.e.* E1 scale row), whereas the second blotch is more elongate along the vertical axis and centered in the E2 scale row, making the impression that the midlateral stripe makes a dorsally directed turn at its posterior end. The arrangement of the bars on the body in essentially the same as described for *A. scitulus* (Říčan & Kullander 2003). Very small spots present on the bases of some body scales in adult specimens. In juveniles the spotted pattern of the body is much more pronounced, with virtually every scale on the body having a spot at its base, including those in the anterior part of the E4 scale row (*i.e.* as in adult *A. scitulus*).

Life coloration. Coloration of life specimens from the rio Uruguai drainage is unknown to us. Staeck (1998a, 1998b, 2003: p. 64) photographed specimens from the rio Iguaçu drainage (Fig. 7). These specimens have a yellow ground coloration with dark vertical bars and a dark horizontal stripe. Several other species of *Australoheros* have a yellowish ground color, but it is best developed in *A. angiru*. The iris is also yellow. The caudal fin has red dorsal and ventral margins and corners. This character is not unique for *A. angiru*, and can also be seen in *A. kaaygua* and in populations of *A. facetus* from the state of Uruguay. Breeding animals have the typical *Australoheros* breeding coloration with the horizontal interruption of the black vertical bars in their dorsal portion between the opercle and the midlateral blotch (Říčan & Kullander 2003; Staeck 1998a: p. 82, 1998b: p. 62, 2003: p. 65). Females in breeding coloration develop a black blotch in the dorsal fin. Staeck (1998b, 2003) describes behavior and spawning under aquarium conditions.

Distribution. *Australoheros angiru* has a disjunct distribution in the rio Iguaçu and in the upper rio Uruguai. One locality is so far known from the middle río Uruguay in Misiones province, Argentina (Fig. 10).

Etymology. The Guaraní word *angirû* means friend, partner (amigo or compañero in Spanish). The etymology is based on the fact that *A. angiru* and *A. kaaygua* have been confused as one species (Říčan & Kullander 2008). New data have however demonstrate that they are two non-sister group species living in the same river drainage (río Iguazú), though not sympatrically.

Notes. Part of *Australoheros angiru* material (MCP 6262) has been previously considered conspecific with *A. kaaygua* (Říčan & Kullander 2008). The authors were aware of the morphological variation within *A. kaaygua* (sensu Říčan & Kullander 2008), but lack of DNA data and of first hand examination of the type series of *A. kaaygua* made them sceptical about describing a new species with an additionally unusual distribution (occuring in the same river basin, río Iguazú as *A. kaaygua*, but not in sympatry, and at the same time also in the río Uruguay). DNA data from the rio Iguaçu populations in Brazil (*A. angiru*) however show no relationship to *A. kaaygua* in the río Iguazú in Argentina (Fig. 2). DNA data from the río Uruguay are so far lacking. A more detailed morphological analysis (Fig. 1) also supports the notion of two unrelated species, with populations of *A. angiru* from both the rio Iguaçu and from the río Uruguay forming a homogenous clade with short intraspecific branch lengths. The sister species of *A. angiru* is *A. minuano*, while that of *A. kaaygua* is *A. tembe* (Fig. 3).

The MCP 6262 lot additionally included two species (Říčan and Kullander, 2008). Nine specimens from this lot are paratypes of *Australoheros forquilha* Říčan and Kullander, 2008. Thirteen specimens from this lot represent *A. angiru* (previously erroneously treated as *A. kaaygua* in Říčan and Kullander, 2008) and were separated into a new lot MCP 46328.

Additional diagnostic characters of *Australoheros angiru* that separate it from all other species except *A. kaaygua* and *A. minuano* are as follows. It is distinguished (in decreasing order of overall similarity; except for species from coastal drainages treated as last) from *A. charrua* and *A. scitulus* by having less scale rows between posterior end of upper lateral line and dorsal fin (ch3 state 2 *vs.* 0 *vs.* 1), less caudal vertebrae (13 *vs*. 14), in being more deep-bodied (50 *vs*. 45% SL), and in having less E0 scales (24 *vs*. >25). Additionally distinguished from *A. charrua* by details in the shape of the midlatral stripe (see description) and by lacking a pinkish body coloration. Additionally distinguished from *A. scitulus* by lacking black blotches on the opercular series, having less anal fin spines (7 *vs*. 8), less dorsal fin spines (16 *vs*. 17), less caudal vertebrae (13 *vs*. 14), in being more deep-bodied (50 *vs*. 45% SL), and in having less pectoral fin rays (12–13 *vs*. 13–14).

Australoheros angiru is distinguished from *A. tembe* by having less scale rows between anterior end of dorsal fin and upper lateral line (ch4 states 1–2 *vs.* 0), by a very narrow or missing caudal base spot, a shorter dorsal fin scale cover (ch1 state 1 *vs.* 0), less scale rows between the posterior end of the upper lateral line and dorsal fin (ch3 state 2 *vs.* 0), by lacking thick lips, by having more anal fin spines (7 *vs*. 6), less caudal vertebrae (13 *vs*. 14), and less caudal peduncle vertebrae (0 *vs.* 3). It is distinguished from *A. guarani*, *A. facetus*, *A. acaroides* and *A. taura* by a large and dominant midlateral blotch (except *A. facetus*), very narrow or missing caudal base spot, and details in the shape of the midlatral stripe (see description).

Australoheros angiru is additionally distinguished from *A. guarani* by a small terminal or subterminal mouth (*vs*. large supraterminal), more anal fin spines (7 *vs*. 6), shorter preorbital distance (21 *vs.* 25% HL), and less C1 gill rakers (6 *vs*. 7). Additionally distinguished from *A. facetus* by a longer dorsal fin scale cover (ch1 state 1 *vs.* 2), more anal fin spines (7 *vs*. 6), less anal fin rays (7 *vs*. 8), less pectoral fin rays (12–13 *vs*. 13–14), and less C1 gill rakers (6 *vs*. 7–8). It is additionally distinguished from *A. acaroides* by a longer dorsal fin scale cover (ch1 state 1 *vs.* 2), shorter caudal peduncle (40% CPD *vs*. 50–60% CPD), by being more deep-bodied (50 *vs*. 45% SL), and having a narrower interorbital distance (35 *vs*. 40–45% HL). It is distinguished from *A. taura* by also lacking a pinkish body coloration, by a small terminal or subterminal mouth (*vs*. large supraterminal), shorter caudal peduncle (40% CPD *vs*. 50% CPD), by being more deep-bodied (50 *vs*. 40% SL), by a narrower interorbital distance (35 *vs*. 40% HL), less pectoral fin rays (12–13 *vs.* 13–14), and less E0 scales (24 *vs*. >25).

Australoheros angiru is distinguished from *A. ykeregua* and *A. forquilha* by a shorter dorsal fin scale cover (ch1 state 1 *vs.* 0), a different scale pattern along anterior border of dorsal fin (ch2 state 0 *vs.* 1), less scale rows between posterior end of upper lateral line and dorsal fin (ch3 state 2 *vs.* 0), very narrow or missing caudal base spot, absence of opalescent spots below orbit, unpaired fins without checker-board spotted pattern, absence of red colored lower head area and opercular membrane, by a small terminal or subterminal mouth (*vs*. large supraterminal), less dorsal fin rays (9 *vs*. 10), less caudal peduncle vertebrae (0 *vs*. 2 *vs*. 2.5), shorter caudal peduncle (40% CPD *vs*. 60% CPD), by being more deep-bodied (50 *vs*. 45 *vs*. 40% SL), with a wider head (55 *vs*. <50% HL), and in having less pectoral fin rays (12–13 *vs*. 13–14). Additionally distinguished from *A. ykeregua* by a large and dominant midlateral blotch, and more anal fin spines (7 *vs*. 6). Additionally distinguished from *A. forquilha* by less scale rows between anterior end of dorsal fin and upper lateral line (ch4 state 1 *vs.* 0), absence of opalescent scale rows on body, and less pectoral fin rays (12–13 *vs*. 13–14).

Australoheros angiru is distinguished from all the Atlantic coast species north of *A. acaroides* and *A. taura* (*A. autrani*, *A. barbosae*, *A. capixaba*, *A. ipatinguensis*, *A. macacuensis*, *A. macaensis*, *A. muriae*, *A. paraibae*, *A. ribeirae*, *A. robustus*, *A. saquarema*) by a longer dorsal fin scale cover (ch1 state 1 *vs.* 2), a large and dominant midlateral blotch, details in the shape of the midlatral stripe (see description), shorter caudal peduncle (40% CPD *vs*. >50% CPD), in being more deep-bodied (50 *vs*. 45% SL), with a narrower interorbital distance (35 *vs*. 40% HL), less pectoral fin rays (12–13 *vs*. 13–14), and less E0 scales (24 *vs.* >25).

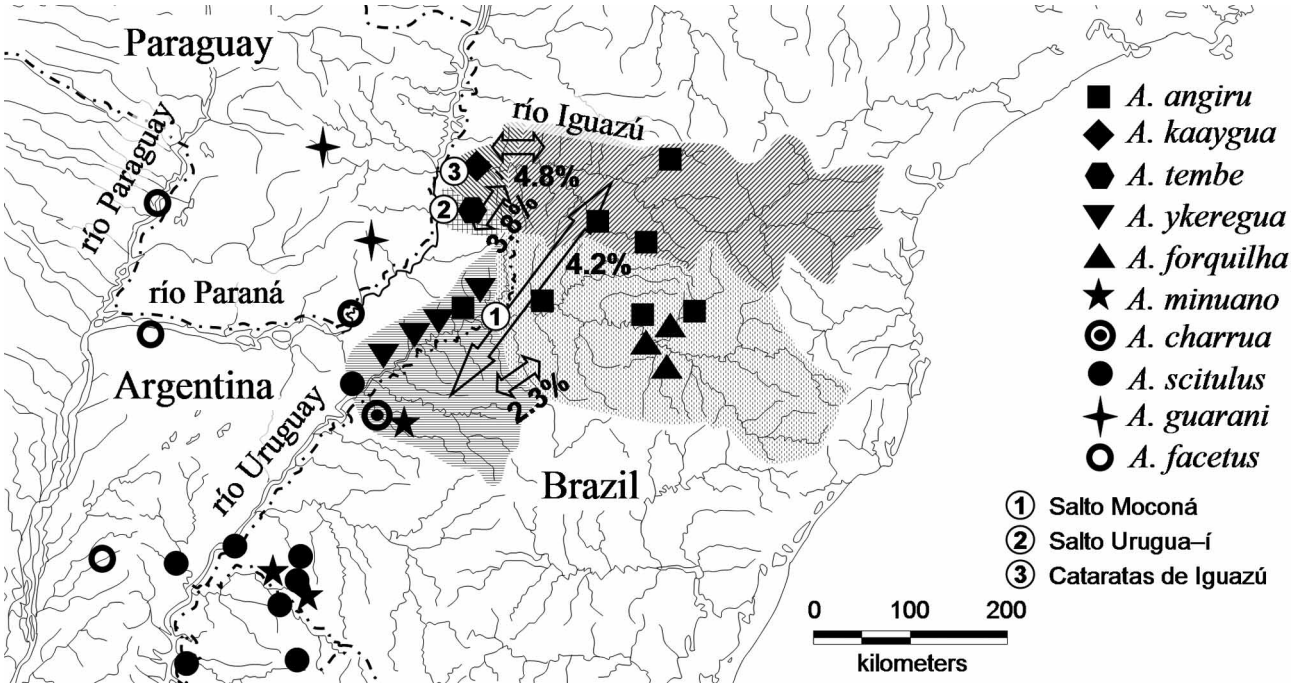

FIGURE 10. Map of the middle Río de la Plata basin. Distributions of the two new species (*A. angiru* and *A. ykeregua*) and their relatives, as well as five areas of endemism are shown. Percent values and corresponding arrows demonstrate sequence divergences in the cyt*b* gene (see Fig. 2) between the species and areas of endemism in the río Iguazú and río Uruguay river drainages (plus the arroyo Urugua–í). Divergence of *A. ykeregua* from its sister species *A. forquilha* is 2.3%. This divergence probably represents the minimum age of the Salto Moconá. Divergence of *A. kaaygua* from its sister species *A. tembe* is 3.8%, and of *A. angiru* from *A. tembe* is similarly 3.6–3.7%. This probably represents the age of the division of the arroyo Urugua–í from the río Iguazú. Divergence of *A. angiru* from its sister species *A. minuano* is 4.2%. This is likely a divergence of the rio Iguaçu and río Uruguay drainages. Divergence of *A. angiru* from *A. kaaygua* (4.8%), two unrelated species endemic to the río Iguazú river drainage, demonstrates an old divergence within the Iguazú drainage basin itself. See Discussion for more detailed description of the biogeography.

Discussion

Biogeography. The cyt*b* data reveal some interesting intraspecific geographical structure within *A. ykeregua*, amounting up to 1.5% divergences. The cytb data sugest that upstream populations (Fig. 2: 13) are potentially ancestral to downstream populations (Fig. 2: 11, 12). This pattern is in good agreement with theoretical prediction since the upstream population does not have a unique haplotype, compared to the downstream populations 11 plus 12. Upstream populations are divided from downstream populations (in this case by river rapids and waterfalls) and the only possible dispersal is downstream. *Australoheros ykeregua* is the only *Australoheros* species common in the tributaries of the río Uruguay in Misiones. This observation has two biogeographical and evolutionary implications (given the presence of waterfalls and a number of rapids on these tributaries and the presence of other *Australoheros* species in the mainstream of the río Uruguay in Misiones and in tributaries further south). First, *A. ykeregua* is the oldest *Australoheros* species in the río Uruguay drainage of Misiones, older than the respective barriers, which are impenetrable for the later immigrating species into the area (*A. angiru*, *A. minuano*, *A. scitulus*, and *A. charrua*). Second, its divergence from its sister species (*A. forquilha*) corresponds to a barrier between them, probably the Salto Moconá, which is not the case for *A. angiru*. *Australoheros angiru* (as presently understood) is partly sympatric with both *A. forquilha* and *A. ykeregua*, but its occupation of the río Uruguay in Misiones is much younger, and we predict that its molecular divergences (presently unknown) of populations below and above Salto Moconá will be much lower than in the case of *A. ykeregua* and *A. forquilha*. The biogeography of *A. angiru* suggests (in the absence of molecular data) that its original distribution area was the rio Iguaçu, and that its presence in the río Uruguay is secondary.

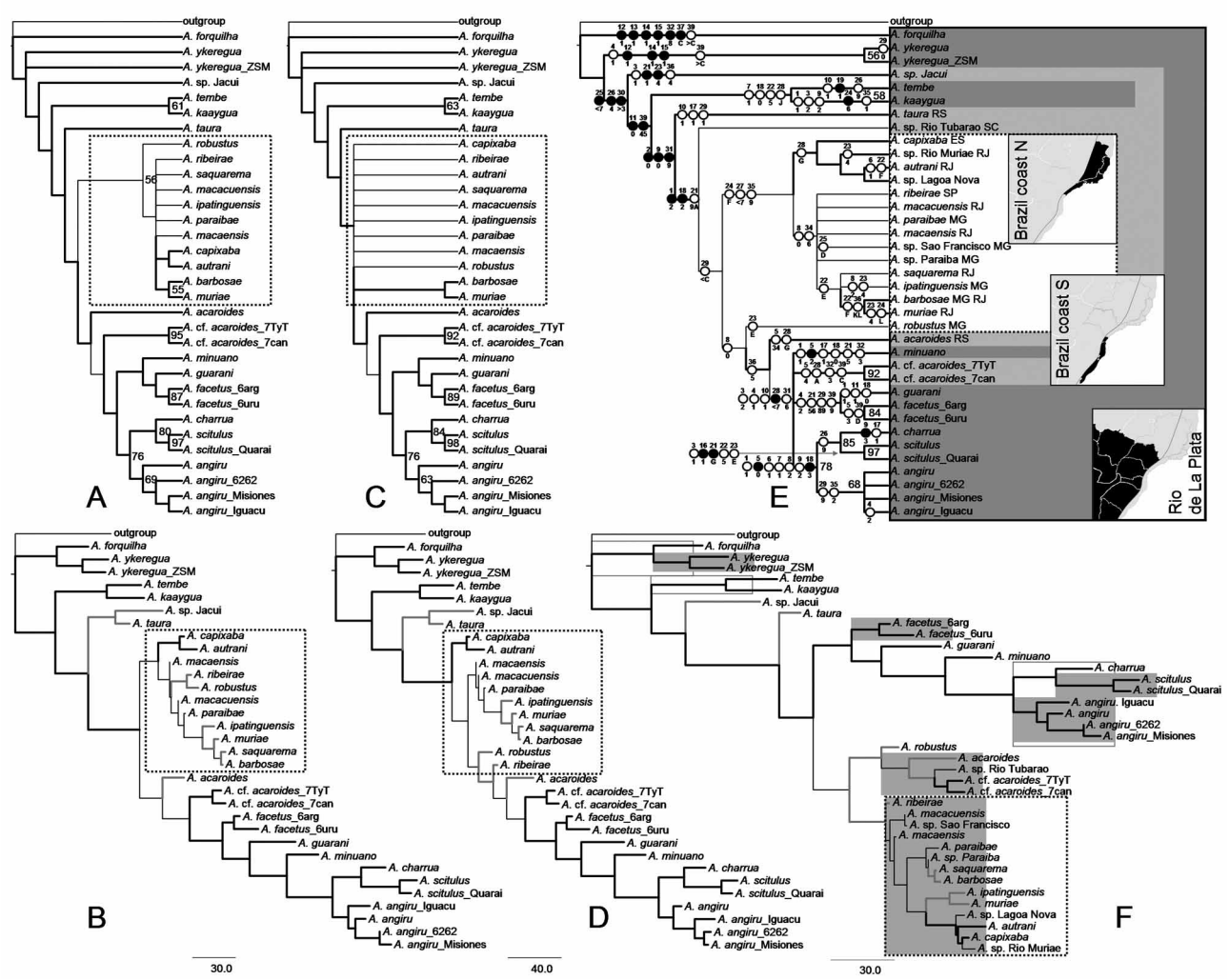

FIGURE 11. Phylogeny of all valid and one putative species of *Australoheros* based on 38 morphological characters. Ottoni and Costa (2008), Ottoni *et al.* (2008), Ottoni and Cheffe (2009) and Ottoni (2010) have diagnosed the Brazilian coastal species by a unique combination of 14 + 12 vertebrae. Our examination of material from some of the drainages (see Figs E and F) instead shows a combination of 13 + 13 vertebrae, which is not unique among *Australoheros*. Our phylogenetic analyses have thus been performed with both combinations (14 + 12 in Figs A and B; 13 + 13 in Figs C – F). The three upper Figs (A, C, E) show maximum parsimony (MP) topologies, the lower three show neighbour joining (NJ) topologies (with branch lengths showing amount of morphological divergence; B, D, F). Numbers at nodes show bootstrap support. Bold black nodes and branches show agreement between all analyses (MP and NJ separately), bold grey nodes and branches agreement between two of three analyses. The interrupted-line boxes show the relationships and branch lengths among the northern Brazilian coastal species. Notable is the collaps of their relationships under the 13 + 13 scenario (Figs C – F) and the markedly short branches separating these species (Figs B, D, F). The short branches separating these species are much more similar to intraspecific variability among other species of *Australoheros* (grey boxes in Fig. F) than to interspecific branch lengths (grey-line boxes in Fig. F). This low differenciation of the northern Brazilian coastal species is also evident from Fig. E, where the morphological matrix (Appendices 1 and 2) is mapped onto the phylogeny (geographical distribution of the species is also shown). Most species, with the exception of the northern Brazilian coastal species, are diagnosed by unique characters or unique combinations of characters. The average number of changes among interspecific pairs described by Říčan and Kullander (2003, 2008, this study) is 98.5, while among intraspecific comparisions it is 20.7. The average for comparisons among the species described by Ottoni and Costa (2008), Ottoni *et al.* (2008), Ottoni and Cheffe (2009) and Ottoni (2010) is 20.5, i.e. corresponding to variation within species of Říčan and Kullander (op. cit.). Based on these considerations we believe that the number of described species from the northern Brazilian coastal drainages is a case of excessive splitting and that the species diversity is actually much lower.

