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# Phylogenetic relationships of species of *Crenicichla* (Teleostei: Cichlidae) from southern South America based on the mitochondrial cytochrome *b* gene

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## Abstract

Phylogenetic analysis using Bayesian inference, likelihood and parsimony methods was conducted on 60 complete mitochondrial cytochrome *b* sequences from 21 species of *Crenicichla*, including all species known from Uruguay (*Crenicichla celidochilus*, *Crenicichla lepidota Crenicichla minuano*, *Crenicichla missioneira*, *Crenicichla punctata, Crenicichla scottii, Crenicichla vittata*), *Crenicichla compressiceps*, *Crenicichla empheres, Crenicichla geayi, Crenicichla iguassuensis*, *Crenicichla macrophthalma, Crenicichla menezesi, Crenicichla notophthalmus*, *Crenicichla regani*, *Crenicichla* cf. *regani*, *Crenicichla semifasciata, Crenicichla sveni, Crenicichla tendybaguassu,* two unidentified species, and also two species of *Teleocichla*. Bayesian analysis resulted in a trichotomy with three major groups: (1) The *C. missioneira* species group (*C. celidochilus, C. empheres*, *C. minuano, C. missioneira*, *C. tendybaguassu,* and an undescribed species analyzed); (2) a group of southern species (*C. iguassuensis, C. punctata, C. scottii, C. vittata*); and (3) a rather heterogeneous group comprising the type species *C. macrophthalma*, members of the *Crenicichla reticulata* species group (*C. geayi*, *C. semifasciata*), members of the *Crenicichla wallacii* species group (*C. compressiceps, C. notophthalmus*, *C. regani, C.* cf. *regani),* members of the *Crenicichla saxatilis* species group (*C. lepidota*, *C. menezesi*, *C. sveni*, *C.* sp.), and two species of *Teleocichla.* Parsimony jackknifing resulted in a quadritomy with: (1) *C. macrophthalma*, (2) *Teleocichla,* (3) the *saxatilis* + *wallacii* group species, and (4) the rest, which include *C. geayi* and *C. semifasciata* as sister group to a dichotomy with the *C. missioneira* group and the remaining southern species. The sequence variation within the *C. missioneira* group is remarkably minor despite considerable morphological differences, supporting the conclusion that it forms an endemic species flock in the Uruguay River basin. Previously proposed species groups within the speciose genus *Crenicichla* (more than 90 species known) are partly corroborated. However, *C. celidochilus* was not previously associated with the *C. missioneira* species group, and *C. vittata* has not previously been associated with *C. scottii, C. iguassuensis*, or *C. punctata. Crenicichla lepidota, C. sveni, C. menezesi* and *C.* sp. represent the *C. saxatilis* group. Species of small size, representing the *C. wallacii* species group and *Teleocichla* are characterized by very long branches, and the position of *Teleocichla* differed considerably between the Bayesian and parsimony trees. This finding does not invalidate *Teleocichla* but rather suggests that the several monophyletic major clades within *Crenicichla* may need nominal recognition. A putative hybrid specimen with a morphology combining components from *C. vittata* and *C. scottii*, but with a cytochrome *b* sequence from *C. scottii* was found in a sample from the Rio Quaraí/Cuareim. Another putative hybrid specimen with a unique morphology but a cytochrome *b* sequence agreeing with *C. scottii* was found in a sample from Maldonado, but no other *Crenicichla* species than *C. scottii* is known from that locality.

**Key words:** Uruguay – hybrids – phylogeny – species flock

## Introduction

The South American cichlid genus *Crenicichla* is the most species-rich cichlid genus with over 90 known species, of which 80 have been formally named (pers. obs.). Species of *Crenicichla* occur in tropical and subtropical regions of cis-Andean South America from the Rio Orinoco and Trinidad in the north to the Rio Paraná basin in the south.

Lucena and Kullander (1992) reported 11 species of *Crenicichla* from the Rio Uruguay drainage in Brazil. Seven of these are endemic to the Rio Uruguay drainage. The large number of species reported from this river was considered exceptional, being about double that for tropical South American rivers of similar size, and the number was recently increased by two more endemic species described by Lucena (2007) from the upper Rio Uruguay drainage. For comparison, Kullander (1986) recorded only eight species of *Crenicichla* from the Amazon River drainage in Peru, four or five of which are endemic. The Peruvian Amazonia is a hotspot for fish diversity, and no additional species of *Crenicichla* have been recorded from Peru since.