As proposed above, the barrier responsible for the divergence of *A. ykeregua* and *A. forquilha* is probably the Salto Moconá on the río Uruguay, just below the mouth of the río Pepirí Guazú, which marks the international boundary between Argentina and Brazil. The divergence between *A. ykeregua* and *A. forquilha* amounts to 2.3% uncorrected distance in the cyt*b* gene. Translated into time units this corresponds roughly to 2.3–3.3 Mya (based on calibration of the cyt*b* gene by Concheiro Pérez *et al.* 2007).

The divergence patterns found in the río Iguazú drainage are even more complex than those in the río Uruguay drainage. The two *Australoheros* species from this drainage are not sister species (*A. kaaygua* and *A. angiru*), and correspondingly their divergence amounts to a higher distance (than in the case of *A. ykeregua* and *A. forquilha*) of 4.8% (*i.e.* 4.8–6.8 Mya). The presence of two separate and non-overlapping fish faunas in the Iguazú again suggests a barrier within the river basin (as the Salto Moconá in the Uruguay river basin). This time, however, each fauna has a different sister group in a separate, but at the same time adjacent river drainage. The sister group of *A. kaaygua* is *A. tembe*, found in the adjacent arroyo Urugua–í river drainage (see Fig. 10) south from the lower río Iguazú where *A. kaaygua* is found. The divergence between the two species is 3.8% (*i.e.* 3.8–5.4 Mya). The sister group of *A. angiru* from the middle Iguaçu river drainage in Brazil is *A. minuano*, found in the middle río Uruguay river drainage, south from the middle rio Iguaçu. The divergence between the two species is 4.2% (*i.e.* 4.2–6.0 Mya). Not only are the relationships of the two non-related species from the río Iguazú drainage (*A. kaaygua* and *A. angiru*) with species in adjacent river drainages to the south (*A. tembe*, *A. minuano*), but also the estimated times of divergence closely match one another (3.8% *vs.* 4.2% divergence). This scenario is complicated by the fact that *A. angiru* occurs not only in the rio Iguaçu basin but also in the upper rio Uruguai basin. Absence of molecular data from the latter populations at the moment prohibits our understanding of additional details responsible for this distribution pattern.

The above described biogeographic and time-frame patterns are likely more than just coincidence. We believe that the fishes are starting to reveal some ancient history of the river drainages themselves. That waterfalls form barriers to dispersal, and that increasing height (and also age?) of the waterfalls increases isolation is evident from our data. Waterfalls in the case of *Australoheros* mostly divide unrelated species from each other. The two highest waterfalls (Cataratas de Iguazú, Salto Urugua–í) divide endemic species (*A. kaaygua* and *A. tembe*) from an unrelated species (*A. guaraní*) (Fig. 3). The same is true vice-versa, since *A. guaraní* is divided from these two species by the equally high Salto Monday in Paraguay (Fig. 10). None of the three species is known from the río Paraná itself below these three waterfalls (where *A. facetus* occurs because there is no barrier for its upstream migration through the río Paraná (see *A. facetus* A24, A25 in Fig. 2; cf. also Table 1). A rather low waterfall (Salto Moconá on the río Uruguay) on the other hand divides two sister species (*A. forquilha* and *A. ykeregua*). Unfortunatelly, we have so far no clue as to the localization of the barrier within the today heavilly dammed rio Iguaçu.

Prominent waterfalls thus in *Australoheros* generally divide unrelated species, while at the same time related species are in most cases separated by drainage divides. This suggests that waterfalls delimit the boundaries of a given fauna, while river captures and drainage translocations are responsible for the evolution of the diversity per se. Our data would thus suggest that the lower río Iguazú and the arroyo Urugua–í were once connected (*A. kaaygua vs. A. tembe*), as was the middle rio Iguaçu with the río Uruguay (*A. angiru vs. A. minuano*). The postulated connection between the lower río Iguazú and the arroyo Urugua–í is additionally supported by several other fish species or species pairs (*Astyanax leonidas*, *Glanidium riberoi*, *Hypostomus myersi*, *H. derbyi*, *Corydoras carlae*, *Crenicichla yaha vs. C.* cf. *yaha* [Casciotta *et al.* 2006b, Piálek *et al.* 2010] *Bryconamericus ikaa vs. B.* cf. *ikaa*) distributed only in the two river drainages. The connection between the middle rio Iguaçu and rio Uruguai is more enigmatic, to our knowledge so far supported only by the distribution of *A. angiru*, and lack of DNA data prohibits our knowledge of additional details of this distribution.

Diversity. Ten species of *Australoheros* are presently known from the Río de la Plata basin (Figs 1, 2, 3, 10) and 13 species from the Atlantic coast drainages of Brazil (Ottoni & Costa 2008; Ottoni *et al.* 2008; Ottoni & Cheffe 2009; Ottoni 2010). Neither the Río de la Plata basin nor the Atlantic coast drainage species of *Australoheros* seem to be a monophyletic group (Fig. 11). The little known *A.* sp. Jacui does not seem to be conspecific with *A. taura* (Ottoni & Cheffe 2009) from the same river drainage, and these two species are probably not related to the remaining species of the Atlantic coast drainages of Brazil (Fig. 11). *Australoheros facetus* seems to have phylogenetic affinities with the remaining species described from the Atlantic coast drainages of Brazil (Ottoni & Costa 2008; Ottoni *et al.* 2008; Ottoni 2010). The interspecific branch lengths between the Atlantic coast species (Ottoni & Costa 2008; Ottoni *et al.* 2008; Ottoni 2010) are much shorter than interspecific branch lengths between the

remaining species, and equal approximately intraspecific branch lengths within *e.g. A. ykeregua*, *A. angiru* or *A. scitulus* (Fig. 11). The Atlantic coast species also lack clear unique diagnostic characters (Ottoni & Costa 2008; Ottoni *et al.* 2008; Ottoni 2010; pers. obs.), which rises questions about the validity and the number of species involved. Under the two-step system of species delimitation employed in the present study (character- and tree-based delimitation), only one species instead of 11 species would be recognized. What is presently understood as *A. facetus* from Argentina and Uruguay shows a much higher diversity (judging from the branch lengths in Fig. 11) than the 11 species from the Atlantic coast of Brazil. Clearly, the *A. facetus* lineage of *Australoheros* (which probably includes the Atlantic coast species of Brazil), requires further study.

The identity of four nominal species, treated variously as synonyms of *A. facetus*, has variously been adressed in studies focusing on species from the Atlantic coast drainages. One of these names, *Heros jenynsii* Steindachner from Montevideo has been synonymized with *A. facetus* (Schindler et al., 2010). Another available name is *Heros acaroides* Hensel from Porto Alegre, Brasil. This nominal species was redescribed by Schindler et al. (2010). Our phylogenetic results (Fig. 11) support its separate status from *A. facetus*. The other two nominal species either have no precise locality (*Heros autochthon* Günther from "Brazil") or the locality is doubtfull (*Chromys oblonga* Castelnau from the rio Tocantins in Goiás, Brazil) and their status remains uncertain.

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APPENDIX 1: Morphological character list. Character 28 is used only in the 32 taxon phylogenetic analysis (Fig. 11).

1. Length of dorsal fin scale cover. states: long, reaching anterior insertion of dorsal fin [0]; intermediate, covering the bases of the middle portion of the hard part of the dorsal fin [1]; short, only covering the bases of the two last spines [2]; outgroup [0] –unordered.

Scale pattern along anterior dorsal fin border. states: scale row terminating with 1 small scale [0]; scale row terminating with 2 small scales arranged horizontally [1]. outgroup [?].

Scale rows between posterior end of upper lateral line and dorsal fin. states. 2 large 1 small or more [0]; 1 large and 1 of almost the same size, 1 additional small from 13–14th dorsal spine [1]; 1 large 1 small, 1 additional small from 13–14th dorsal spine [2]; 1 large 1 small, 1 additional small from 9th spine [3]. outgroup [0] –unordered.

Scale rows between anterior end of dorsal fin and upper lateral line. states. 5 [0]; 4 [1]; 3 [2]. outgroup [0]. –unordered.

Abdominal bars. states. 3 in all developmental steps and also in adults [0]; 4 in about 50% of juveniles, 3 in all adults [1]; 4 in about 50% of juveniles, 4 about 50% of adults [2]; 4 in all juveniles, 4 in more than 80% of adults, but only in less than 20% completely separated [3]; 4 in all juveniles, 4 in more than 80% of adults, completely separated in more than 80% of adults [4]. outgroup [0] -unordered

Distinct and dominant midlateral stripe between operculum and midlateral spot continuous, not fragmented into spots. states. no [0]; yes [1]. outgroup [?].

Large, dominant and well circumscribed midlateral blotch in juveniles and adults: no [1]; yes [0]. outgroup [0].

Caudal base spot. states: distinct, rounded spot [0]; weakly developed [1]; very narrow or completely missing [2]. outgroup [?]-unordered.

Midlateral stripe posterior from the midlateral blotch. states: running in scale rows 0 and E1 as anterior of the blotch [0]; The midlateral stripe runs in scale rows E0, E1 and E2 posterior to the midlateral blotch—*i.e.* the midlateral stripe gets wider posterior of the midlateral blotch [1]; midlateral stripe bend upwards posterior from the midlateral blotch—the blotch posterior to the midlateral stripe is centered in the same scale row as the midlateral bar (*i.e.* E1 scale row), and the last blotch is high on the body [2]; midlateral stripe bend upwards posterior from the midlateral blotch—the midlateral blotch is centered in the E1 scale row, while the next posterior blotch is centered in the E2 scale row and the blotch in the last body bar is centered in the E3 scale row. The midlateral stripe does not run in the 0 scale row posterior from the midlateral blotch [3]; outgroup [1] –unordered.

Midlateral stripe. states: without distinct borders [0]; clearly bordered [1]; outgroup [?]

Spots in scales arranged into stripes (at least one) also ventral from the 0 scale row. states: no [1]; yes, at least in the posterior part of the body [0]; outgroup [?]

Opalescent line below the circumorbital series. states: absent [0]; present [1]. outgroup [0].

Opalescent scales on body and head. states: absent [0]; present [1]. outgroup [0].

Checkerboard spotted unpaired fins (*i.e.* soft part of dorsal, caudal and soft part of anal fins). states: absent [0]; present [1]. outgroup [0].

Red ventral part of head, preoperculum and opercular membrane. states: absent [0]; present [1]. outgroup [0].

Opercular spots. states: absent [0]; present [1].

Pink body coloration. states: absent [0]; present [1].

Mouth position and size. states: mouth proportionally large, terminal [0]; mouth proportionally large, pointing down, lower jaw proportionally shorter [1]; mouth proportionally large, pointing up, lower jaw projecting in front of upper [2]; mouth very small, terminal or slightly pointing down [3]. –unordered.

Species develops thick lips. no [0]; yes [1].

Anal pterygiophores. Range 11–15. Frequency bins spaced at 0.2. states: 11.0–11.2 [0]; 11.2–11.4 [1]; ... [2,3,4,5,6,7,8,9,A,B,C,D,E,F,G,H,J,K]. –ordered.

Anal spines. Range 5–9. Frequency bins spaced at 0.2. states: 5.0–5.2 [0]; 5.2–5.4 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E,F,G,H,J,K]. –ordered.

Anal rays. Range 6–9. Frequency bins spaced at 0.2. states: 6.0–6.2 [0]; 6.2–6.4 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E,F,G,H,J,K]. –ordered.

Dorsal spines. Range 14–18. Frequency bins spaced at 0.2. states: 14.0–14.2 [0]; 14.2–14.4 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E,F,G,H,J,K]. –ordered.

Dorsal rays. Range 7–12. Frequency bins spaced at 0.2. states: 7.0–7.2 [0]; 7.2–7.4 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E,F,G,H,J,K,L,M,N,P,Q]. –ordered.

Dorsal total. Range 24–27. Frequency bins spaced at 0.2. states: 24.0–24.2 [0]; 24.2–24.4 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E]. –ordered.

Caudal vertebrae. Range 12–15. Frequency bins spaced at 0.2. states: 12.0–12.2 [0]; 12.2–12.4 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E]. –ordered.

Caudal peduncle vertebrae. Range -2-(+3.5). Frequency bins spaced at 0.2. states: -2-(-1.8) [0]; -1.8-(-1.6) [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E,F,G,H,J,K,L,M,N,P,Q]. –ordered.

Caudal peduncle length / caudal peduncle depth. Range 0.28–0.74. Frequency bins spaced at 0.2 states. 0.28–0.30 [0]; 0.30–0.32 [1]; … ...[2,3,4,5,6,7,8,9,A,B,C,D,E,F,G,H,J,K,L,M,N].]. –ordered.

Body depth / SL. Range 0.40–0.53. Frequency bins spaced at 0.1. states: 0.40–0.41 [0]; 0.41–0.42 [1]; ...[2,3,4,5,6,7,8,9,A,B,C]. –ordered.

Head width / HL. Range 0.44–0.64. Frequency bins spaced at 0.2. states: 0.44–0.46 [0]; 0.46–0.48 [1]; ...[2,3,4,5,6,7,8,9]. –ordered.

Interorbital distance / HL. Range 0.22–0.46. Frequency bins spaced at 0.2. states: 0.22–0.24 [0]; 0.24–0.26 [1]; ...[2,3,4,5,6,7,8,9,A,B]. –ordered.

Preorbital distance / HL. Range 0.10–0.36. Frequency bins spaced at 0.2. states: 0.10–0.12 [0]; 0.12–0.14 [1]; ...[2,3,4,5,6,7,8,9,A,B,C]. –ordered.

Pectoral fin length / SL. Range 0.24–0.36. Frequency bins spaced at 0.2. states: 0.24–0.26 [0]; 0.26–0.28 [1]; ...[2,3,4,5]. –ordered.

Ventral fin length / SL. Range 0.22–0.48. Frequency bins spaced at 0.2. states: 0.22–0.24 [0]; 0.24–0.26 [1]; ...[2,3,4,5,6,7,8,9,A,B,C]. –ordered.

Pectoral fin rays. Range 12–14. Frequency bins spaced at 0.2. states: 12.0–12.2 [0]; 12.2–12.4 [1]; ...[2,3,4,5,6,7,8,9]. –ordered.

E0 scales. Range 23–26. Frequency bins spaced at 0.2. states: 23.0–23.2 [0]; 23.2–23.4 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E]. –ordered.

L1 scales. Range 13–19. Frequency bins spaced at 0.4. states: 13.0–13.4 [0]; 13.4–13.8 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E]. –ordered.

L2 scales. Range 6–11. Frequency bins spaced at 0.2. states: 6.0–6.2 [0]; 6.2–6.4 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E,F,G,H,J,K,L,M,N,P,Q]. –ordered.

C1 gill rakers. Range 5–9. Frequency bins spaced at 0.2. states: 5.0–5.2 [0]; 5.2–5.4 [1]; ...[2,3,4,5,6,7,8,9,A,B,C,D,E,F,G,H,J,K]. –ordered.

APPENDIX 2. Morphological character matrix.

outgroup	0?000?1?1? ?000000?0K ??CQEER?90 63???EEQA
'A forquilha '	0100101110 1111100106 699HCCPF32 48226ECDC
'A ykeregua '	0101100110 110110010? 4AAHD{789}{HJKL}?01 5610BCAG{EF}
'A ykeregua ZSM '	0101100110 1101100101 478H89KE52 263369BFF
'A sp Jacui '	0110?00110 100000?101 1A4F54KC53 552364A98
'A tembe'	0000101111 0000000011 559949RK43 4622?9???
'A charrua'	1001011231 0000001307 A8BDA9A665 66434BAC5
'A kaaygua'	1?20{123}01120 0000000{02}0? 429604?J34 47331C7H4
'A angiru'	1021011221 0000000306 979C75D593 653425BA4
'A angiru 6262'	1021011221 0000000305 96AB64C?95 654602A82
'A angiru Misiones'	1021011221 0000000305 96A954779{345} 65{34}{456}{012}{2345}{AB}{89A}{234}
'A angiru Iguacu'	1022011221 0000000308 989B63339{345} 65{34}{456}{012}{2345}{AB}{89A}4
'A scitulus'	1011011221 000001030C H5EA9AB764 434359AA5
'A scitulus Quarai'	1011011221 000001030B G5E9999674 664459AF4
'A minuano'	1021200011 0000001008 6CAD949664 5333058D4
'A guarani'	1022100101 1000000005 599C74D795 873444979
'A facetus 6arg'	2022300001 0000000206 6A9E94C595 6534859FD
'A facetus 6uru'	2022300001 0000000205 5C9FA6A584 543487ACE
'A facetus 7TyT'	2021400001 0000000208 A8BB76FA65 53335677C
'A facetus 7can'	2021400001 0000000209 A8AB65FA45 4233476BC
'A capixaba'	????{12}0010? 000000020? 997G84?G53 9?359B9H?
'A taura'	????{12}00101000000110?{56789}9{ABCDE}{ABCDEFGHJ}?4?C13 9?33{56789}{ABCDEFGHJKLMNPQ}{789}{ABCDEFGHJK}?
'A ribeirae'	2???{12}000?0000000020?{56789}99{ABCDE}?4?9{789A}{234} {9AB}??{567}9{56789ABCDE}{789ABC}{56789ABCDE}?
'A autrani'	2???{12}10100000000020?{ABCDE}{FGHJK}{56789}{FGHJKLMNPQ}?4?J{56789A}{45} {9ABC}?{01234}{456}9{ABCDEFGHJKLMPQ}{56789ABC}{0123456789ABCDEFGHJK}?
'A saquarema'	2???{12}???????????0?0?9E9{FGHJK}?4?{6789ABC}{45678}{45} {AB}?{123456}{789}9{ABCDEFGHJK}{ABC}{56789ABCDE}?