Lucena and Kullander (1992) assigned the species from the Rio Uruguay drainage to species groups, reflecting assemblages of similar and presumably closely related species, but did not provide a formal classification or phylogenetic analysis. One of these species groups, labelled the *Crenicichla missioneira* group consisted of five species, three of which were sympatric in the middle Rio Uruguay (*Crenicichla minuano*, *missioneira*, *Crenicichla tendybaguassu*), and two other species sympatric in the upper Rio Uruguay (*Crenicichla igara*, *Crenicichla jurubi*). The sympatric species differed among themselves principally in the length of the jaws and the thickness of the lips. Among species from the middle Rio Uruguay drainage, *C. missioneira* has the typical piscivorous appearance of most species of *Crenicichla*, with long snout, long jaws and the lower jaw projecting slightly. The syntopic *C. minuano* has a short snout and shorter lower jaw and the third syntopic species, *C. tendybaguassu*, has somewhat long snout but the upper and lower lips are enlarged anteriorly. These three species are very similar in colour pattern. *Crenicichla igara* and *C. jurubi* from the upper Rio Uruguay drainage share an unusual spotted colour pattern among

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themselves, and differ by the former having long snout and long lower jaw, the latter having a short snout and short lower jaw. The differences in trophic structures suggested that the species involved might be evolved in sympatry by disruptive selection on trophic morphology, or perhaps represent a single plastic species. The second species group, the *Crenicichla scottii* group, included three allopatric species, *C. scottii*, *Crenicichla gaucho* and *Crenicichla prenda*, replacing each other along the Rio Uruguay. These three species are similar in mouth shape, but differ slightly in colour pattern. One additional endemic species, *Crenicichla celidochilus,* was demonstrated to have a novel growth pattern and sexual dimorphism, and was not assignable to any particular species group. Species of *Crenicichla* are typically sexually dichromatic, and females commonly possess an ocellated blotch in the dorsal fin. Most species of *Crenicichla* possess a juvenile colour pattern with horizontal stripes which is modified or replaced by other markings in adults. By contrast, *C. celidochilus* is sexually monomorphic except that juvenile females do possess a dorsal-fin ocellus, the adult colour pattern is similar to that of juveniles, and the growth pattern expressed in adults is similar to that of juveniles.

We have since searched for independent data particularly to reduce the options for explanation of the species richness and the highly restricted sympatric occurrence of presumed closely related species of *Crenicichla* in the Rio Uruguay drainage which reflects a case of riverine species flock. Recent collections from Uruguay include samples enabling genetic analysis of syntopic *C. missioneira* and *C. minuano* as well as *C. celidochilus*, and all other species occurring in Uruguay, viz. *C. scottii*, *Crenicichla punctata*, *Crenicichla vittata* and *Crenicichla lepidota*.

With that material, supplemented by samples from the Brazilian and Argentinian portions of the Rio Uruguay, we are able in this paper to examine relationships of species of the *C. missioneira* group, their phylogenetic position within the genus *Crenicichla*, and their status as a species flock. Further, we test if the groupings of species of *Crenicichla* defined on morphology could be corroborated with molecular data.

## Materials and Methods

Whole specimens or small pieces of muscle tissue or fin clips were preserved in 95% ethanol in the field or from aquarium sources. Samples are listed in Table S1, and Uruguayan sampling sites are shown in Fig. 1. Voucher specimens are preserved in the collection of the Swedish Museum of Natural History, Stockholm (NRM) and the Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre (MCP); detailed collecting data are available from the Global Biodiversity Information Facility (http://data.gbif.org).

Morphometric and meristic data were obtained as described in Kullander (1986), and morphometric and meristic data on *C. scottii* and *C. vittata* are the same as in Lucena and Kullander (1992).

Total genomic DNA was extracted from tissue samples using the QiAmp DNA Mini Kit (QIAGEN Inc., Valencia, CA, USA). The complete mitochondrial cytochrome *b* was amplified using primers FishCyt*b*-F, Cyt*b*I-7F, Cyt*b*I-3R and TrucCyt*b*-R, and protocols as described by Sevilla et al. (2007), with illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare Life Sciences, Little Chalfont, UK). Subsequent PCR products were checked on a minigel, and then purified using enzymes Exonuclease I (Exo) and Shrimp Alkaline Phosphatase (SAP) according to protocol for PCR product clean-up (MBI Fermentas Inc., Burlington, ON, Canada). Sequencing reactions were performed using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) with the same primers as used for amplications. Sequence reaction products were purified using the Dyex 96 kit (QIAGEN). Sequencing was done by using an ABI 3130x1 (Applied Biosystems) automatic sequencer. Sequences were assembled using SeqMan and aligned with Megalign (LaserGene v. 7.1, DNASTAR Inc., Madison, WI, USA) using the Clustal W method with the Fast-Approximate option. The alignment comprises the entire cytochrome *b*, 1137bp, with a TAA stop codon.

Mitochondrial cytochrome *b* was chosen because it is known to perform well in analyses of cichlid interrelationships at species and genus level (Farias et al. 2001). Sixty-two sequences were obtained (GenBank accession numbers GQ199902–GQ199963; http://www.ncbi.nlm.nih.gov/Genbank/), and analyzed together with a *Crenicichla regani* sequence from GenBank (GenBank accession number AF370646). A putative *Teleocichla monogramma* sequence from GenBank (accession number AF370648) was excluded after it was found not to group with the ingroup. *Cichla* was selected as outgroup in phylogenetic analyses based on the sister-group relationship between *Crenicichla* and *Cichla* in Kullander (1998). To understand why several different lengths have been reported for cytochrome *b*, we downloaded from GenBank all cytochrome *b* sequences that were tagged as complete, and inspected them visually.

Sequences were analyzed using parsimony, maximum likelihood and Bayesian inference methods. Trees were captured with TreeView 1.6.6 (Page 2001) and subjected to graphical improvement in Adobe Illustrator 11.0. Dambe 5.0.23 (Xia and Xie 2001), Mega 4.1 (Kumar et al. 2008), and the homogeneity test in PAUP*4 (Swofford 2001) was used to calculate base frequencies and to test for saturation.

Parsimony analysis was made with PAUP* 4 (Swofford 2001), and the heuristic search method with default settings. The parsimony jackknife analysis was performed with 300 pseudoreplicates, five random addition sequences per pseudoreplicate, tree bisection and reconnection (TBR) branch swapping, character deletion frequency 37%.

MrModelTest 2.2 (Nylander 2004) was used to obtain an evolutionary model that best fit the data and used in Bayesian inference and maximum likelihood analyses. Maximum likelihood analysis was conducted using PAUP* 4 under the GTR + I + G model selected by MrModeltest. Bayesian inference was analyzed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), using the same model. Markov chain Monte Carlo analysis was run for 1 million generations, sampled at an interval of 100 generations, at which time the average standard deviation of split frequencies had fallen under 0.01 and convergence assumed. The earliest 25% of samples were discarded as 'burn-in'.

## Results

The 60 *Crenicichla* sequences represent 46 haplotypes. Both specimens of *C. vittata* have the same haplotype. All four specimens of *C. celidochilus* have the same haplotype. Three specimens of *C. minuano* from Durazno share the same haplotype, whereas the specimen from Artigas has a haplotype differing in four positions, two of which shared with *C. tendybaguassu*. *Crenicichla tendybaguassu* has only four unique bases compared with *C. minuano*. The geographical range of samples of *C. lepidota* is greater than the other species analyzed, and includes seven haplotypes, one of which differing from the remainder in 2% of the bases. *Crenicichla missioneira* has six haplotypes, *C. scottii* has 11.

Performance of the chi-square test in PAUP*4 yielded a homogeneous base composition ($p = 1.000$) in the total dataset, with frequencies A = 0.25%, G = 0.33%, C = 0.14% and T = 0.28. The saturation test in Dambe (Xia and Xie 2001) indicates insignificant level of saturation at codon positions 1 and 2, and moderate saturation at codon position 3. This low level of saturation was not expected to confound phylogenetic analysis, and as Källersjö et al. (1999) showed, even highly saturated sequences can retain local phylogenetic signal. The third codon position was therefore retained in all analyses.