'A macacuensis'	2???{12}00000000000020?{ABCDE}{ABCDE}9{FGHJK}?4?{3456789A}{6789}{456}{789}?{123456}{67}9{ABCDEFGHJKLMNPQ}{56789}{56789ABCDE}?
'A ipatinguensis'	2???{12}00200000000020?9E4{FGHJK}?4?{78}{789A}{123}{78}?{345}{3456789ABC}9{ABCDE}{789}{56789ABCDE}?
'A barbosae'	2???{12}00100000000020?{ABCDE}{FGHJK}9{FGHJK}?4?{3456789AB}{456789}{345}{9ABC}?{2345}{234567}{ABCDE}{KLMNPQ}{56789}{56789ABCDE}?
'A paraibae'	2???{12}00000000000020?{ABCDE}{ABCDE}{56789}E?4?{678}{23456}{234}{9A}?{123}{23456}{56789}{FGHJKLMNPQ}{789}{56789ABCDE}?
'A macaensis'	2???{12}00000000000020?{ABCDE}{ABCDE}9{FGHJK}?4?{ABCDEF}{45678}{3456}{9ABC}?{2345}{2345678}9{ABCDEFGHJKLMNPQ}{789}{ABCDEFGHJK}?
'A robustus'	2???{12}00000000000020?{ABCDE}{56789}E{56789}?4?{ABC}{345}{3456}{9ABC}?{34}{2567}{56789}{FGHJKLMNPQ}{789}{56789}?
'A muriae'	2???{12}00{01}00000000020?{56789ABCDE}{FGHJK}4{LMNPQ}?4?9{3456789A}{345678}{89ABC}?{2345}?{ABCDE}{LMNPQ}{789}{FGHJKLMNPQ}?
'A acaroides'	2???{34}00{01}?0 000000020? 99BB74?G54 A?22628E4
'A sp Sao Francisco'	2???{12}00?00 000000020B AAAHD44??? ?????????
'A sp Lagoa Nova'	2???{12}00?00 000000020B 9B7E627??? ?????????
'A sp Paraiba'	2???{12}00?00 000000020C 9C9E945??? ?????????
'A sp Rio Muriae'	2???{12}00?00 000000020A 994F566??? ?????????
'A sp Rio Tubarao'	2???{12}00?00 0000000207 A79C74G??? ?????????

Paper IV

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Multilocus phylogeny of *Crenicichla* (Teleostei: Cichlidae), with biogeography of the *C. lacustris* group: Species flocks as a model for sympatric speciation in rivers

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ABSTRACT

First multilocus analysis of the largest Neotropical cichlid genus *Crenicichla* combining mitochondrial (cyt*b*, ND2, 16S) and nuclear (S7 intron 1) genes and comprising 602 sequences of 169 specimens yields a robust phylogenetic hypothesis. The best marker in the combined analysis is the ND2 gene which contributes throughout the whole range of hierarchical levels in the tree and shows weak effects of saturation at the 3rd codon position. The 16S locus exerts almost no influence on the inferred phylogeny. The nuclear S7 intron 1 resolves mainly deeper nodes. *Crenicichla* is split into two main clades: (1) *Teleocichla*, the *Crenicichla wallacii* group, and the *Crenicichla lugubris*–*Crenicichla saxatilis* groups ("the TWLuS clade"); (2) the *Crenicichla reticulata* group and the *Crenicichla lacustris* group–*Crenicichla macrophthalma* ("the RMLa clade"). Our study confirms the monophyly of the *C. lacustris* species group with very high support. The biogeographic reconstruction of the *C. lacustris* group using dispersal-vicariance analysis underlines the importance of ancient barriers between the middle and upper Paraná River (the Guaíra Falls) and between the middle and upper Uruguay River (the Moconá Falls). Our phylogeny recovers two endemic species flocks within the *C. lacustris* group, the *Crenicichla missioneira* species flock and the herein discovered *Crenicichla mandelburgeri* species flock from the Uruguay and Paraná/Iguazú Rivers, respectively. We discuss putative sympatric diversification of trophic traits (morphology of jaws and lips, dentition) and propose these species flocks as models for studying sympatric speciation in complex riverine systems. The possible role of hybridization as a mechanism of speciation is mentioned with a recorded example (*Crenicichla scottii*).

1. Introduction

Crenicichla is the most species rich genus within the Neotropical Cichlidae (e.g. Kullander and Lucena, 2006; Casciotta et al., 2010; Kullander et al., 2010; Piálek et al., 2010). At present 85 species are considered valid (http://www.fishbase.org) but possibly half as many species are known and remain to be formally described (Stawikowski and Werner, 2004; http://www.cichlidae.com). *Crenicichla* has a widespread distribution in cis-Andean South America, ranging from Trinidad and the Orinoco basin to the Negro River in Patagonia, Argentina (Casciotta, 1987; Kullander et al., 2010), with a comparatively high diversity in the subtropical regions of South America (the *Crenicichla lacustris* group). Kullander (1988) described several rheophilic species inhabiting the Brazilian and Guiana shield tributaries of the lower Amazon as a new genus, *Teleocichla* (seven valid species), but other authors (Ploeg, 1991; López-Fernández et al., 2010) considered *Teleocichla* an ingroup of *Crenicichla*.

Crenicichla is traditionally divided into five species groups (Kullander, 1981, 1982, 1986; Ploeg, 1991; Stawikowski and Werner, 2004; Kullander et al., 2010): the *C. lacustris* group (with 28 valid species), the *Crenicichla lugubris* group (15), the *Crenicichla reticulata* group (9), the *Crenicichla saxatilis* group (25), and the *Crenicichla wallacii* group (7); the classification of the type species *Crenicichla macrophthalma* in respect to these groups remains unclear. The species groups are mostly defined by the color pattern, several meristic characters, and geographic distribution. The monophyly of the proposed species groups is uncertain, and their interrelationships are at present virtually unknown. So far, the phylogenetic relationships within *Crenicichla* were studied only by Kullander et al. (2010) who provided a partial and largely unresolved phylogeny of the genus, based on a single mitochondrial marker (cyt*b*), and separated a new *Crenicichla missioneira* species group from the *C. lacustris* group.

Most of the species groups of *Crenicichla* are largely sympatric, with distribution being centered in the Amazon and Orinoco drainages. The *C. lacustris* species group is, however, allopatric, distributed in the Río de la Plata basin (the Paraná and Uruguay Rivers) and in the Atlantic coastal drainages. The Uruguay River drainage is inhabited by 11 endemic or nearly endemic species of this group

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in two species complexes (Lucena and Kullander, 1992; Lucena, 2007): (1) the *C. missioneira* complex including *Crenicichla celidochilus*, *Crenicichla empheres*, *Crenicichla hadrostigma*, *Crenicichla igara*, *Crenicichla jurubi*, *Crenicichla minuano*, *C. missioneira*, *Crenicichla tendybaguassu*; (2) the *Crenicichla scottii* complex with *Crenicichla gaucho*, *Crenicichla prenda*, and *C. scottii* (the last also entering the lower Paraná River). The Paraná River drainage itself hosts 10 endemic species of this species group (Casciotta et al., 2010; Piálek et al., 2010): *Crenicichla haroldoi*, *Crenicichla hu*, *Crenicichla iguassuensis*, *Crenicichla jaguarensis*, *Crenicichla jupiaensis*, *Crenicichla mandelburgeri*, *Crenicichla niederleinii*, *Crenicichla tesay*, *Crenicichla yaha*, and *Crenicichla ypo*. Another species of the *C. lacustris* group, *Crenicichla vittata*, occurs both in the Paraná and Uruguay River basins. The coastal drainages of Brazil and Uruguay are inhabited by six endemic species (Kullander and Lucena, 2006): *Crenicichla iguapina*, *C. lacustris*, *Crenicichla maculata*, *Crenicichla mucuryna*, *Crenicichla punctata*, and *Crenicichla tingui*.

The aim of our study is to provide the first large-scale multilocus phylogeny of *Crenicichla* (including *Teleocichla*) with a special focus on the historical biogeography and possible speciation modes of the diverse *C. lacustris* group, in the latter case using almost complete taxon sampling. While the reasons for the pronounced diversity of *Crenicichla* remain unstudied we will argue that two sets of factors are likely responsible for the high diversity of the *C. lacustris* species group in the subtropical region of the Brazilian shield in particular.

The first factor is likely the complex geological and biogeographical history of the area. This factor recently gained support in several studies. Albert and Carvalho (2011) have found in their Brooks parsimony analysis (BPA) of 43 South American freshwater ecoregions using species-level phylogenies of 32 fish clades that while in the Amazon and other regions of northern South America the analysis recovers continuous areas as monophyletic, this was not the case in the La Plata and Atlantic coastal drainages. In the Amazon and northern South America the major biogeographic patterns thus appear to have been established in association with the formation of the modern basin boundaries during the Neogene. By contrast, biogeographic patterns of fish clades in the La Plata basin and Atlantic coastal drainages are either older than the present basin configuration thus reflecting past river configurations (e.g. Říčan et al., 2011), or are younger, indicating a history with more geodispersal (i.e. erosion of barriers to dispersal; e.g. Ribeiro, 2006; Menezes et al., 2008; Torres and Ribeiro, 2009), or perhaps with more extinction (e.g. Malabarba, 1998). Říčan et al. (2011) have found indications for past drainage configurations and explained the diversity and endemism in the cichlid genus *Australoheros* in the La Plata basin predominantly by the orogeny of the present drainage divides. Migration barriers on the other hand mostly divided unrelated faunal elements further supporting the notion that changes in watershed boundaries, not major rapids and waterfalls are the primary responsible force driving diversification. Rapids and waterfalls however seem significant in promoting additional diversification within drainages.

As a second factor offering possible explanation of the large diversity of *Crenicichla* are indications for the existence of species flocks similar to those known from lacustrine habitats in the lakes of the East African Rift Valley (e.g. Salzburger and Meyer, 2004; Kocher, 2004), Cameroon (Schliewen, 2005) or Middle America (e.g. Barluenga et al., 2006; Geiger et al., 2010). The cichlid species flocks, contrary to previous evidence, however appear not to be limited to lacustrine habitats, but are also present in complex riverine habitats such as in the *C. lacustris* species group in the upper La Plata basin (the Paraná and Uruguay River drainages), in *Crenicichla* and *Teleocichla* in the large Amazonian rapids (e.g. Kullander, 1988) or in *Steatocranus* and *Nanochromis* cichlids in the mighty Lower Congo rapids in Africa (e.g. Schwarzer et al., 2011). *Crenicichla* (including *Teleocichla*) appears to be a genus prone to undergo complicated speciation patterns in complex riverine habitats, and its diversity in the La Plata basin seems to be augmented by the historical complexity of the area itself.

2. Material and methods

2.1. Taxon sampling

Our study focuses on the phylogeny of *Crenicichla* at two levels and our taxon sampling reflects this goal. On the large-scale level of *Crenicichla* phylogeny, representatives of all species groups were sampled (including *Teleocichla*). As most species groups (with the notable exception of the *C. lacustris* group) are largely sympatric in the Amazon basin and northern South America and their species have very often large distribution areas, even a relatively small geographic area can provide a representative species sampling. At the level of the *C. lacustris* group we have included almost all known species, many with multiple samples from different localities and our sampling is thus well balanced taxonomically and geographically.

In total our study includes sequences of 169 terminals representing 43 valid species (including outgroups). Sequences of 134 specimens representing 30 species are newly sequenced and the remaining obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank). Most of the novel samples were obtained during field expeditions to the Misiones province (Argentina) and adjacent drainages in Paraguay in 2007, 2009, and 2010. Several additional samples were acquired from the aquarium trade (Supplement Table 1). Voucher specimens for the *C. lacustris* group species are deposited in the Museo Argentino de Ciencias Naturales (MACN) and Asociación Ictiológica La Plata (AI) under the catalog numbers given in Supplement Table 1.

Within the *C. lacustris* group we encountered several ambiguities in determination of the sampled specimens. The specimens of the *C. missioneira* complex (especially *C. missioneira* and *C. minuano*), diagnosis of which is based mainly on proportions in jaw lengths, often displayed intermediate states. The ordination analyses of Lucena and Kullander (1992) show, in addition, a large-scale overlap between both species and *C. tendybaguassu*. Following Lucena and Kullander (1992), we thus name specimens with a prognathous lower jaw as *C. missioneira*, and those with isognathous jaws or a prognathous upper jaw as *C. minuano*, although we find a continuum between the two extremes. Similarly, *C. mandelburgeri* and *C. niederleinii* were distinguished by the E1 number of scales in the row immediately above that containing the lower lateral line (44–56 vs. 56–65; see Kullander, 2009).

2.2. Outgroup selection

Several successive outgroups based on the studies of Smith et al. (2008) and López-Fernández et al. (2010) were used to root our phylogeny. The outgroup taxa included *Acarichthys*, *Astronotus*, *Biotoecus*, *Crenicara*, *Dicrossus*, *Geophagus*, and *Satanoperca* (Supplement Table 1). *Cichla*, a postulated sister group of *Crenicichla* based on morphological characters (Kullander, 1998), was also included among the outgroup taxa although it is invariably recovered as only distantly related to *Crenicichla* in all molecular or combined morphological-molecular analyses (e.g. Farias et al., 1999, 2000, 2001; Sparks, 2004; Smith et al., 2008; López-Fernández et al., 2010).

2.3. DNA isolation, PCR, and sequencing

We used three mitochondrial (cyt*b*, ND2, 16S) and one nuclear (ribosomal protein S7 intron 1, "S7-i1" hereinafter) loci. All four markers are widely used in the phylogenetic studies of cichlid

fishes (e.g. Wimberger et al., 1998; Farias et al., 1999, 2000, 2001; Willis et al., 2007; Říčan et al., 2008; Musilová et al., 2009; Kullander et al., 2010; López-Fernández et al., 2010), which enabled us to combine our dataset with sequences from previous studies.

Genomic DNA was extracted from ethanol-preserved gill or fin tissue using the JETQUICK Tissue DNA Spin Kit (Genomed) following standard protocol. The primers and reaction conditions of PCR amplification for all loci are given in Table 1. Each PCR reaction volume of 25 μl contained 12.5 μl of Combi PPP Master Mix (Top-Bio, http://www.top-bio.cz), 1.5 μl of each primer (10 pmol/μl), and 1 μl of extracted DNA. PCR reactions were performed in a Bioer XP Thermal Cycler and PCR products were purified using the JETQUICK PCR Purification Spin Kit (Genomed). Sequencing reactions were performed following standard protocol with the use of primers listed in Table 1, and the products were analyzed in an ABI 3730XL automated sequencer (Applied Biosystems; both steps done by Macrogen Inc., Korea). Contiguous sequences of the gene segments were created by assembling DNA strands (forward and reverse) using BioLign 4.0.6.2 (Hall, 2001). All sequences were submitted to GenBank under Accession Nos. JF519856–JF520391 (Supplement Table 1).

2.4. Alignment

Sequences were edited in BioEdit 7.0.9 (Hall, 1999), and aligned using MUSCLE ver. 3.8 (Edgar, 2004) with default settings. The 16S and S7-i1 markers were additionally realigned (option "refine"; no subjective "by-eye" treatment was applied to the resulting alignments). BMGE software (Criscuolo and Gribaldo, 2010) was used to investigate the informativeness of the 16S and S7-i1 datasets in order to identify sites with ambiguous alignment or mutational saturation effect. Gaps were treated as integral parts of these two loci and therefore no default cut-off of characters was applied (value of the option changed to "g 1.0"). Separate alignments of individual loci were assembled together into a final phylogenetic matrix by a computer program created in Borland Delphi (Borland Delphi for Microsoft Windows, version 10, 2005. Borland Software Corporation), written by the first author.

2.5. Phylogenetic methods

We arbitrarily defined significant support values above which we consider a node to be "well supported"; they are 0.95 for posterior probability in Bayesian analysis, 75% for bootstrap values (both maximum parsimony and maximum likelihood analyses), and 1 for Bremer support.

To obtain a time estimate for several of the discussed cladogenetic events we translated uncorrected pairwise divergences in the cyt*b* gene into time units. With respect to considered higher evolutionary rates in geophagine cichlids (e.g. Farias et al., 1999, 2000, 2001; Smith et al., 2008) we have used a 2% divergence rate per My (Pereyra and García, 2008) instead of a 1% divergence rate used in other Neotropical cichlid fish groups (e.g. Concheiro Pérez et al., 2007).

Uncorrected pairwise divergences were counted in PAUP* with the use of the command "showdist".

2.5.1. Maximum parsimony (MP)

MP tree construction was done in PAUP* ver. 4.0b10 (Swofford, 2003). Heuristic searches were performed to find the most parsimonious tree(s) using tree bisection-reconnection (TBR) branch-swapping, and 100 random sequence addition replicates with equal weight for all sites.

Node support was estimated using nonparametric bootstrapping (Felsenstein, 1985), and by Bremer support (BS; Bremer, 1988, 1994) and partitioned Bremer support indices (PBS; Baker and DeSalle, 1997; Baker et al., 1998). Bootstrapping was performed with 1000 total pseudoreplicates and TBR branch-swapping with 10 random sequence addition replicates per pseudoreplicate. BS and PBS were computed using a Borland Delphi based software, written by the first author, implementing the algorithm described by Baker and DeSalle (1997) and utilizing PAUP* to perform the search of constrained MP trees. Relative PBS values were computed as a ratio between a PBS value and the sum of absolute values of all PBS with the same sign for the given node.

The PBS indices can be substantially biased and incorrect if the dataset is incomplete, lacking an entire character partition for some taxon (pers. obs.). We therefore prepared a reduced dataset containing exclusively taxa with all four loci available (see Supplement Table 1); this dataset with 133 taxa and 3183 characters was used for the PBS analyses.

2.5.2. Bayesian analysis (BA)

MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was used for the Bayesian inference of

Table 1
Primers, PCR conditions, alignment characteristics, and estimated substitution models for loci used in phylogenetic analyses. F = forward primer, R = reverse primer, A = amplifying primer, S = sequencing primer; Ts/Tv = transition/transversion ratio.

Locus	Primers			PCR conditions	Alignment length	Pars. informative chars excluding outgroup	Ts/Tv	Nucleotide-substitution model
	Name	Type	Sequence					
cyt *b*	BaccytB-R	R-AS	CCGGCCTCCGGCTTACAAGGCCG	94 °C, 15 s; 50–55 °C, 30 s; 72 °C, 50–70 s	1049	426 (41%)	3,01	GTR + I + Γ
	CytBl-1F	F-A	CGATTCTTCGCATTCCACTTCCT					
	CytBl-3R	R-A	GGGGTAAAGTTGTCTGGGTCTCC					
	CytBl-7F	F-A	CTAACCCGATTCTTTGCCTTCCACTTCCT					
	FishcytB-F	F-AS	ACCACCGTTGTTATTCAACTACAAGAAC					
	GLUDG	F-AS	CGAAGCTTGACTTGAARAACCAYCGTTG					
	GLuDG.L	F-AS	TGACTTGAARAACCAYCGTTG					
	H15915	R-AS	AACTGCAGTCATCTCCGGTTTACAAGAC					
	L14725	F-AS	CGAAGCTTGATATGAAAAACCATCGTTG					
	TruccytB-R	R-AS	CCGACTTCCGGATTACAAGACCG					
ND2	ASN	R-AS	CGCGTTTAGCTGTTAACTAA	94 °C, 15 s; 50 °C, 30 s; 72 °C, 90 s	1047	435 (42%)	2,33	GTR + I + Γ
	ILE	F-AS	CCGGATCACTTTGATAGAGT					
16S	16SAR	F-AS	CGCCTGTTTATCAAAAACAT	94 °C, 15 s; 49 °C, 30 s; 72 °C, 45 s	549	113 (21%)	1,97	GTR + I + Γ
	16SBR	R-AS	CCGGTCTGAACTCAGATCACGT		526[a]	109 (21%)[a]	2.04[a]	GTR + I + Γ[a]
S7-i1	S7-1F	F-AS	TGGCCTCTTCCTTGGCCGTC	94 °C, 15 s; 60 °C, 30 s; 72 °C, 45 s	545	52 (10%)	1,43	HKY + Γ
	S7-2R	R-AS	AACTCGTCTGGCTTTTCGCC					
All					3190	1135 (31%)	2,46	

[a] Locus 16S modified (characters with more than 10% of gaps removed).

phylogeny. An optimal model of evolution for each locus according to Akaike criterion was selected using MrModelTest 2.2 (Nylander, 2004). The Bayesian analysis using the Markov chain Monte Carlo simulation was run with unlinked parameters (except for branch length and topology) for 5 and 8.5 million generations for single loci and the complete dataset, respectively. Trees were sampled and saved every 100 generations (50,000 and 85,000 trees saved per run, respectively). Several independent analyses, each comprising two runs with four chains, were performed using the computational facilities of the Computational Biology Service Unit of Cornell University (http://cbsuapps.tc.cornell.edu).

The first 25–50% of trees from each run before reaching equilibrium were discarded as burn-in. Convergence between the two runs was estimated with the use of: (1) diagnostic criteria produced by the "sump" command in MrBayes; (2) graphical exploration of MCMC convergence in the AWTY online program (Wilgenbusch et al., 2004); (3) graphical visualization and diagnostics in Tracer ver. 1.5.0 (Rambaut and Drummond, 2007). The remaining trees were used for reconstruction of the 50% majority-rule consensus tree with posterior probability (PP) values of the relevant branches displayed by the "sumt" command.

2.5.3. Maximum likelihood (ML)

PhyML 3.0 (Guindon and Gascuel, 2003) was used to reconstruct ML phylogenetic trees. The computations were partially executed online (http://www.atgc-montpellier.fr/phyml). Separate ML analyses of single loci were performed with the same models as selected for the BA, the multilocus analysis was done with one general model (GTR + I + Γ) for all sites. Both analyses were run with empirical estimation of base frequencies. To evaluate statistical branch supports, nonparametric bootstrapping was used with 1000 replicates for single loci and 100 replicates for the complete dataset.

2.5.4. Saturation of loci

To estimate the saturation level of each locus, (1) the expected transition/transversion (Ts/Tv) ratio was estimated in PAUP* by the command "lscore" (model F84, computed from the neighbor-joining tree obtained in PAUP*); (2) saturation plots of uncorrected pairwise divergences were constructed in MS Excel.

2.6. Biogeographic analysis of the C. lacustris species group

In order to interpret the inferred phylogeny of the *C. lacustris* group in terms of biogeography, we used the RASP software (Reconstruct Ancestral State in Phylogenies; Yu et al., 2011). This software tool evaluates the alternative ancestral ranges at each node in a tree statistically, accounting for uncertainties both in phylogenetic inference and in biogeographic optimization. The software complements DiVA (Ronquist, 1997) including the utilities based on methods of Nylander et al. (2008) and Harris and Xiang (2009).

In total 10 areas of endemism (Resende, 2003; Zaniboni Filho and Schulz, 2003; Albert and Carvalho, 2011) were used for the biogeographic reconstruction of the *C. lacustris* species group: (A) Northern coastal rivers, (B) Southern coastal rivers, (C) Lower Uruguay, (D) Middle Uruguay, (E) Upper Uruguay, (F) Lower Paraguay, (G) Lower Paraná, (H) Middle Paraná, (I) Iguazú, and (J) Upper Paraná. The areas are defined by endemism in most cases and are delineated primarily by watershed boundaries. Within the thus delineated hydrogeographic basins significant changes in landscape physiognomy, often accompanied by significant migration barriers further delimit smaller areas. The barriers are in the form of large rapids and/or significant waterfalls. The Iguazú Falls (Cataratas del Iguazú, C. do Iguaçu) delimit the Iguazú from the Middle Paraná (H/I), the Apipé Falls (Saltos de Yacyretá-Apipé; today replaced by the Yacyretá hydroelectrical dam) the Lower Paraná from Middle Paraná (G/H), the Guaíra Falls (Saltos del Guairá, Salto das Sete Quedas do Guaíra; today replaced by the Itaipu hydroelectrical dam) the Middle Paraná from Upper Paraná (H/J), the Salto Grande falls (today replaced by the Salto Grande dam) the Lower Uruguay from Middle Uruguay (C/D), and the Moconá Falls (Saltos del Moconá, Salto do Yucumã) the Middle Uruguay from Upper Uruguay (D/E).

For the purpose of the RASP reconstruction, an additional run of Bayesian analysis of the multilocus dataset including 118 taxa (Supplement Table 1) and using the same models as in Section 2.5.2, was performed in MrBayes (with unlinked parameters, except for the branch length and topology, 8 mil. generations with 3 mil. burn-in, sampled each 5000 generations).

3. Results

3.1. Alignment characteristics

The complete dataset includes 602 sequences of individual genes (534 of which are new) representing 169 taxa and 3190 characters. The alignment characteristics as well as the nucleotide-substitutions models inferred for each dataset are listed in Table 1. Translation of the coding sequences (cyt*b* and ND2) into amino acids displayed no stop codons or frame shifts. The BMGE software did not identify any sites with ambiguous alignment or mutational saturation effect in 16S and S7-i1 loci. Saturation plots reveal a very weak saturation of the third codon position of ND2 and a stronger saturation in the cyt*b* (not shown), as do values of expected Ts/Tv ratios (Table 1).

3.2. Tree reconstruction

Bayesian and ML analyses of the combined dataset yielded robust and almost identical phylogenetic hypotheses on the relationships within *Crenicichla*. There are no significant conflicts between the topologies obtained from analyses of the complete dataset by the three different methods (see next section). The BA topology (Fig. 1) differs slightly from the ML topology within three species complexes (the *C. missioneira*, *C. scottii*, and *C. mandelburgeri* complex), and within the species *C. lacustris*. The MP analysis resulted in a very large number of equally parsimonious trees (length 6470; consistency index excluding uninformative characters 0.34; retention index 0.85). The node supports obtained from MP/ML bootstrap and Bayesian analyses (all computed both separately for each locus and for the combined dataset) as well as Bremer and partitioned Bremer supports for the MP tree (not shown) are given in Table 2.

3.3. Contributions of individual loci to the combined tree topology and congruence

All phylogenetic analyses were applied to the combined dataset and to each locus separately in order to examine the contribution of each locus to the inferred phylogeny (Table 2). In addition, the influence of individual loci on the final hypothesis was studied, using a relationship between the relative value of the partitioned Bremer support (see Section 2.5.1) and the cumulative branch length of each node measured from the tree root (Fig. 2). This comparison, in congruence with Table 2, revealed that deep nodes (i.e. relationships between species groups) are supported mainly by the S7-i1 and ND2 loci, while intermediate and terminal nodes (corresponding roughly to interspecific and intraspecific relationships) are supported mainly by the ND2 and cyt*b* loci. The contributions of individual loci in terms of PBS values fully agree with the observed saturation in cyt*b* sequences.

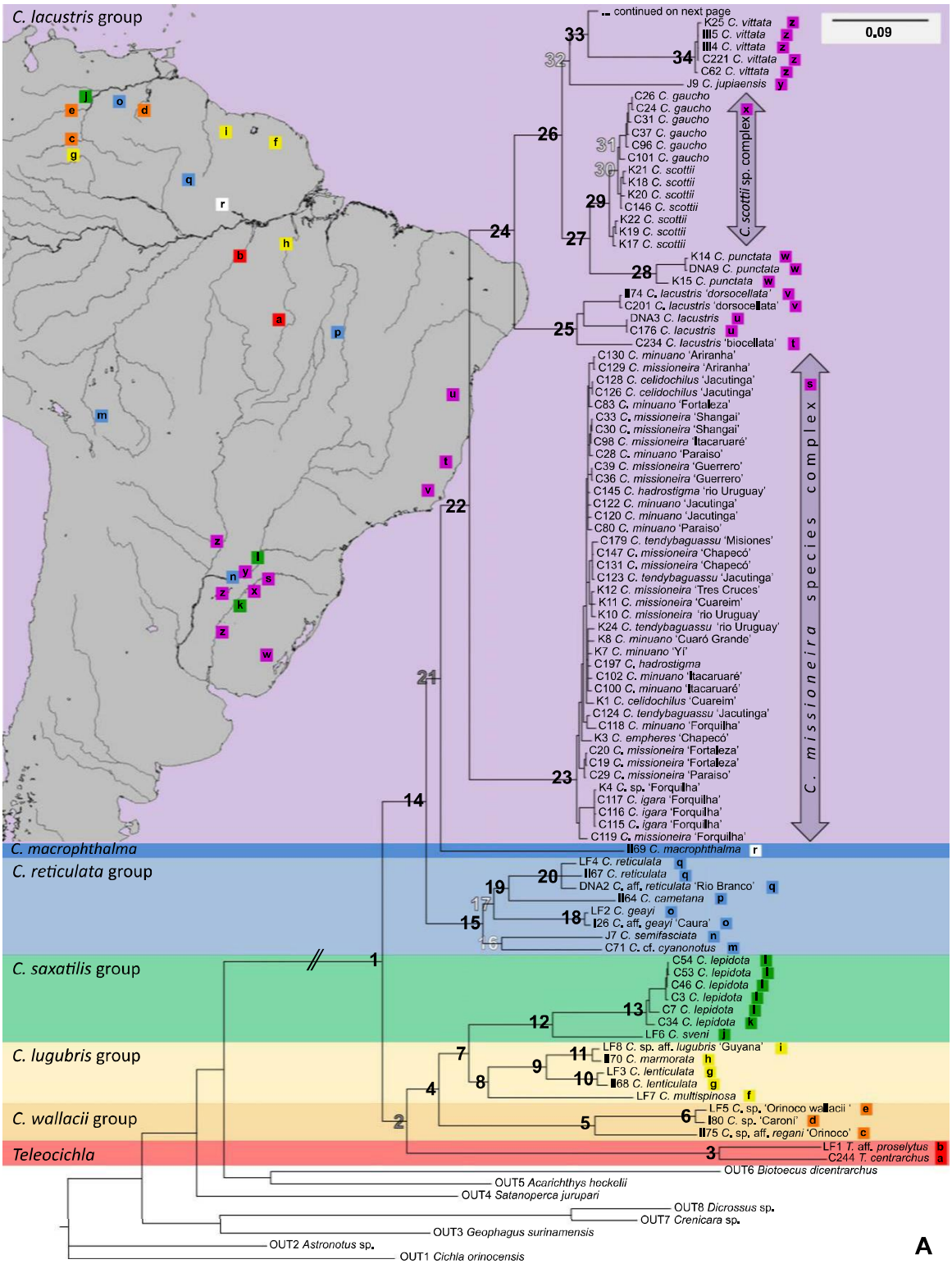

Fig. 1. Phylogenetic relationships of *Crenicichla* inferred from BA analysis of the combined dataset. Nodes with black numbers are well supported (PP ⩾ 0.95), gray numbers indicate nodes well supported in the dataset with reduced or removed 16S locus, white numbers indicate nodes with PP < 0.95. Specimens primarily determined as *C. niederleinii* are indicated by E1 counts as part of their taxon names.

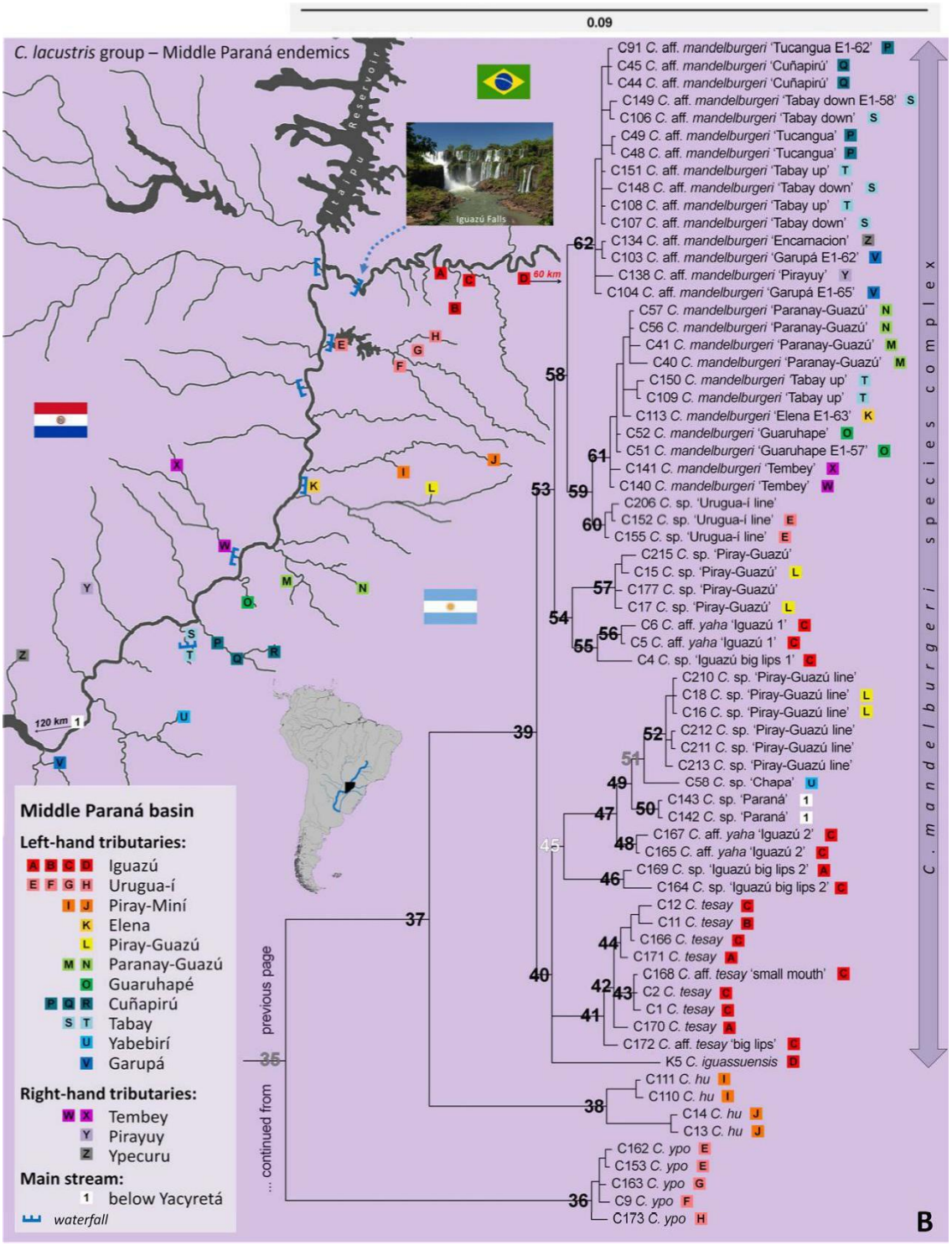

Fig. 1 (*continued*)

The results show negligible or even slightly negative effect of the 16S marker on the support values of the multilocus phylogeny, especially at deep phylogenetic levels (Fig. 2, Table 2). The alignment of the 16S sequences was therefore further modified by removing those segments with more than 10% of gaps (corresponding mainly to the "loop" segments with weak evolutionary constraints), and a new Bayesian analysis of the modified 16S locus was computed, as were new combined analyses either with the

Table 2
Node supports obtained by different phylogenetic methods. Node numbers refer to Fig. 1. "x" indicates conflict between tree inferred by the respective method/locus and BA multilocus tree presented in Fig. 1, numbers in parentheses express the support value of the alternative topology; "–" indicates unresolved nodes or a weakly supported alternative topology (no conflict); "NA" means not applicable due to missing sequences relevant for this node.