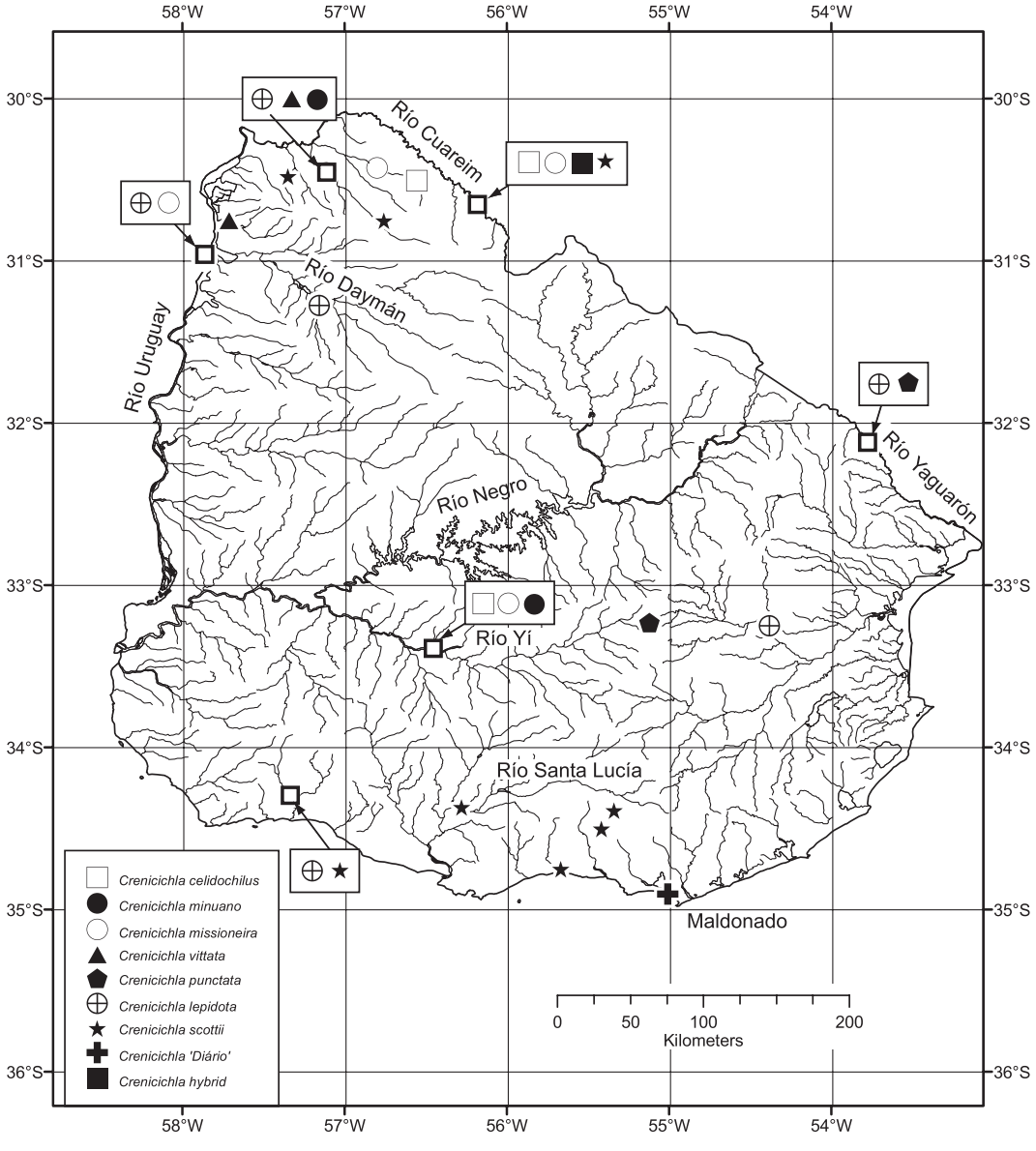


Fig. 1. Map of Uruguay showing Uruguayan sampling localities

Of 1137 nucleotide positions, 514 are variable and 432 are parsimony-informative. The parsimony, maximum likelihood and Bayesian inference analyses produced trees with almost identical topologies (Figs 2 and 3). The maximum likelihood topology, not shown here, is identical to the parsimony tree except for resolving the collapsed *C. lepidota* clade above the Cerro Largo specimen, and identical with the Bayesian tree in the *missioneira* group topology except for higher resolution within *C. missioneira*. The Bayesian tree shows a trichotomy with three major clades. Within one clade, with very low bootstrap support (79%), it recovers monophyletic groups of the hypothesized *Crenicichla saxatilis* group represented by four or more species (*C. lepidota, Crenicichla menezesi, Crenicichla sveni, C.* sp.), the *Crenicichla reticulata* group (*Crenicichla geayi*, *Crenicichla semifasciata*), and the *Crenicichla wallacii* group (*Crenicichla compressiceps, Crenicichla notophthalmus*, *C. regani*, and *C.* cf. *regani*). A group of southern species (*Crenicichla iguassuensis, C. vittata*, *C. punctata,* C. *scottii*), and the *C. missioneira* group (*C. celidochilus, Crenicichla empheres, C. minuano, C, missioneira, C. tendybaguassu*, *C.* sp. 'Forquilha'), make up the other two major well-supported clades.

The parsimony jack-knife tree (Fig. 2), with a basal quadritomy, has only weak support (64% jackknife frequency) for combining the southern clade and the *C. missioneira* group as sister groups, and places the *C. reticulata* group as sister group to these with only 54% support. In the quadritomy, the *C. wallacii* and *C. saxatilis* groups are sister groups in one, weakly supported clade (59%), and *Crenicichla macrophthalma* and *Teleocichla* make up the remaining two.

There is considerable nucleotide divergence between major groups of *Crenicichla,* between about 10% and 18% nucleotide divergence. Within clades of closely related species it ranges up to 9.9% within the *C. saxatilis* group (*C. sveni* versus *C. lepidota*) and 16.5% in the *C. wallacii* group (*C.* cf. *regani* versus *C. compressiceps*).

There is little sequence variation between the species of the *C. missioneira* group (less than 1% nucleotide divergence, except in the upper Uruguayan species *C. empheres* (1.1–1.3%)

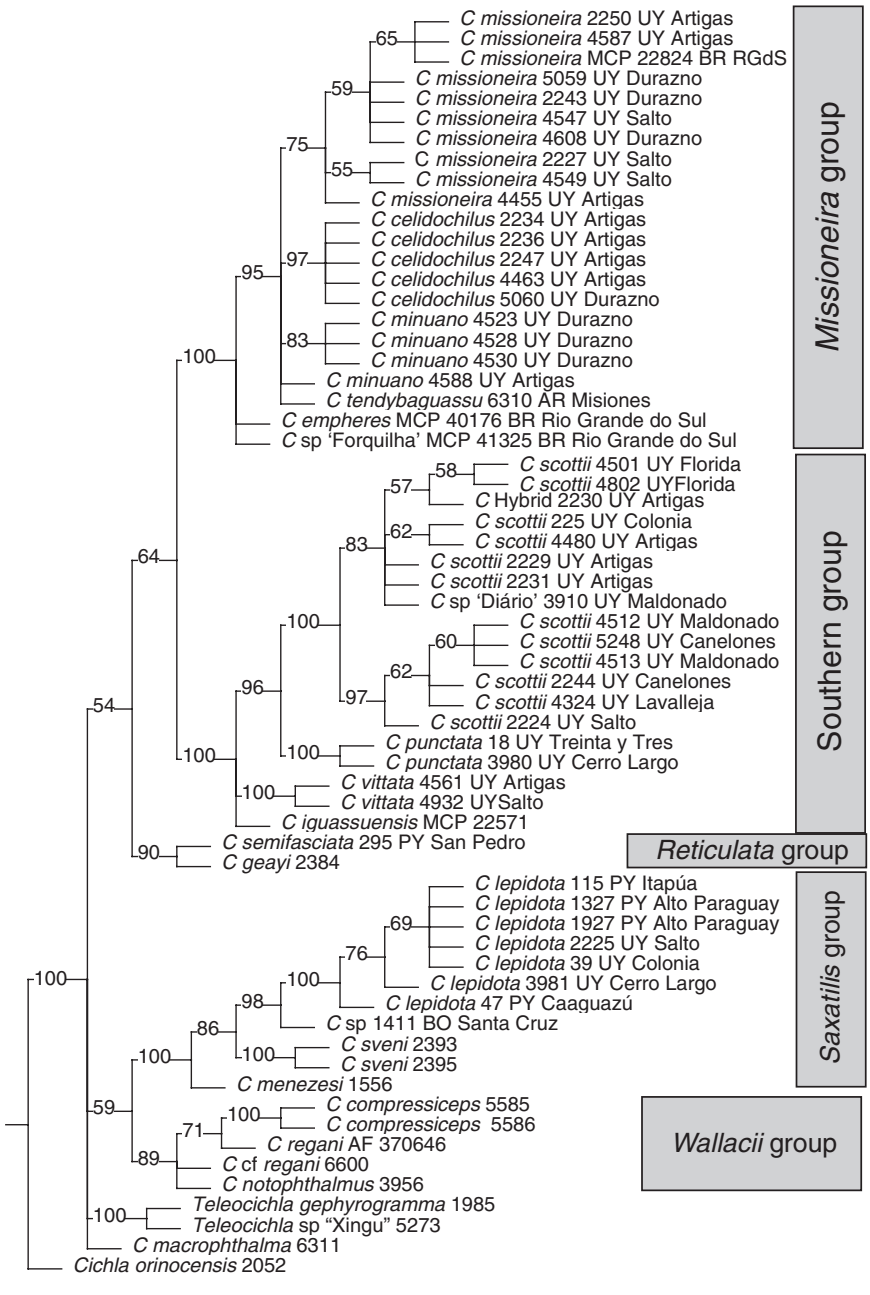


Fig. 2. Cladogram of relationships among southern South American species of *Crenicichla* based on parsimony analysis of the nucleotide sequence of the complete mitochrondrial cytochrome *b* gene. Majority-rule consensus tree (cut-off 50%) summarizing result of parsimony jack-knife analysis of cytochrome *b* alignment (obtained using PAUP*4 with 300 pseudoreplicates, five random additions per replicate, TBR branch swapping, and character deletion frequency 37%). Labels at nodes indicate jack-knife frequency. Samples are identified by NRM tissue numbers or MCP catalogue numbers, and locality were known. AR = Argentina, BO = Bolivia, BR = Brazil, PY = Paraguay, UY = Uruguay, RGdS = Rio Grande do Sul

and *C*. sp. 'Forquilha' (1.8–2.1%). The parsimony analysis collapses the group into a trichotomy, whereas the Bayesian tree shows a pectinate branching with *C*. sp. 'Forquilha', *C*. *empheres* and the remainder as successive branches. The two samples of *C*. *punctata* analyzed differ by 2.4% nucleotide divergence. Between the *C*. *reticulata* group species *C*. *semifasciata* and *C*. *geayi*, it is 12.8%. Samples of *C*. *scottii* form two clades with mixed geography and at most 1.2% nucleotide divergence.

## Discussion

### Cytochrome *b* variation in cichlids

All sequences reported here include 1137 nucleotide base pairs, corresponding to 379 amino acids. Nucleotide frequencies are almost identical to those reported for cytochrome *b* across the Cichlidae by Farias et al. (2001) (A = 25%, G = 32%, C = 14%, T = 29%). The 514 variable and 432 parsimony-informative sites are less than reported for the Cichlidae as a whole by Farias et al. (2001) (37% invariable sites, 579 parsimony informative), but our taxonomic sampling is more restricted. The start codon is ATG, and the stop codon is TAA. This agrees with observations of Martin and Bermingham (1998), Quenouille et al. (2004) and Hulsey et al. (2004), all for Central American cichlids. Other studies of cichlids report different lengths, e.g. 1143 bp by Concheiro et al. (2007) for Central American cichlids and Říčan and Kullander (2006) for South American cichlids, 1138 bp by Farias et al. (2001) for African and Neotropical cichlids, 1111 bp by Musilová et al. (2008) for South American cichlids, and 'approximately 1040 bp' by Roe et al. (1997) for Central American cichlids. Of the 295 cichlid cytochrome *b* sequences labelled as complete in GenBank, 249 are 1137 bp long and end in TAA except in two ending in TAG. Ten sequences are contained in complete

mitochondrial genomes, where cytochrome *b* is 1137 bp long, ending in TGA. Thirty-six sequences are longer than 1137 bp, but of these 24 have TAA and five have TGA at positions 1135–1137, followed by 1–4 bases from tRNA-Thr. Three sequences stand out having TGGC, TAGA or TAGC at positions 1135–1138. Four sequences have three or six insertions preceding the TAA end. It seems thus that the cichlid cytochrome *b* is fairly conservative with a length of 1137 bp and usually a TAA stop codon, less commonly TGA or TAG. The TGG variant, unless an error, may represent an incomplete stop codon T** completed by post-transcriptional polyadenylation (cf. Rüber et al. 2007), and the GG bases belong to the tRNA-Thr sequence.