Node	Taxa included	Description	Bayes							MP bootstrap					MP bootstrap						PBS (reduced dataset)				
			All	All[a]	All[b]	cyt*b*	ND2	16S	S7-i1	All	cyt*b*	ND2	16S	S7-i1	All	cyt*b*	ND2	16S	S7-i1	BS	All	cyt*b*	ND2	16S	S7-i1
1	C244–C91	Genus *Crenicichla*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	100	99	100	100	99	100	100	100	96	99	42	90	21	41	15	14
2	C244–C54	TWLuS clade	0.92	0.96	0.99	1.00	x (0.98)	–	1.00	57	80	–	–	86	–	–	–	–	82	–	–	–	–	–	–
3	C244–LF1	Genus *Teleocichla*	1.00	1.00	1.00	1.00	NA	1.00	1.00	100	100	NA	100	100	100	100	NA	100	100	66	NA	NA	NA	NA	NA
4	II75–C54	WLuS clade	0.99	1.00	0.95	–	–	–	–	71	–	36	25	–	–	54	–	–	–	3	4	−2	5	0	1
5	II75–LF5	*C. wallacii* group (W)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	100	100	100	100	92	100	100	100	100	89	59	60	25	17	15	3
6	I80–LF5	Internal W node	1.00	1.00	1.00	1.00	NA	1.00	0.99	100	100	NA	100	64	100	100	NA	100	62	52	NA	NA	NA	NA	NA
7	LF7–C54	LuS clade	1.00	1.00	0.99	0.94	0.76	0.70	–	94	74	60	38	–	92	89	–	–	–	9	3	−5	7	−1	1
8	LF7–LF8	*C. lugubris* group (Lu)	0.98	1.00	0.99	–	NA	–	1.00	81	–	NA	–	93	57	–	NA	–	89	1	NA	NA	NA	NA	NA
9	II68–LF8	Internal Lu node	1.00	1.00	1.00	1.00	1.00	1.00	0.96	100	100	98	100	65	100	98	99	99	63	21	39	13	13	9	4
10	II68–LF3	*C. lenticulata*	1.00	1.00	1.00	1.00	NA	0.62	–	100	100	NA	55	–	100	100	NA	70	–	27	NA	NA	NA	NA	NA
11	II70–LF8	Internal Lu node	1.00	1.00	1.00	1.00	NA	1.00	x (0.99)	100	99	NA	97	–	100	100	NA	99	–	23	NA	NA	NA	NA	NA
12	LF6–C54	*C. saxatilis* group (S)	1.00	1.00	1.00	1.00	NA	1.00	1.00	100	98	NA	91	100	100	96	NA	90	98	20	NA	NA	NA	NA	NA
13	C34–C54	*C. lepidota*	1.00	1.00	1.00	1.00	1.00	1.00	–	100	100	100	96	–	100	100	100	99	–	45	117	45	50	17	5
14	C71–C91	RMLa clade	1.00	1.00	1.00	–	0.97	0.51	1.00	97	47	83	45	98	71	–	52	–	95	3	8	−6	5	4	4
15	C71–LF4	*C. reticulata* group (R)	1.00	1.00	1.00	1.00	1.00	0.96	0.75	100	99	99	70	52	100	91	96	64	–	23	25	12	13	1	−1
16	C71–J7	Internal R node	0.89	0.94	0.79	0.99	0.90	–	x (1.00)	44	66	75	–	x (79)	60	65	–	–	–	2	4	7	2	−2	−3
17	I26–LF4	Internal R node	0.84	0.80	–	–	0.90	–	x (1.00)	67	–	79	–	x (79)	–	–	–	–	–	–	2	−3	3	1	1
18	I26–LF2	*C. geayi*	1.00	1.00	1.00	1.00	NA	1.00	1.00	100	100	NA	100	98	100	100	NA	100	98	45	NA	NA	NA	NA	NA
19	II64–LF4	Internal R node	0.99	0.98	0.98	–	0.92	–	–	53	–	88	–	–	–	–	–	–	–	–	2	−2	2	1	1
20	DNA2–LF4	*C. reticulata*	1.00	1.00	1.00	1.00	1.00	1.00	x (0.97)	100	100	100	98	–	100	100	100	98	–	27	51	23	24	6	−2
21	II69–C91	*C. macrophthalma* + La	0.93	0.95	0.95	–	0.63	–	1.00	42	32	52	–	–	–	–	–	–	–	0	–	–	–	–	–
22	C119–C91	*C. lacustris* group (La)	1.00	1.00	1.00	0.61	0.98	–	–	96	58	73	40	–	83	65	59	–	–	8	5	−2	5	−1	2
23	C119–C130	*C. missioneira* complex	1.00	1.00	1.00	1.00	1.00	0.96	x (0.99)	100	100	100	99	–	100	100	100	97	–	40	86	34	46	4	2
24	C234–C91	Internal La node	1.00	1.00	1.00	1.00	1.00	0.58	–	100	99	95	35	–	100	96	98	–	–	20	23	10	16	−1	−2
25	C234–II74	*C. lacustris*	1.00	1.00	1.00	1.00	1.00	0.80	–	100	100	100	88	–	100	100	100	75	–	41	39	10	31	0	−2
26	K15–C91	Internal La node	1.00	1.00	1.00	1.00	1.00	1.00	x (0.99)	100	99	99	81	–	100	97	100	62	–	26	33	14	12	6	2
27	K15–C26	Internal La node	1.00	1.00	1.00	1.00	1.00	0.86	x (0.99)	100	98	100	56	–	100	97	99	62	NA	16	NA	NA	NA	NA	NA
28	K15–K14	*C. punctata*	1.00	1.00	1.00	1.00	NA	NA	NA	100	100	NA	NA	–	100	100	NA	NA	NA	18	NA	NA	NA	NA	NA
29	K17–C26	*C. scottii* complex	1.00	1.00	1.00	1.00	NA	NA	NA	100	97	NA	NA	–	100	100	NA	NA	NA	8	NA	NA	NA	NA	NA
30	C146–C26	*C. scottii* complex	0.91	0.92	0.92	0.72	1.00	1.00	x (0.99)	–	53	100	99	–	65	64	100	99	–	1	53	25	20	10	−1
31	C101–C26	*C. gaucho*	0.81	0.81	–	0.88	–	0.80	x (0.99)	–	75	–	54	–	67	82	–	50	–	0	0	3	−3	2	−1
32	J9–C91	Internal La node	0.80	0.87	0.80	0.55	0.60	–	–	52	42	38	–	–	–	–	50	–	–	0	–	–	–	–	–
33	C62–C91	Internal La node	0.99	0.97	0.99	0.79	0.96	–	–	69	50	67	–	–	60	51	55	–	–	0	4	3	3	0	−2
34	C62–K25	*C. vittata*	1.00	1.00	1.00	1.00	1.00	1.00	–	100	100	100	100	–	100	100	100	100	–	36	86	37	37	9	2
35	C173–C91	Internal La node	0.90	0.87	0.96	0.82	0.62	–	–	84	65	–	–	–	57	–	60	–	–	0	2	1	1	1	−1
36	C173–C162	*C. ypo*	1.00	1.00	1.00	1.00	1.00	0.95	–	100	100	100	97	–	100	100	100	81	–	54	54	32	19	2	0
37	C13–C91	Internal La node	1.00	1.00	1.00	1.00	1.00	0.99	–	100	100	99	72	–	100	100	97	51	–	19	22	15	5	2	−1
38	C13–C111	*C. hu*	1.00	1.00	1.00	1.00	1.00	1.00	–	100	100	100	100	–	100	100	100	99	–	35	36	15	14	7	0
39	K5–C91	*C. mandelburgeri* complex	1.00	1.00	1.00	1.00	1.00	–	–	100	100	99	–	–	100	100	99	–	–	14	22	15	5	2	−1
40	K5–C210	Internal La node	0.99	0.99	0.99	0.95	–	–	–	49	78	–	–	–	–	56	–	–	–	–	1	4	−4	1	0
41	C172–C12	Internal La node	1.00	1.00	1.00	1.00	1.00	–	–	100	100	91	–	–	100	100	90	–	–	11	12	9	2	1	0
42	C170–C12	Internal La node	1.00	0.98	0.99	0.96	–	–	–	69	70	–	–	–	77	73	–	–	–	2	2	3	−2	1	−1
43	C1–C168	Internal La node	1.00	0.98	1.00	1.00	1.00	–	–	100	95	87	–	–	99	95	88	–	–	5	5	4	1	1	−1
44	C171–C12	Internal La node	0.95	0.96	0.92	–	0.87	–	–	88	–	68	–	–	57	–	64	–	–	1	1	2	−1	1	−1
45	C164–C210	Internal La node	0.85	0.84	0.88	0.73	–	–	–	54	89	–	–	–	64	78	–	–	–	1	1	3	−4	1	0
46	C164–C169	*C.* sp. 'Iguazú big lips 2'	1.00	1.00	1.00	1.00	1.00	0.75	–	100	100	100	63	38	100	100	99	63	–	10	14	10	3	2	−1
47	C165–C210	Internal La node	1.00	1.00	1.00	1.00	1.00	0.82	–	99	94	99	57	–	100	90	97	54	–	7	11	7	3	1	−1
48	C165–C167	*C.* aff. *yaha* 'Iguazú 2'	1.00	1.00	1.00	1.00	–	0.92	0.96	100	98	–	69	63	99	86	–	–	–	4	4	4	−2	2	0
49	C142–C210	Internal La node	1.00	1.00	1.00	–	1.00	–	–	67	x (80)	90	–	–	86	–	75	–	–	2	3	2	0	1	0
50	C142–C143	*C.* sp. 'Paraná'	1.00	1.00	1.00	1.00	0.97	–	–	100	100	95	–	–	100	98	64	–	–	6	6	5	0	1	0
51	C58–C210	Internal La node	0.84	0.81	1.00	–	–	x (0.97)	–	–	–	–	–	–	57	–	–	–	–	1	1	3	−2	0	0
52	C213–C210	*C.* sp. 'Piray-Guazú line'	1.00	1.00	1.00	1.00	0.99	–	–	92	93	91	–	–	92	76	84	–	–	3	3	4	0	1	−3

53	C4–C91	Internal La node	1.00	1.00	1.00	0.61	1.00	–	–	55	56	84	–	–	86	–	85	–	–	2	3	2	3	0	−2
54	C4–C215	Internal La node	1.00	1.00	1.00	1.00	0.99	–	–	85	88	75	65	–	92	82	73	–	–	3	4	5	−1	1	−1
55	C4–C6	Internal La node	1.00	1.00	1.00	0.95	1.00	0.68	–	91	67	90	–	–	97	68	85	65	–	5	5	2	0	2	0
56	C5–C6	*C.* aff. *yaha* 'Iguazú 1'	1.00	1.00	1.00	0.96	1.00	–	–	99	83	100	–	–	100	76	99	–	–	6	6	2	4	1	−1
57	C17–C215	*C.* sp. 'Piray-Guazú'	1.00	1.00	1.00	1.00	1.00	–	–	100	100	99	–	–	100	99	96	–	–	9	9	7	2	1	−1
58	C155–C91	Internal La node	1.00	0.98	0.99	0.91	0.68	■	■	69	61	69	■	■	88	54	57	■	■	2	2	3	−1	1	−1
59	C155–C57	Internal La node	1.00	1.00	1.00	1.00	0.96	–	–	55	83	78	–	–	83	76	74	–	–	3	5	6	0	1	−2
60	C155–C206	*C.* sp. 'Urugua-í line'	1.00	1.00	1.00	0.93	–	–	–	92	87	–	–	–	84	57	–	–	–	1	2	3	−2	1	0
61	C140–C57	*C. mandelburgeri*	1.00	1.00	1.00	0.99	–	–	–	80	85	–	–	–	86	78	–	–	–	2	2	3	−2	1	−1
62	C104–C91	*C.* aff. *mandelburgeri*	1.00	1.00	1.00	0.98	0.99	–	–	86	74	92	–	–	95	70	85	–	–	3	5	4	1	1	−1

[a] Locus 16S modified (characters with more than 10% of gaps removed).
[b] Without the 16S locus.

modified 16S partition, or entirely without it (Table 2). This treatment of the 16S locus increased the overall support for several nodes at the deep level in the combined analysis of all data, but the resulting support values are practically identical to the combined analysis based only on the remaining three loci (cyt*b*, ND2, S7-i1; Table 2).

We used the arbitrarily defined significant support values (Section 2.5) to detect important conflicts between trees inferred by different methods or from different data partitions. No conflicts were found among trees derived by different phylogenetic methods from the combined dataset, but several conflicts between single-locus and multilocus hypotheses were detected (Table 2). Most of them were observed between the nuclear S7-i1 marker and the three mitochondrial genes. Due to the rather low resolution of the S7-i1 locus at terminal nodes, most of the conflicts are caused by only one or two nucleotide substitutions. The only alternative topology based on a coding locus was observed for node 2 (=TWLuS clade; see Section 3.4) in the ND2 Bayesian analysis: *Teleocichla* was recovered as a basal lineage in the RMLa clade (=node 14; see Section 3.4), not in the TWLuS clade as in the combined tree. This discordance is likely explained by absence of ND2 sequences of some of the related taxa, in combination with a long branch of *Teleocichla*. These factors probably lower the overall value of PP for node 2 which is otherwise strongly supported by the S7-i1 and cyt*b* loci.

3.4. The phylogeny of Crenicichla

All phylogenetic analyses support the monophyly of *Crenicichla* (incl. *Teleocichla*; node 1; Fig. 1) with high support values in all methods (i.e. 1.00 in BA, 100 in both bootstrap analyses, 42 in BS). *Crenicichla* is split into two major clades (Fig. 1A): (1) the "TWLuS clade" (node 2) including the *C. wallacii*, *C. lugubris*, and *C. saxatilis* species groups plus *Teleocichla*), and (2) the "RMLa clade" (node 14) including the *C. reticulata* and *C. lacustris* species groups plus *C. macrophthalma* (for the clade support see Table 2). *Teleocichla* is found as a species group within *Crenicichla*, thus rendering *Crenicichla*, as presently understood, paraphyletic.

The TWLuS clade (node 2) is supported in combined BA analyses with a removed (PP 0.99) or modified 16S dataset (0.96; see previous section). The RMLa clade (node 14), *C. wallacii* group (5), the *C. saxatilis* group (12), the *C. reticulata* group (15), *C. lacustris* group (22), and also *Teleocichla* (3) receive high support values from all multilocus analyses. The somewhat lower support of the *C. lugubris* group (node 8), compared to the other species groups, is caused by attraction of the basal *Crenicichla multispinosa* towards the *C. saxatilis* group in the cyt*b* partition.

The *C. lacustris* group (22; Fig. 1) shows a basal division between the *C. missioneira* species complex (23) and the clade (24) called "Southern" by Kullander et al., (2010; see Section 4.1). The inferred relationships within the *C. missioneira* complex vary among individual loci and do not seem to form any distinct subclades with a reasonable support, nor do they present any reliable species-level taxonomy (see Section 4.2.1).

The first lineage to separate from node 24 is the coastal species *C. lacustris* with a deep phylogenetic structure (25), followed by node 27, composed of *C. punctata* (28) and the *C. scottii* species complex (29), and then by *C. jupiaensis* (another upper Paraná River species, *C. jaguarensis*, is also recovered in the same clade; U. Schliewen, unpublished results) and a subtree subordinate to node 33. Three species are subsequently split off from this subtree: basal *C. vittata* (34), *C. ypo* (36), and *C. hu* (38). The remaining, strongly supported complex (39) comprises several valid species (*C. mandelburgeri*, *C. tesay*, *C. yaha*, *C. iguassuensis*, and possibly also *C. niederleinii*) as well as several undescribed forms from the middle Paraná River basin including its major tributary, the

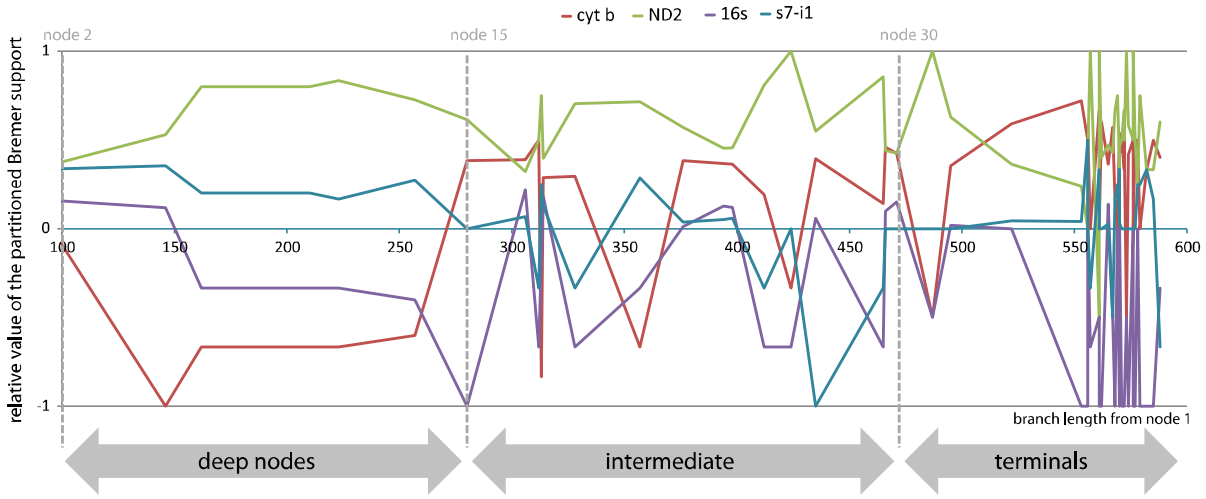

Fig. 2. Dependence of relative PBS values on the cumulative branch length from the tree root for all loci. The division between deep, intermediate and terminal nodes is arbitrarily assigned to nodes 15 and 30 in the phylogeny (Fig. 1).