### Species groups of *Crenicichla*

Kullander (1981, 1982, 1986, 1988, 1990a,b, 1991, 1997), Kullander and Lucena (2006), Lucena and Kullander (1992) and Ploeg (1991) recognized several major species groups within *Crenicichla* based on colour pattern, meristics, snout shape and other external morphological features, viz., the *C. saxatilis*, *C. wallacii*, *Crenicichla lacustris*, *Crenicichla lugubris*, and *C. reticulata* groups, as detailed below. In addition, Lucena and Kullander (1992) distinguished the *C. missioneira* and *C. scottii* groups as smaller potentially monophyletic units. A major divide among species of *Crenicichla* is evident in scale counts because there is a natural break with one large-scaled assemblage having up to about 70 scales in a row along the side, and another small-scaled group (the *C. lugubris* group) having about 80 or more scales (Kullander 1988, 1991). All species analyzed here belong to the large-scaled group, except *C. vittata* which has been associated with the otherwise Amazonian small-scaled species by Kullander (1991) and Ploeg (1991).

The present analysis reveals a clear phylogenetic structure within the genus and provides molecular support for major groups except the *C. lugubris* group which was not represented, as the only included species, *C. vittata,* is apparently a member of a distinct southern assemblage. It enforces the view that *Crenicichla*, with more than 90 species, includes several distinct lineages as already implied by the recognition of morphologically defined species groups. There is still a lack of anatomical studies and phylogenetic analytic approaches based on morphology.

Both the Bayesian and the parsimony analysis resulted in three major clades, but the topology is different. Most of the difference is due to differing degrees of resolution, but the position of some species varies. Two major clades are strongly supported in both analyses, one containing *C. scottii, C. punctata*, and *C. iguassuensis*, which traditionally have been grouped under the *C. lacustris* group, and *C. vittata*; the other containing the *C. missioneira* group herein shown to include also *C. celidochilus*. The *reticulata* group is recovered, but in different positions. Both analyses support the *C. saxatilis* and *C. wallacii* groups.

Within the small-scaled group of *Crenicichla*, Kullander (1991, 1997) distinguished the *C. lugubris* group with more than 100 scales in a lateral row, short, blunt snout, nostril close to the margin of the postlabial skin fold, and a distinctive ontogenetic colour pattern transformation; and the *C. acutirostris* group with 76–111 scales, and long-pointed snout, to which *C. vittata* (78–85 scales) conforms. The small-scaled species were treated as a single group, the *C. lugubris* group, by Ploeg (1991). The inclusion of *C. vittata* in a clade of southern species (*C. scottii, C. punctata, C. iguassuensis*) provides a novel relationship hypothesis for *C. vittata* among southern species to which it geographically belongs, supported by a shared colour pattern character state (see below), and suggests that the subdivision of the genus into small-scaled and large-scaled species may not be phylogenetically valid. All other small-scaled species of *Crenicichla* are found exclusively in the Amazon and Orinoco basins, and the Guianas. None of these were available for inclusion in the present analysis.

Kullander (1990a) identified a group of small species, 50–75 mm SL (versus 100–300 mm in other species) characterized by serrated supracleithrum, reduced predorsal squamation and vertebral numbers, and low nostril margin, but did not conclude monophyly. This is the *C. wallacii* group of Ploeg (1991) minus species of *Teleocichla*. *Teleocichla* consists of several small bottom-living species occurring in rapids of rivers tributaries to the lower Rio Amazonas, sharing several uniquely derived characters with *Crenicichla* but departing considerably from that genus in specializations related to rheophily (Kullander 1988). Ploeg (1991) suggested that *Teleocichla* would be nested within *Crenicichla*, and referred the species of *Teleocichla* then known to his *C. wallacii* group. The *C. wallacii* group is represented here by *C. compressiceps, C. regani, C.* cf. *regani*, and *C. notophthalmus*, which form a monophyletic group (Figs 2–3).

Our two sequences of *Teleocichla* are nested in one larger clade in the Bayesian analysis (Fig. 3), while in the parsimony analysis they are found unresolved at the base of the tree (Fig. 2). The *Teleocichla* sequences are highly divergent, perhaps reflecting higher evolutionary rate. As Bayesian analysis is insensitive to long-branch attraction, it is conceivable that their basal position in the parsimony tree is an artefact of the method of analysis. Denser taxon sampling will be needed to clarify the position of *Teleocichla*, but we note that Ploeg's (1991) inclusion of *Teleocichla* within the *C. wallacii* group is not supported by the present analysis (Figs 2–3). *Crenicichla* and *Teleocichla* are sister groups in the analysis of Farias et al. (2001). The positions obtained here for *Teleocichla* within *Crenicichla* do not necessarily invalidate this genus. The analysis clearly demonstrates that there are distinct monophyletic lineages within *Crenicichla* and it may be justified to recognize more genera. Yet, our taxon sampling at this time is insufficient for nomenclatural action.

The *Crenicichla saxatilis* species group (Ploeg 1991; as *C. lepidota* group in Kullander 1982, 1986) includes numerous species with low meristics and a distinct colour pattern with an ocellated humeral blotch in most species. They occur chiefly in the Amazon and Orinoco basins, but also in the Guianas, coastal streams in Uruguay and Rio Grande do Sul and the Paraná and São Francisco basins. *Crenicichla lepidota* and similar species included here, *C. sveni*, *C. menezesi*, and *C.* sp., belong to the *C. saxatilis* group, and come out as a monophyletic group (Figs 2–3).

Most species of *Crenicichla* are horizontally striped. Species with vertical bars, blunt snout and firmly implanted teeth were referred to a separate genus *Batrachops* up until Kullander (1986) who found tooth fixation and snout shape variable among species with a colour pattern with vertical bars across the sides, and consequently synonymized *Batrachops* with *Crenicichla*. This group, approximating the *reticulata* group of Ploeg (1991), may still be recognized by the shared colour pattern including vertical bars. *Crenicichla semifasciata*, from

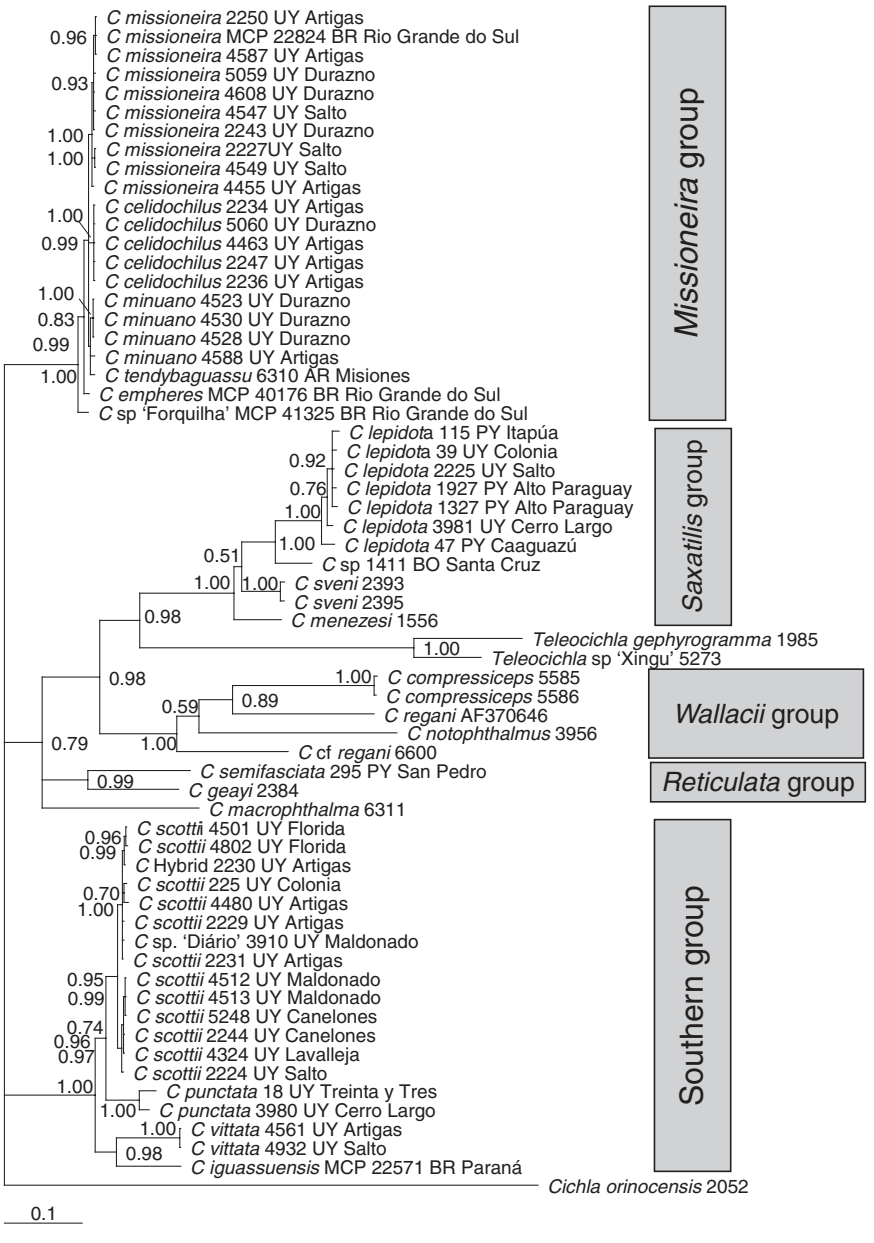


Fig. 3. Majority-rule consensus phylogram of relationships among southern South American species of *Crenicichla* based on Bayesian inference analysis of the nucleotide sequence of the complete mitochrondrial cytochrome *b* gene. Figures at nodes are bootstrap frequencies (values below 50 not shown). The scale bar represents substitutions per site. Samples are identified by NRM tissue numbers or MCP catalogue numbers, and locality were known. AR = Argentina, BO = Bolivia, BR = Brazil, PY = Paraguay, UY = Uruguay, RGdS = Rio Grande do Sul

the Rio Paraguay drainage, and *C. geayi*, from the Rio Orinoco drainage, represent the *C. reticulata* group in the present analysis and are recovered as sister species, although in variable position in the two analyses (Figs 2–3).