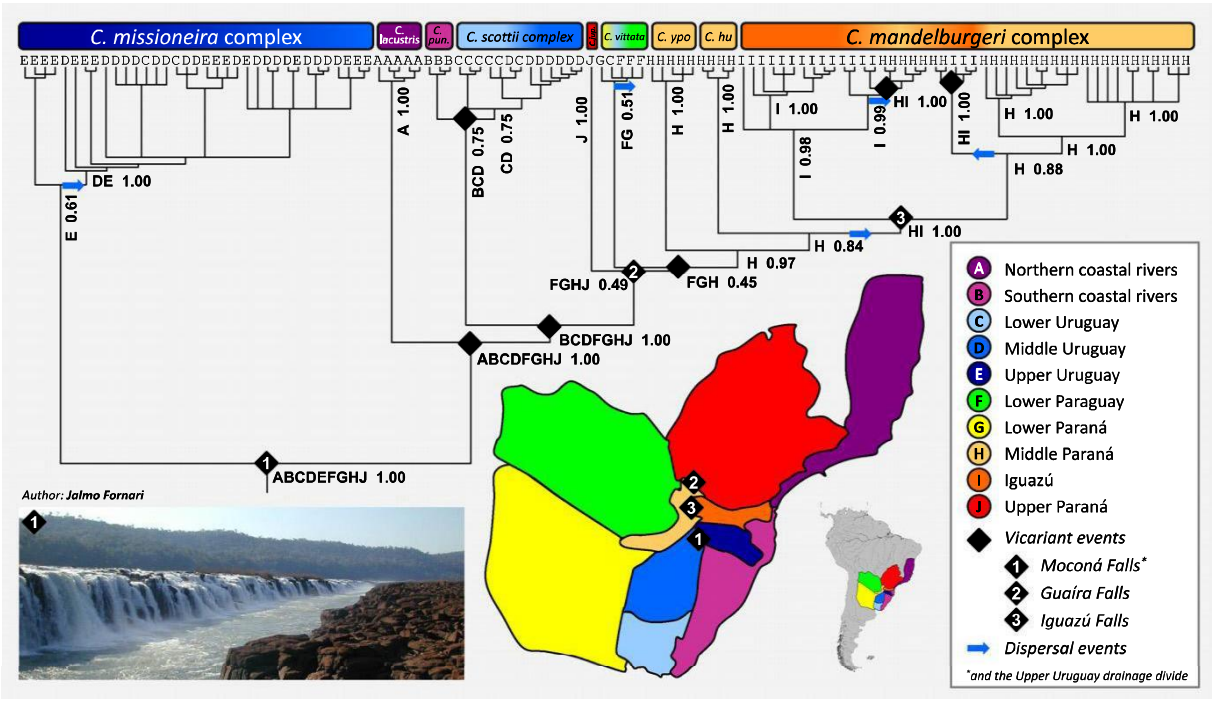

Fig. 3. Biogeographical reconstruction of ancestral areas (RASP analysis; see text).

Iguazú River (Fig. 1B). This clade, called "the *C. mandelburgeri* species complex" hereinafter (see Section 4.2.1), represents a third species complex within the *C. lacustris* group.

3.5. Biogeographic reconstruction of the C. lacustris species group

The biogeographic interpretation of relationships among areas of endemism (Fig. 3) reconstructs virtually all basal nodes as vicariant events. The common ancestor is thus hypothesized as having been widely distributed in all the present drainages except the Iguazú (I). The first vicariance separated the Upper Uruguay (E; isolated by the Moconá Falls and the upper Uruguay River drainage divide) from the wide ancestral distribution, the second the Northern coastal rivers (A), the third the Southern coastal rivers (B) together with the Lower and Middle Uruguay (CD) (followed by vicariance between the coastal and the Uruguay areas). The next vicariance separated the Upper Paraná (J; until recently isolated at the Guaíra Falls, but today semipermeable due to the Itaipu dam; Casciotta et al., 2007; Júlio et al., 2009) from the rest (FGH), followed by the last basal vicariance between the Lower Paraguay–Lower Paraná (FG) and the Middle Paraná (H; until recently probably separated at the Apipé Falls). The terminal clade, including *C. ypo*, *C. hu* and the *C. mandelburgeri* complex is thus reconstructed as originally endemic to the Middle Paraná (H). The *C.*

mandelburgeri complex is the only clade present in the Iguazú (I) following dispersal from the Middle Paraná (H). An initial vicariant event between the Iguazú (I) and Middle Paraná (H) is reconstructed in the basal node of this complex, but secondary dispersal and vicariant events suggest semipermeability of the barrier between the two areas and/or river captures in this area. Additional dispersals in the *C. lacustris* group are limited to two instances; one in the *C. missioneira* complex and one in *C. vittata* (see Fig. 3).

4. Discussion

4.1. Phylogeny

Our study resulted in a robust phylogenetic hypothesis of *Crenicichla*, at present the largest genus among the Neotropical Cichlidae (Fig. 1). It confirms monophyly of all species groups within *Crenicichla*, including *Teleocichla*. Our results differ substantially in several regards from the only available phylogeny of *Crenicichla* (Kullander et al., 2010). Their phylogeny was unresolved at deep nodes (between the species groups of *Crenicichla*), and their BA and MP tree topologies differ from each other. Their most important result was the postulated independence of two main clades of the *C. lacustris* group (their "Southern" and "Missioneira" groups). We have reanalyzed the dataset from the Kullander's et al. study and below explain that the main result and other conflicts with our study are largely analytical artifacts of the cited study:

(1) The first important drawback of the Kullander et al. (2010) study was an insufficient taxon sampling; especially the absence of the *C. lugubris* group seems to be crucial. When this species group (e.g. *C. lenticulata*, *C. lugubris* 'Guyana', *C. marmorata*, *C. multispinosa*) is added to the Kullander et al. (2010) cyt*b* dataset (results not shown), their phylogeny becomes resolved at the basal nodes (although with a weak support) and in agreement with our study, including the TWLuS (PP 0.87, *C. wallacii* group at the base of this clade) and RMLa (0.55) clades. Within the RMLa clade, the *C. lacustris* group is recovered as monophyletic (PP 0.72; contrary to the independent "Southern" and "Missioneira" groups postulated by Kullander et al. (2010).

(2) The unresolved topology of the Kullander et al. (2010) study was additionally caused by conflicting positions of two long-branch ingroup taxa (*Teleocichla* and *C. macrophthalma*) attracted towards a remote outgroup, and these multiple LBA artifacts collapsed the tree topology. The only outgroup taxon in Kullander et al. (2010) study, *Cichla*, has on morphological grounds been postulated as a sister group of *Crenicichla* (Kullander, 1998), but since then refuted by all molecular and combined molecular-morphological studies as closely related to *Crenicichla* (e.g. Smith et al., 2008; López-Fernández et al., 2010).

(3) The Kullander's et al. (2010) study was based on a single DNA marker, the cyt*b*. The authors mentioned "moderate saturation at codon position 3" in this gene (also detected in our study), but did not try to correct for the saturation.

The only other study with marginal phylogenetic information on the relationships within *Crenicichla* is that of López-Fernández et al. (2010), focused on the phylogeny of the whole Neotropical cichlid clade (using five DNA markers). They included only eight specimens of *Crenicichla* representing four species groups plus *Teleocichla*. The relationships within *Crenicichla* are practically identical to our results, except for the exchanged position between *Teleocichla* and the *C. wallacii* group within the TWLuS clade.

Our results are also compatible with Ploeg (1991) who divided *Crenicichla* into six (including *Teleocichla*) main species groups based on an intuitive analysis of the morphological characters. There are however several differences: Ploeg (1991) placed *C. vittata* into the *C. lugubris* group and *C. scottii* into the *C. reticulata* group, both contrary to our results.

4.2. Systematics and taxonomy

4.2.1. Genera and species groups

As already suggested (López-Fernández et al., 2010), *Teleocichla* Kullander 1988 is an ingroup of *Crenicichla*. *Crenicichla* is thus clearly in a need of taxonomical revision. The best strategy is to split it into several genera, which is however beyond the scope of the present paper. The potential for such taxonomical changes is there since the species groups (putative genera) are long isolated evolutionary units and most of them are largely diagnosable using morphological characters.

Within the *C. lacustris* group Lucena and Kullander (1992) and Lucena (2007) described seven new species from the upper and middle Uruguay River drainages in Brazil, identifying them as the *C. missioneira* complex. Kullander et al. (2010) discovered that some of these endemic species are very similar genetically, based on the cyt*b* gene, but they explain an identical haplotype present in two specimens referred to as *C. minuano* and *C. tendybaguassu* as caused by misdetermination of the former, thus in general advocating monophyly of the described species. Our results, based on a much larger taxon sampling from all parts of distribution of the *C. lacustris* group, support the close relatedness of the *C. missioneira* complex: *C. celidochilus*, *C. empheres*, *C. hadrostigma*, *C. minuano*, *C. missioneira*, *C. tendybaguassu* (Fig. 1A, node 23), and possibly also *C. jurubi* (not present in our dataset). We, however, demonstrate that the species are not monophyletic based on the examined loci and thus impossible to separate using sequence data, contrary to Kullander et al. (2010). This species complex clearly requires further study using additional molecular markers.

Our detailed study of the middle Paraná/Iguazú River drainages in Misiones (Argentina) reveals the presence of another monophyletic species complex within the *C. lacustris* species group, the *C. mandelburgeri* complex (Fig. 1B, node 39), which includes four described (*C. mandelburgeri* Kullander 2009, *C. tesay* Casciotta and Almirón 2009, *C. yaha* Casciotta et al. 2006, *C. iguassuensis* Haseman 1911) and several potential but yet undescribed species. We have recently described two successive sister species of this complex (*C. ypo* Casciotta et al. 2010, *C. hu* Piálek et al. 2010), which are sympatric with other members of the complex. One more species, *C. niederleinii* (Holmberg 1891), whose identity (and non-conspecifity with *C. mandelburgeri*, see below) remains to be established, also seems to belong here.

4.2.2. Species-level taxonomy

Within the nominal species *C. lacustris* (node 25), we recover three deeply isolated allopatric lineages. Two of these lineages agree with the nominal taxa *C. biocellata* Ihering 1914 and *C. dorsocellata* Haseman 1911, that were synonymized with *C. lacustris* (Castelnau 1855) by Ploeg (1991; followed by Kullander, 2003; Kullander and Lucena, 2006). Ploeg agreed that "*C. lacustris* shows a considerable variability in several characters", admitting that he did not examine the two type specimens of *C. dorsocellata*. Under the concept of three species they can be distinguished by the presence, location, and coloration of dots on the body and fins (Jens Gottwald, pers. comm.; unfortunately, coloration of the dots cannot be examined in preserved specimens). Uncorrected pairwise divergences (cyt*b*) between *C. lacustris* s.str. and "*biocellata*" is 7.4%, between *C. lacustris* s.str. and "dorsocellata" is 5.1–5.6%, and between "*biocellata*" and "*dorsocellata*" is 6.3–6.8% (Fig. 1A).

These distances indicate several million years of isolation (see Section 2.5) and support the existence of several species.

Our results also point out that diagnosis of several taxa are incongruent: (1) Specimens of *C. mandelburgeri* from two of the type localities (C140, C141, Tembey River [holotype locality]; C138, Pirayuy River [paratype locality]) were recovered as paraphyletic toward the *C.* sp. 'Urugua-í line', an endemic lineage of the Urugua-í River differing in higher number of scales in the lateral line E1 (44–56 vs. 53–64), and in the general coloration pattern. (2) *C. mandelburgeri* cannot be distinguished morphologically from the insufficiently described *C. niederleinii*, a species that was claimed to have different E1 counts (44–56 vs. 56–65), size, and coloration pattern in adult specimens (Kullander, 2009). We thus name our samples *post hoc*, based on the molecular phylogeny, as *C. mandelburgeri* and *C.* aff. *mandelburgeri* (see Fig. 1B). At the present stage of knowledge, we cannot exclude mitochondrial introgression of *C. mandelburgeri* into *C. niederleinii* nor a less probable ancestral polymorphism (for the complex taxonomic history of *C. niederleinii* see Kullander, 1981). (3) Several species from the *C. missioneira* complex, at least *C. minuano* Lucena and Kullander 1992 and *C. missioneira* Lucena and Kullander 1992 are in our analyses not distinguishable from each other in both morphological and molecular characters (see Section 4.2.1, Fig. 1A, and also Lucena and Kullander, 1992).

Kullander et al. (2010) suggested that two specimens among their samples could be interspecific hybrids between *C. scottii* (Eigenmann 1907) and *C. vittata* Heckel 1840. The only novel sample of *C. scottii* in our dataset, C146 from Entre Ríos Province (Argentina) clusters with *C. scottii* GenBank cyt*b* sequences, and forms a monophyletic clade with *C. gaucho* Lucena and Kullander 1992 in all mitochondrial loci, while the nuclear S7-i1 sequence of C146 specimen groups with *C. vittata*. This observation has two possible explanations: (1) our specimen is in fact a *C. scottii*-like hybrid between *C. scottii* and *C. vittata*, and the hybridization process is indicated by both parental parts of the genome persisting; or (2) *C. scottii* originated as an interspecific hybrid between *C. vittata* and *C. gaucho*. The latter scenario would find some biogeographic support as the distribution of *C. scottii* falls between areas of its putative parent species. Although based on a single sequence, this finding suggests that *C. gaucho* should be considered in hypotheses on possible hybridization between *C. scottii* and *C. vittata*.

4.3. Biogeography of the C. lacustris group and of SE South America

The *C. lacustris* group is endemic to the Río de la Plata basin (the Paraná and Uruguay River drainages) and the adjacent Atlantic coastal drainages. It is also allopatric with virtually all other *Crenicichla* species groups (except two species of the *C. saxatilis* group and one species of the *C. reticulata* group; Piálek et al., 2010) that inhabit mainly the Amazon and Orinoco basins (Fig. 1A). Within the distribution of the *C. lacustris* group the highest diversity is found in the middle Paraná River and its tributaries (the Iguazú River being the most significant) and in the Uruguay River. Our biogeographic reconstructions also depict the Middle Paraná–Iguazú and Uruguay areas of endemism as historically and geographically most complex (Fig. 3).

The biogeography of *Crenicichla* in SE South America supports the complex biogeographic patterns of freshwater fishes in this area recovered by Albert and Carvalho (2011). In both studies are the La Plata and Atlantic coast faunas non-monophyletic with highly complex relationships both within river drainages and between adjacent river drainages. The BPA of Albert and Carvalho (2011) places all drainages SE of the Amazon except the Upper Uruguay (see below) into two clades of areas, and the postulated paleodrainage divide between them runs exactly through the areas which have the most interesting biogeographic patterns in *Crenicichla* (as well as in *Australoheros*; see Říčan et al., 2011). This most interesting area is centered on the Upper Uruguay and Iguazú, their drainage divide and the divides with the adjacent Atlantic coast drainages to the east and the divides and waterfalls between the Paraná and Middle Uruguay drainages to the west.

4.3.1. The Upper Uruguay

The first of the *C. lacustris* group species flocks (the *C. missioneira* flock) is reconstructed as having been ancestrally endemic to the Upper Uruguay and the vicariance between the Upper Uruguay and all remaining areas of endemism (Fig. 3) is reconstructed as the basalmost split in the *C. lacustris* group analysis. The BPA of Albert and Carvalho (2011) also places the Upper Uruguay in a very basal position from the rest of the La Plata basin and Atlantic coastal drainages (actually as basal to the Amazon/Orinoco), which suggests different faunal affinities, different paleodrainage patterns, and/or large-scale extinctions.

A complex biogeography in the Upper Uruguay was also found in the cichlid genus *Australoheros* (Říčan et al., 2011). One species in the Upper Uruguay (*Australoheros angiru*) is shared with the upper Iguazú River across the drainage divide between the two river basins. The sister species of *A. angiru* (*Australoheros minuano*) is found in the Middle Uruguay below the Moconá Falls. Another species of the Upper Uruguay (*Australoheros forquilha*) is the sister species of the Middle Uruguay *Australoheros ykeregua*, the two species being again separated by the Moconá Falls. The divergences between the species of the Upper and Middle Uruguay have been dated at min. 2.3–3.3 mya in the *A. forquilha*–*A. ykeregua* pair, and 4.2–6.0 mya in the *A. angiru*–*A. minuano* pair (based on 0.7–1% divergence rate; Concheiro Pérez et al., 2007). The divergence between the *C. missioneira* complex and the rest of the *C. lacustris* group is at least 6–8 mya (based on 13.1–15.3% sequence divergence and 2% divergence rate). At least based on these two cichlid genera these dates seem to set the timeframe for the evolution of the endemic faunas of the Upper Uruguay. The youngest date most probably represents the age of the Moconá Falls. The two older dates reflect more complex biogeographic patterns that involve not only the Moconá Falls, but also the drainage divide of the Upper Uruguay and adjacent drainages. The two older dates thus probably represent biogeographic configurations that predate the establishment of the present drainage basins in the area.

Confirming different past configurations of the drainage divide of the Upper Uruguay are also faunal affinities with the Southern coastal rivers. Several fish species occur only in the Uruguay River and in the coastal Jacuí River, e.g. *Bryconamericus patriciae* (Silva, 2004), *Cnesterodon brevirostratus* (Lucinda, 2005), *Hypostomus aspilogaster* and *Hypostomus commersonii* (Reis et al., 1990; the latter occurring also in the Paraná River).

The geological history of the Upper Uruguay River is not known in any detail and thus insufficient to shed light on its paleocourse or the establishment of its present drainage divide. The Upper Uruguay River flows in an E–W direction in parallel to the Iguazú River with a drainage divide also with the Middle Paraná and the Atlantic coastal drainages. The boundary with the rest of the Uruguay River is situated at the Moconá Falls. The Moconá Falls are located in a distinct bend of the Uruguay River where it abruptly changes course from roughly the E–W in the upper section to N–S in the middle and lower sections (Fig. 1A, locality "s"). The almost 2 km long Moconá Falls presently act as an effective barrier prohibiting upstream migration. The Moconá Falls create a chasm of about a 10 m drop perpendicular both to the river's course (just barely crossing it from one side to the other) as well as to the Sierra de Misiones, which separates the Uruguay from the Paraná River.

4.3.2. The Iguazú/Middle Paraná

The second of the *C. lacustris* group species flocks (the *C. mandelburgeri* flock) is endemic to the Iguazú/Middle Paraná with a vicariance between the two river basins coincident with the origin of the flock (Fig. 3). Prior to the evolution of the *C. mandelburgeri* complex and prior to the evolution of its two successive outgroups (*C. ypo* and *C. hu*) the lineage has been evolving only in the tributaries of the Middle Paraná (Fig. 3). The Iguazú River has additionally been colonized by the *C. lacustris* group as the last major river drainage and is also the only area absent from the postulated wide ancestral distribution of the group (Fig. 3). This biogeographic reconstruction finds support in the BPA of Albert and Carvalho (2011) where the Iguazú River is found in a clade containing all Atlantic coastal drainages plus São Francisco and Parnaíba Rivers, but not in a clade containing the Paraná River. This relationship suggests geodispersal (Albert and Carvalho, 2011) and thus a different paleocourse of the Iguazú (towards the coast, not into the Paraná), which also would explain its absence in the ancestral area of the *C. lacustris* group. All data thus seem to indicate that the *C. mandelburgeri* complex colonized the Iguazú River only after its flow-reversal into the Paraná. The colonization was then almost immediately followed by separation of the faunas, possibly indicating the origin of the Iguazú Falls (Fig. 3).

The possible date for origin of the Iguazú Falls is based on the basal vicariance within the *C. mandelburgeri* complex dated at ca 1–1.5 mya (based on the observed maximum divergence of 3.12% within the *C. mandelburgeri* complex and a 2% divergence rate). The colonization of the Iguazú by the *C. mandelburgeri* complex (of the *C. lacustris* group) might have happened directly from the Middle Paraná prior to the erosive force having created the falls or through river captures on a changing watershed divide, e.g. from the Urugua-í River immediately to the south of it (Fig. 1B) with which the Iguazú River shares several species or species pairs endemic just to these two rivers (*Astyanax leonidas*, *Glanidium riberoi*, *Hypostomus myersi*, *Hypostomus derbyi*, *Corydoras carlae*, *Australoheros kaaygua* vs. *Australoheros tembe*, *C. yaha* vs. *C.* cf. *yaha* [Casciotta et al., 2006; Piálek et al., 2010]; *Bryconamericus ikaa* vs. *B.* cf. *ikaa*). Two cases of secondary dispersal between the Iguazú and Middle Paraná and its tributaries have occurred (Fig. 3). In one case (between nodes 45 and 47, Fig. 1B) the dispersal is from the Iguazú into the Paraná River (thus possibly over the falls), but the other instance (between nodes 53 and 54, Fig. 1B) is against the Iguazú Falls and the only possibility is thus contact through headwaters (geodispersal; see the map in Fig. 1B).

Biogeography of the genus *Australoheros* (Říčan and Kullander, 2008; Říčan et al., 2011) suggests that the postulated reversal of the Iguazú River likely occurred in steps, with an yet unidentified barrier within the river basin (as the Salto Moconá in the Uruguay river basin). This barrier is postulated to have originally divided the two endemic and non-overlapping *Australoheros* faunas in the Iguazú (*A. kaaygua* and *A. angiru*; plus their sister groups from adjacent drainages) from each other. The relationships of these two species also suggest that the paleo-Iguazú River had different drainage divides, since the sister group of *A. kaaygua* in the part above the falls for at least 100 km is *A. tembe*, an endemic species of the Urugua-í River (to the south, tributary of the Middle Paraná, divided from it by the large Urugua-í fall), while the more upstream species (*A. angiru*) is shared with the Upper Uruguay and its sister species is in the Middle Uruguay (*A. minuano*; see Section 4.3.1). Contrary to the colonization of the Iguazú River by the *C. mandelburgeri* complex the colonization of *Australoheros* probably occurred through changes in the paleodrainage divides with the Uruguay River, where the genus has the highest diversity, the species in question their closest relatives (Říčan et al., 2011), and which permitted its earlier colonization of the Iguazú River than in the case of *Crenicichla*.

As a final note on the Middle Paraná this river section seems to be naturally divided into two biogeographically distinct sections. The northern tributaries of the Middle Paraná (Iguazú, Urugua-í, Piray-Miní, and possibly also the Piray-Guazú River and the opposite tributaries in Paraguay; see Říčan and Kullander, 2008) have species endemic to each individual tributary that are not found in the mainstream of the Middle Paraná (Fig. 1B). On the contrary, the southern tributaries of the Middle Paraná (from the Paranay-Guazú and Tembey Rivers to the south) do not posses tributary endemics, and the species are present in the mainstream of the Middle Paraná. Both the northern and the southern tributaries have waterfalls close above their mouths into the Middle Paraná, but in the southern tributaries the falls do not separate endemic species while in the northern tributaries they do (and some such as the Piray-Miní do not have waterfalls at all). This peculiar observation is well worth further study.

4.3.3. The Upper Paraná

Like the Moconá Falls, the once mighty Guaíra Falls also seem to be responsible for an ancient vicariance in *Crenicichla*. These waterfalls used to divide the Upper Paraná from the rest of the Paraná/Paraguay River drainage, and the same pattern is seen in our biogeographical reconstruction of the *C. lacustris* group (Fig. 3). According to Albert and Carvalho (2011) this reconstruction may not apply to the whole fauna of the Upper Paraná because their BPA analysis places the Upper Paraná in a clade with the adjacent Northern coastal drainages. This conflict between *Crenicichla* and the Albert and Carvalho' BPA suggests that the Upper Paraná may not be one homogenous biogeographic area, similarly as the Uruguay and Iguazú Rivers.

4.3.4. The Atlantic coastal rivers

Also the final complex result of our biogeographic analysis, the non-monophyly of the coastal *Crenicichla* fauna (Figs. 1A and 3; *C. lacustris*, *C. punctata*) is supported by the BPA of Albert and Carvalho (2011). In both analyses, the Southern coastal rivers are not joined with the Northern coastal rivers, but with the Lower–Middle Uruguay and other Río de la Plata drainages (except the Upper Uruguay, the Iguazú, and the Upper Paraná, see above). The headwaters of the Upper Uruguay and Iguazú (see above) are also situated in this zone of division between the Southern and Northern coastal drainages (Figs. 1 and 3).

The complex geomorphological history of the contact area of the upper Uruguay River, the Iguazú River, and the adjacent drainages seems to generate biogeographical complexity and species diversity and endemism. Data available at present (bases on the only two fish groups so far studied in detail, i.e. *Crenicichla* and *Australoheros*) indicate that there is no clear dichotomy between the diversification-promoting roles of migration barriers like waterfalls and large rapids on one hand and drainage divides on the other. They probably acted together and were often directly linked. However, the role of the changing drainage divides seems to be stronger than the role of the waterfalls since the former preceded the formation of the latter in all instances. Areas rich in waterfalls and large rapids nevertheless indicate more profound and less visible forces and continue to be fascinating clues for discovery.

4.4. Species flocks as a model for sympatric speciation in rivers

Our study supports the existence of at least two species flocks within the *C. lacustris* group which are, except for their occurrence in complex riverine habitats, very similar to the lacustrine species flocks in the lakes of the East African Rift Valley (e.g. Salzburger and Meyer, 2004; Kocher, 2004), Cameroon (Schliewen, 2005), and Middle America (e.g. Barluenga et al., 2006; Geiger et al., 2010). The lacustrine cichlid species flocks have been established

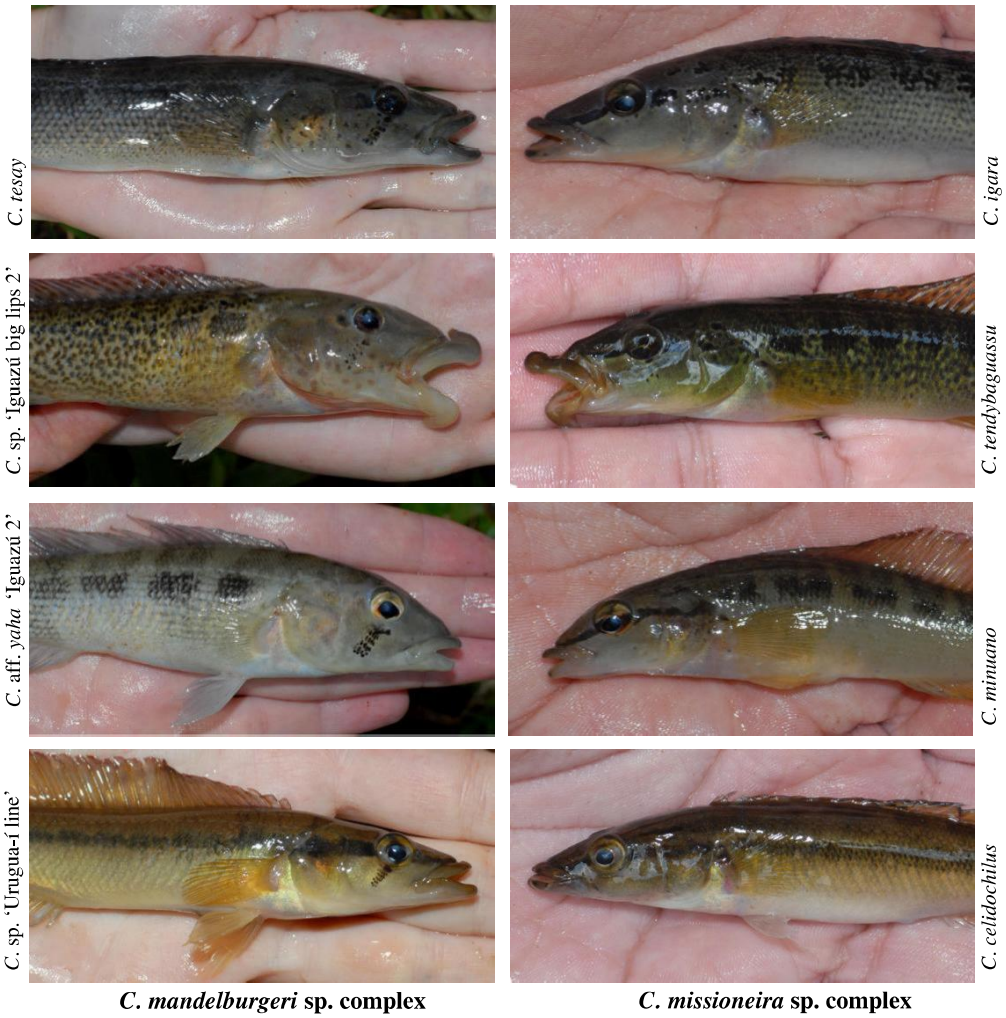

Fig. 4. Overview of several color patterns and eco-morphological variations within *C. mandelburgeri* and *C. missioneira* species flocks (see text).

as evolutionary model systems (Kocher, 2004; Seehausen, 2006). In contrast, the possibility of riverine cichlid species flocks has remained poorly studied. A few postulated species complexes in riverine habitats should be noted: the serranochromine cichlids of southern African rivers (which may however have originally radiated under lacustrine conditions in the now extinct Lake palaeo-Makgadikgadi; Joyce et al., 2005), *Steatocranus* and *Nanochromis* cichlids in the mighty Lower Congo rapids (e.g. Schwarzer et al., 2011), *Crenicichla* and *Teleocichla* in the large Amazonian rapids (e.g. Kullander, 1988), and two complexes of the *C. lacustris* group in SE South America (Lucena and Kullander, 1992; Kullander et al., 2010; this study).

A species flock is, according to Salzburger and Meyer (2004) and in the sense of Mayr (1942, 1984) and Greenwood (1984), commonly referred to a monophyletic assemblage of closely related species that coexist in the same area with a high level of endemicity. Both the *C. mandelburgeri* and *C. missioneira* complexes fulfill the above criteria. The diversity of the two species complexes may suggest the first instance of possible sympatric speciation in a riverine habitat within Neotropical cichlids.

Despite the fact that the *C. missioneira* and *C. mandelburgeri* complexes are separated from each other for several millions of years (at least 6–8 mya based on cyt*b* sequence divergences between the clades of 13.1–15.3% and a 2% divergence rate), are not closely related, and have been evolving in biogeographically separate areas, they both have developed a striking resemblance between their species (Fig. 4).

The coloration patterns within the two species complexes can be roughly classified as follows: (1) species with a prominent lateral band (*C.* sp. 'Urugua-í line', *C.* sp. 'Piray-Guazú line', *C.* sp. 'Chapa' of the *C. mandelburgeri* complex vs. *C. celidochilus* of the *C. missioneira* complex); (2) with bars or double-bars (*C. mandelburgeri*, *C.* aff. *mandelburgeri*, *C. niederleinii*, *C.* sp. 'Piray-Guazú' vs. *C. hadrostigma*); or (3) with a row of rectangular blotches on the upper part of flank, sometimes dissolved in a kind of marbling in the hind part of body, the general body background with or without dots (all other species; see also Lucena and Kullander, 1992; Lucena, 2007).

Both complexes also developed several very similar head morphologies: (1) species with prognathous upper jaw or isognathous jaws and small mouth (e.g. *C.* aff. *yaha* vs. *C. minuano*, *C. jurubi*); (2) with prognathous lower jaw and large mouth (e.g. *C. tesay* vs. *C. missioneira*, *C. igara*); and (3) with lobed lips and prognathous upper jaw (*C.* sp. 'Iguazú big lips' vs. *C. tendybaguassu*). There are

also differences in dentition between several species: e.g. *C. igara* is distinguished from *C. jurubi* (both of the *C. missioneira* complex) by pointed vs. molariform pharyngeal teeth (Lucena and Kullander, 1992). Similar differences in dentition are also found in *C.* aff. *yaha* 'Iguazú 1' and *C.* aff. *yaha* 'Iguazú 2' (with molariform vs. pointed teeth, respectively) from the *C. mandelburgeri* complex.

These morphologically distinct species within each complex live often sympatrically and even syntopically and form mixed-species flocks (schools): they have been repeatedly caught together at the same time and in the same spot using gillnets or hook-and-line (pers. obs.).

Within the *C. mandelburgeri* flock, molecular phylogenetic analyses support the hypothesis of a close relationship of the syntopic forms differing in mouth arrangement (Fig. 1B): (1) the samples of *C.* 'Iguazú big lips 1' and of *C.* aff. *yaha* 'Iguazú 1' (three specimens from one locality in total) form a clade (node 55), (2) the specimens (from another locality) of *C.* 'Iguazú big lips 2' and *C.* aff. *yaha* 'Iguazú 2' (Fig. 4) form two successive splits (node 45) with very little molecular divergence between them, and (3) specimens of *C.* aff. *tesay* 'big lips' and *C.* aff. *tesay* 'small mouth' (both subadults) are comprised in the monophyletic *C. tesay* lineage. It thus seems that diversification in color patterns is generally older than the variation in trophic traits (syntopic forms distinguished by mouth arrangement share the same coloration pattern).

In the *C. missioneira* complex, we can find similar ecomorphological variation among syntopic forms with the same coloration pattern as well: (1) *C. missioneira*/*C. minuano*/*C. tendybaguassu*; (2) *C. igara*/*C. jurubi*/*C. empheres* (see Lucena and Kullander, 1992); in this case we, however, lack compelling molecular evidence about the species' relationships.

The astonishing resemblance between forms of both species complexes (Fig. 4) suggests that the mouth morphologies may develop repeatedly in geographically isolated habitats of a similar type. Such situation is well-known from African lake cichlids (e.g. Sturmbauer et al., 2003) and the common explanation is that closely related morphological forms likely evolve by disruptive evolution of trophic traits connected with exploitation of different food resources (e.g. Kocher, 2004). The relation between the mouth arrangement (jaws and lips characteristics, dentition) and the feeding preferences of the species in *C. missioneira* complex was already proposed by Lucena and Kullander (1992). Also, the proximate causes of the jaw or dentary remodeling in cichlids are known (Liem, 1973; Meyer, 1990a): a jaw can be rebuilt even within one generation (Meyer, 1990b). There is hence a legitimate question regarding the conservativeness of the resulting structure. However, it is interesting to note that no other *Crenicichla* species group except the *C. lacustris* is known to develop thick lips.

The evolutionary radiations observed in the species flocks of *Crenicichla* might involve the same steps as in Lake Malawi, but the order seems to be different. In Lake Malawi the three stages of the radiation are: (a) adaptation to distinct rocky and sandy habitats, (b) radiation of trophic morphologies within each habitat which are genus specific, and (c) diversification of male color patterns within each lineage (Kocher, 2004). In the *C. lacustris* flocks: (a) sexual selection on color pattern seems to precede (b) adaptation to distinct habitats and (c) radiation in trophic morphologies. Additionally, in the species flocks in the *C. lacustris* group the radiation in trophic morphologies is probably not associated with distinct macrohabitats, since different trophic morphologies form mixed schools (like bird mixed foraging flocks). Contrary to these differences in the trajectories of evolution of species flocks of Lake Malawi and the *C. lacustris* group the time scales within which they have evolved are quite comparable. The haplochromines underwent radiations after they colonized Lakes Malawi and Victoria over the past 1–2 My (Meyer et al., 1990; Verheyen et al., 2003), similarly to the *C. missioneira* and *C. mandelburgeri* species flocks in the Uruguay and Paraná/Iguazú Rivers (2.30% and 3.12% max. divergence in the *C. missioneira* and *C. mandelburgeri* complex, respectively, i.e. 1–2 My). In the *Crenicichla* species flocks the trigger for their radiation comparable to the colonization of Lakes Malawi and Victoria by the haplochromines so far remains unknown. The situation is especially puzzling within the *C. missioneira* complex where the striking morphological diversity is not linked with corresponding molecular diversity at the observed loci despite that the complex was separated from the rest of the *C. lacustris* group by the basal vicariance at least 6–8 mya (Fig. 3). A much deeper diversification would be expected (Fig. 1A; see the relatively long branch at node 23), and, consequently, some kind of bottleneck seems to have preceded the present diversification of the complex (see Section 4.3).

The *Crenicichla* species complexes apparently represent an early stage of evolution. In both species flock models (the haplochromines and *Crenicichla*) reconstructing the recent history of these radiations is complicated by the fact that many species still share the ancestral genetic polymorphisms (Moran and Kornfield, 1993; Nagl et al., 1998), with possible influence of hybridization. Sequencing of commonly used genomic markers hence does not provide sufficient resolution to unravel the multi-layer and possibly reticulated phylogenetic network among the nascent species. Therefore, other additional methods (e.g. microsatellites, AFLP fingerprinting, NGS sequencing of larger portions of a genome like MHC complexes etc.) must be applied, hand-in-hand with thorough morphological analyses of the used samples, to uncover the details of diversification within these highly interesting species complexes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.09.006.

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The following passage (pages 77–87) is a manuscript in review and it was removed from this version of the thesis that is open to public. The bibliographic information as well as the abstract of this publication follows:

Paper V

Casciotta, J., Almirón, A., Aichino, D., Gómez, S., Piálek, L., Říčan, O., in review. *Crenicichla taikyra* (Teleostei: Cichlidae), a new species of pike cichlid from the middle río Paraná, Argentina. Submitted to Zootaxa.

Abstract

Crenicichla taikyra, new species, is described from the middle río Paraná, Argentina. *Crenicichla taikyra* is distinguished from the other species of the genus by the following combination of characters: lower pharyngeal tooth plate stout, bearing molariform teeth, ascending arm of premaxilla longer than the dentigerous arm, posterior edge of preoperculum serrated, a well developed suborbital stripe, and absence of scattered dark dots on flanks. Molariform teeth on pharyngeal jaws is a derived character among *Crenicichla* species, however this character state has appeared several times in unrelated species.

The following passage (pages 89–126) is a manuscript in preparation and it was removed from this version of the thesis that is open to public. The bibliographic information as well as the abstract of this publication follows:

Paper VI

Piálek, L., Doubnerová, K., Petrusek, A., Casciotta, J., Almirón, A., Říčan, O., in preparation. Parallel evolution: Repeated origin of morphological species in Neotropical cichlids (*Crenicichla*) revealed by phylogenomics.

Abstract

The parallel evolution of convergent forms is a well documented phenomenon, especially in adaptive radiations. Among the best studied cases of these evolutionary phenomena are the *Anolis* lizards of the Caribbean islands, the stickleback fishes from postglacial lakes, and the cichlid fishes in African rift and crater lakes. Recently we have found a similar case of parallel evolution of convergent forms in two species flocks of the South American cichlid genus *Crenicichla* in the geomorphologically complex rivers of the southern Brazilian shield highlands. Here, we present a detailed analysis of one of these species flocks (the *Crenicichla missioneira* complex in the Uruguay River basin) and we document a new type of recurrent evolution of convergent forms. We demonstrate using phylogenomic analyses employing the ddRADseq method that most of the eight recognized species of this complex are polyphyletic and that in fact these morphological species have evolved repeatedly many times in local sympatric radiations endemic to upland portions of tributaries to the Uruguay river. The described morphological species in this complex are thus not real biological species but rather ecomorphological types. The *C. missioneira* species complex thus demonstrates that there is indeed a real-life distinction in the debate whether species are real unique biological entities (philosophical individuals) or defined philosophical classes. Our results show that pure reliance on solely morphological aspects of biodiversity can and does confuse these two kinds of species.

Results summary

Newly described species

The above mentioned high diversity of *Crenicichla* in the La Plata River basin seems to be considerably undervalued; three new species from the middle Parana River drainage have been recently described by our team (and about six other evolutionary lineages from the same area are being investigated as putative new taxa). *Crenicichla ypo* (**PAPER II**) was described from the Urugua–i River, a left-hand inlet of the middle Paraná River in Misiones province, Argentina. This tributary, the next one south of the Iguazu River, was separated from the main stream of the Paraná by a large waterfall, that was partly destroyed during the construction of the Urugua–i reservoir; the drainage is further inhabited by another (discovered by our team and still undescribed) congener temporarily called as "*C.* sp. n. Urugua–i line", a slender-bodied species with a prominent longitudinal stripe that is morphologically entirely distinct from the described *C. ypo*. *Crenicichla ypo* is recognized by 6 to 8 irregular blotches along the upper lateral line, absence of scattered dark spots on flanks, low number of E1 scales, and a slightly prognathous lower jaw. Females have a distinctive coloration of the dorsal fin, with a wide black longitudinal stripe on the distal portion with an equally wide red stripe below it.

Another new species, *C. hu*, was described from the arroyo Piray–Miní, again a left-hand tributary of the Paraná River just south of the Urugua–i River (**PAPER I**). *Crenicichla hu* is easily distinguished by the dark coloration (dark grey or dark brown to black), a color pattern consisting of 7 to 9 black irregular blotches on the flank, and 47–54 scales in the E1 row. Adult females have a differently colored dorsal fin from *C. ypo* with an irregular color pattern formed by wide black and white longitudinal stripes and blotches. The newly described taxon shares its habitat in the lower portion of the river with *C. mandelburgeri*, a species widespread in tributaries of the lower-middle Paraná River that lack significant waterfall barriers close to their mouths (with some exceptions) and which are on the other hand typical for the middle-upper Paraná reaches (including the Urugua–i and Iguazu in Missiones).

Crenicichla taikyra n. sp. (**PAPER V**) is described from the main course at the southern terminus of the middle Paraná River, collected in a stony environment. *Crenicichla taikyra* is distinguished from the other species of the genus by a stout lower pharyngeal tooth plate bearing molariform teeth, ascending arm of premaxilla longer than the dentigerous one, posterior edge of preoperculum serrated, a well developed suborbital stripe, and absence of scattered dark dots on flanks. Molariform teeth on pharyngeal jaws is a derived character among *Crenicichla* genus, however only three other species are known to have this character (from which two belong to the *C. lacustris* sp. group: *C. yaha* and *C. jurubi*). Among the *C. lacustris* group *C. taikyra* is most similar to *C. yaha* (also inferred as a sister species by phylogenomic analysis, and as a close relative based on

mitochondrial phylogeny; see further in the text) and its more derived durophagous dentition thus seems to be directly derived from the less developed condition in *C. yaha*.

Phylogeny of the genus *Crenicichla*

The first molecular phylogenetic analysis of the genus *Crenicichla* is the subject of **PAPER IV**. Our analysis combined three mitochondrial and one nuclear gene sequences of 169 specimens and yielded a robust phylogenetic hypothesis: *Crenicichla* is split into two main clades: (1) *Teleocichla*, the *Crenicichla wallacii* group, and the *Crenicichla lugubris–Crenicichla saxatilis* groups; (2) the *Crenicichla reticulata* group and the *Crenicichla lacustris* group–*Crenicichla macrophthalma*. Influence of particular markers on the inferred phylogeny was also investigated revealing a contribution of ND2 gene throughout the whole range of hierarchical levels in the tree with weaker effects of saturation at the 3rd codon position compared to cytochrome *b*. Our study further confirmed the monophyly of the *C. lacustris* species group with very high support and recovers two endemic species flocks within the *C. lacustris* group, the *Crenicichla missioneira* species flock and the herein discovered *Crenicichla mandelburgeri* species flock from the Uruguay and Paraná/Iguazu Rivers, respectively. The phylogeny further showed much higher diversity within the *C. mandelburgeri* species complex than expected.

The species of the *C. missioneira* and *C. mandelburgeri* flocks display in each group an extremely wide range of morphologies and coloration patterns which are similar between these flocks, yet the two complexes are strongly monophyletic and are not immediate sister groups (separation of their evolutionary lineages is estimated to 6–8 mya based on cyt *b*; **PAPER IV**). Most species thus resemble forms from the unrelated species complex rather than closely related species within their complex (Fig. 4 in **PAPER IV**; Fig. 1 in **PAPER VI**; **front cover**), and only subtle morphological characters (e.g. the lack of a suborbital stripe in the *C. missioneira* species group) hint at the fact that there are indeed two distinct species complexes, each limited to a different river basin. The striking similarities in morphology and in coloration patterns across the two species groups have thus evolved in parallel, multiple times, and similar morphologies and colorations are found in both groups in similar habitats (**PAPER VI**). For example, gregarious species from each group that feed in a large extent by grazing on periphyton (*C. hadrostigma* and *C.* sp. n. Iguazu) both have a similar mouth and head morphology (head short, lower jaw shorter than upper and whole head thus inclined ventrally), teeth morphology, but surprisingly, they also have a very similar (and unique) coloration pattern, composed of double vertical bars on a green body with a spot behind the dorsal portion of the head (Fig. 1 in **PAPER VI**, bottom pair).

Speciation mechanisms

Crenicichla missioneira species flock

Within the *C. missioneira* species complex, traditional mitochondrial and nuclear markers provide very low resolution regarding evolutionary relations between ecomorphological forms (i.e. described species; **PAPER IV**). Therefore a recently developed phylogenomic method, Double Digest Restriction-site Adjacent DNA Sequencing (ddRADseq) was used to reconstruct the history of the rapid diversification within this species flock (**PAPER VI**). The preliminary results revealed that most of the described species (morphotypes) are polyphyletic, clustering by drainage or tributary instead of by the given species. This pattern is visible especially in the higher elevations of individual tributaries and/or in those isolated by a major waterfall. In the lower sections of the basin without significant barriers there are widespread lineages which indicates dispersal. Geographical proximity of tributaries is further reflected in phylogenetic proximity of their fauna. The interpretation is thus that the morphological species are evolving repeatedly in parallel; this is clearly evident in the inferred phylogenies where in four different tributaries (Forquilha, Pepiri, Yaboti, and Soberbio Rivers) several different morphotypes form a sympatric monophyllum.

The most common parallel evolution that we observed is in the *C. minuano-missioneira-tendybaguassu* triplet of forms differing solely in their mouth morphology. This triplet has evolved at least three times independently (in the Pepiri, Yaboti, and Soberbio tributaries). The weakest case among these is Soberbio, which is also the shortest and smallest tributary with the least elevation gradient. On the other hand, the monophyllum of these three forms from Pepiri River (a tributary flowing into the main stream Uruguay close above the Moconá Falls, further isolated by several rapids) received a strong support not only from the ddRADseq based inference but also in the otherwise weakly resolved mitochondrial phylogeny (unpublished results).

In the Forquilha River a sympatric evolution of the *C. minuano-missioneira-igara* triplet was revealed; we are however missing samples of several other species from this river. Such finding however offers a sufficient explanation of the Lucena and Kullander's note regarding different appearance of specimens from this tributary (see the note in Introduction – *C. lacustris* species group). Finally, it was subtle color pattern differences unique to different tributaries that were shared across morphotypes in the Uruguay River tributaries that first suggested to us the possibility that we might be witnessing local speciation and parallel evolution of the head-jaw defined morphotypes.

Crenicichla mandelburgeri species flock

In the *C. mandelburgeri* species complex, most of the morphological forms were basically recovered as monophyletic evolutionary lineages, with several proposed relationships lacking considerable support (**PAPER IV**). The intra-flock multilocus phylogeny is however based solely on mitochondrial loci, as the nuclear marker S7-i1 used in the study (as well as several other tested nuclear markers)

showed almost no variability within this complex (see also Fig. 2 in **PAPER IV**). Phylogeny of the *C. mandelburgeri* flock was therefore also studied using the ddRADseq (**Fig. 1**; unpublished preliminary results). Here it may be appropriate to point out that the ddRAD tags sequences originate virtually exclusively from nuclear DNA (statistically, every 60,000th restriction fragment is of the mitochondrial origin based on the lengths ratio; but given the limited absolute number of restriction sites in the mitochondrion the real count of size-selected tags is probably zero). The ddRADseq tree gained strong bootstrap support for all nodes (**Fig. 1**). Specimens from all river systems are resolved as monophyletic lineages (with one dispersal downstream over the Iguazu Falls) reflecting thus clearly relationships between drainages; similarly, mapping of the most considerable morphological changes of the mouth/teeth arrangement is unambiguously parsimonious (**Fig. 1**).

The ddRADseq phylogeny was compared to the mitochondrion based inference (cyt *b*, ND2) with corresponding taxon sampling (**Fig. 1**); the comparison revealed several considerable discrepancies between the topologies. In three cases, a couple of species/forms from the same drainage which are distinct and morphologically diagnosable one from another (as well as from other species of the genus; and also forming separate and unrelated mitochondrial lineages) form a monophyletic cluster in the ddRADseq, sometimes one form making the other paraphyletic. These couples are *C. hu* - *C. mandelburgeri* (from the Piray–Mini River), *C. ypo* - *C.* sp. n. Urugua–i line (Urugua–i River), and *C.* sp. n. Piray–Guazu - *C.* sp. n. Piray–Guazu line (Piray–Guazu River). Since in all these examples the nuclear lineage contains morphologically distinct phenotypes, we consider one of the species in each of the pairs being evolved by hybridization. In the other case of differences between the displayed trees, one specimen of *C.* sp. n. biglips (monophyletic in the ddRADseq tree) clusters with *C. yaha* (a syntopically living species) in the mitochondrial tree. Such case we preliminary classify as introgression and expect to identify more similar examples with increased taxon sampling. Conflicts between the ddRADseq and mitochondrial trees thus, in our opinion, reveal putatively hybridized or introgressed evolutionary lineages and these phenomena seems to be more common than generally expected. We are about to study the speciation mechanisms within the *C. mandelburgeri* species flock as a part of our future projects.

Historical biogeography

The biogeographic reconstruction of the *C. lacustris* group (**PAPER IV**) using dispersal-vicariance analysis supported complex biogeographic patterns of freshwater fishes in this area. The La Plata and Atlantic coast faunas are non-monophyletic with highly complex relationships both within river drainages and between adjacent river drainages. The postulated paleodrainage divide between them runs exactly through the areas which have the most interesting biogeographic patterns in *Crenicichla* as well as in another cichlid genus, *Australoheros* (**PAPER III**). This most interesting area is centered on the upper Uruguay and Iguazu Rivers, their drainage divide and the divides with the adjacent Atlantic coast drainages to the east and the divides and waterfalls between the Paraná and middle

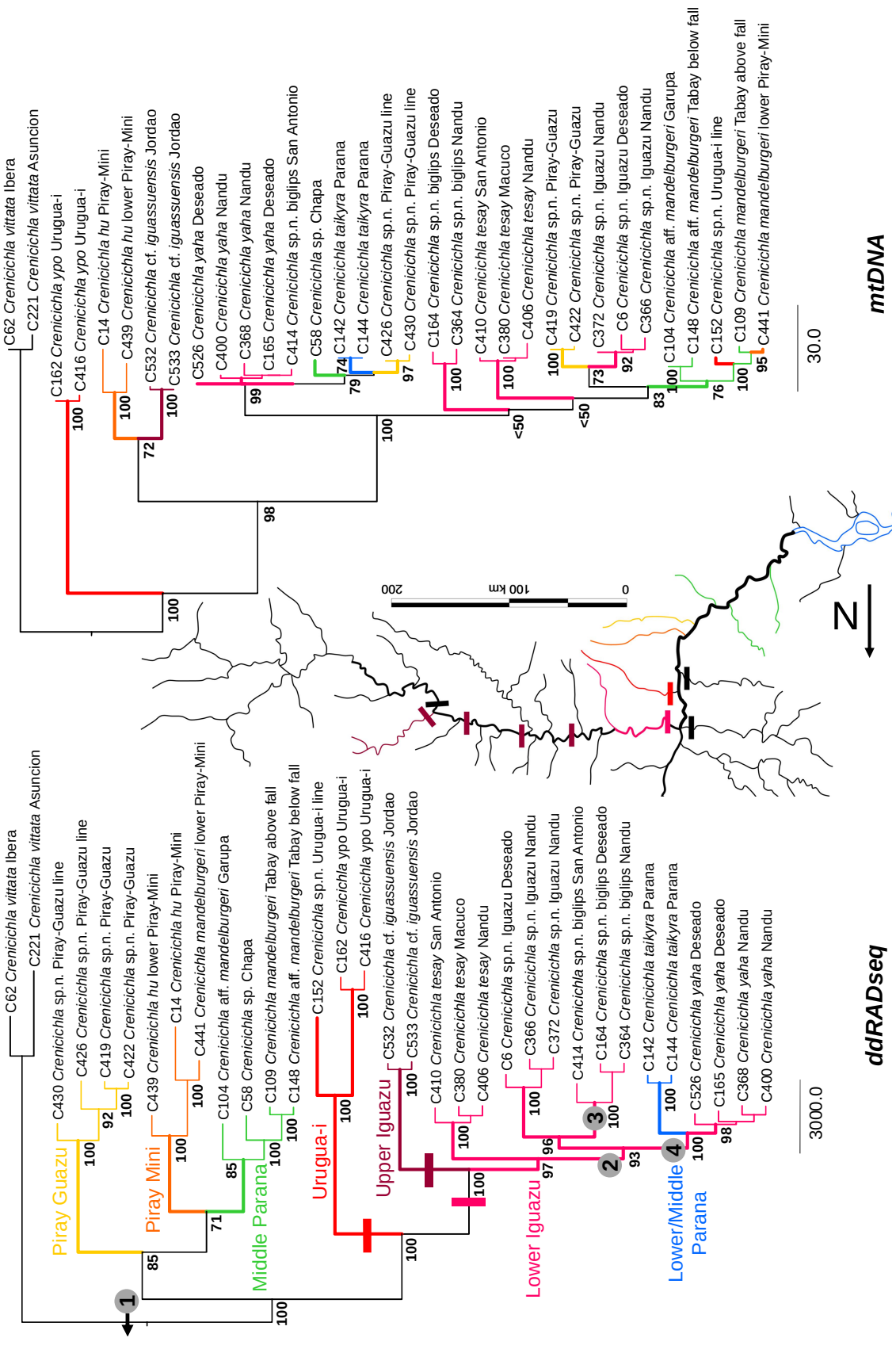

Fig. 1. **ddRADseq (left) and mitochondrial (cyt *b*, ND2; right) phylogenetic trees inferred by Maximum Parsimony (MP).** Support values display MP bootstraps for significant nodes. Relevant drainages in the map and their phylogenetic lineages differ by color; the solid bars depict waterfalls. Gray numbers in circles in the ddRADseq tree display mapping of important morphological characters: Mouth size, 1 – big mouth, 2 – small mouth; 3 – thick lips; 4 – molariform teeth.

Uruguay drainages to the west. Regarding different biology and distribution patterns of both genera their biogeography analyses are partially complementary establishing thus a more complete picture about the history of the area.

The results underlined the importance of ancient barriers between the middle and upper Uruguay River (the Moconá Falls) and between the middle and upper Paraná River (the Guaira Falls); another important barrier is hypothesized within the Iguazu (**PAPER III**), of which there are several in the form of large waterfalls, that are however all (except for the Iguazu Falls) flooded by a succession of several hydroelectrical dams. Prominent waterfalls (like the most famous 70 meters high Iguazu Falls or the Urugua-i Falls) generally divide endemic taxa since they form barriers to dispersal and the increasing height of the waterfalls increases isolation. This suggests that waterfalls delimit the boundaries of a given fauna, while river captures and drainage translocations are responsible for the (older) evolution of the diversity per se. The distribution patterns of *Australoheros* species in the Uruguay and Iguazu River drainages further pointed to historical connections between today isolated river drainages (the lower Iguazu River with the arroyo Urugua-i, and the middle Iguazu River with the upper Uruguay River).

Conclusions

Species diversity of *Crenicichla*, the most-specious genus of Neotropical cichlids, is very high in the southern part of its distribution area – in the La Plata River basin (the Uruguay, Paraná and Iguazu Rivers) and adjacent coastal drainages. Three new species from the middle Paraná River drainage were described during work on this thesis, and other five putative species from the *C. mandelburgeri* complex are currently in the process of description. The actual diversity within the *C. missioneira* species complex from the Uruguay River basin can hardly be expressed in traditional taxonomic categories.

Two sets of factors were pointed out as likely responsible for the species richness: the innate diversification potential of the genus, and the geological complexity of the upland rivers of the La Plata River basin. Besides the generally prevailing allopatric diversification, two other main speciation mechanisms occurring within *Crenicichla* species flocks were revealed. First, repeated origin of analogous morphological species taking place in different tributaries was evidenced by phylogenomic approach utilizing ddRAD tags high-throughput sequencing. Such scenario can be synonymized with sympatric speciation within these drainages; a process likely initiated and/or driven by utilization of different trophic niches. Second, our preliminary results drew attention to hybridization and introgression as potentially a surprisingly common speciation mechanism within the *C. mandelburgeri* species complex.

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