Most southern species of *Crenicichla*, including those of the Paraná River drainage, have been referred to a group called the *C. lacustris* group (Kullander 1982; Ploeg 1991), for which no diagnostic character has been proposed. There is no obvious distinguishing character for these species, except that they were mostly misidentified as *C. lacustris* in the past. Kullander and Lucena (2006) suggested that coastal members of this group had three distinct components: (1) *Crenicichla punctata*, which occurs in tributaries of the Laguna dos Patos including Uruguayan tributaries of the Lagoa Mirím/Laguna Merín, is most similar to *C. maculata*, which replaces *C. punctata* in drainages north of the Laguna dos Patos; both species possess a series of dark blotches along the middle of the side. (2) *Crenicichla lacustris*, *Crenicichla tingui*, and *Crenicichla iguapina*, all with a wide dark lateral band, occur allopatrically between the Itapocu and Jequitinhonha rivers. (3) *Crenicichla mucuryna* from the Rio Mucurí, with vertical bars, was thought to possibly be related to the species in the Rio Paraná rather than to *C. lacustris*. Of the coastal taxa, only *C. punctata* was available for the present analysis. *Crenicichla iguassuensis* from the Rio Iguaçu, in the Rio Paraná drainage, has traditionally been referred to the *C. lacustris* group.

*Crenicichla scottii* has also been referred to the *C. lacustris* group (Kullander 1981) or the *C. reticulata* group (Ploeg 1991), but was treated as a member of a *C. scottii* group by Lucena and Kullander (1992), including also *C. gaucho* and *C. prenda,* both from the Rio Uruguay. The group was loosely diagnosed referring to a combination of a broad contrasted lateral band, a suborbital stripe consisting of black dots, and a proximally positioned caudal ocellus.

The type species of *Crenicichla*, *C. macrophthalma*, was included in the *C. lacustris* group as the only Amazonian species by Ploeg (1991), and that group was not diagnosed by

any synapomorphies. *Crenicichla macrophthalma* has a distinctive morphology, characterized in particular by the very large eyes, and it does not fit with any of the morphologically defined groups. Resolution of the phylogenetic position of *C. macrophthalma* is a priority matter in *Crenicichla* taxonomy, because it determines the options for naming constituent clades.

The position of *C. macrophthalma* remains enigmatic, unresolved either at the base of the tree (Fig. 2), or unresolved at the base of a clade with *C. wallacii*, *C. reticulata*, *C. saxatilis* group species and *Teleocichla* (Fig. 3), i.e. species groups that also occur in the Amazon basin.

Kullander and Lucena (2006) provided one potential synapomorphy shared by *C. celidochilus, C. missioneira* group species, *C. reticulata* group species and *C. vittata*, viz., orange to red abdominal sides in females (in the monomorphic *C. celidochilus* in both sexes). We have now observed this colouration also in females of *C. scottii*, but the character state is not observed in *C. punctata*. This colour pattern synapomorphy suggests that of the alternative positions for the *reticulata* group in the two trees (Figs 2–3), the better hypothesis may be the resolved sister-group position to southern species as obtained in the parsimony analysis (Fig. 2). We propose here to call the group including *C. vittata, C. scottii, C. punctata* and *C. iguassuensis*, the 'southern group', instead of using a species name for a label, but in general it reflects the *C. lacustris* group of Kullander (1982) and Ploeg (1991), but with molecular support. The *C. missioneira*, group, which is recovered in both parsimony and Bayesian analyses, and discussed further below, might be included in a major group along with the southern group as indicated in the parsimony analysis (Fig. 2), but there is no molecular support for its association with the southern group in the Bayesian analysis (Fig. 3).

Whereas several monophyletic groups are well supported here, the fraction of the genus analyzed is minor, and the possibility remains that taxa from other regions may be part of the southern clade (*C. scottii, C. punctata, C. vittata, C. iguassuensis*) which also includes several southern species not available for analysis. Morphologically, *C. punctata* is most similar to *C. maculata* from coastal Rio Grande do Sul. *Crenicichla gaucho* and *C. prenda* from the Brazilian portion of the Rio Uruguay are most similar to *C. scottii*.

### The *Crenicichla missioneira* species group

The *C. missioneira* species group, as defined by Lucena and Kullander (1992) is endemic to the Rio Uruguay and characterized by a smooth preopercle and details of the colour pattern. The species originally included by Lucena and Kullander (1992) were *C. minuano, C. missioneira*, and *C. tendybaguassu*, which occur sympatric in the middle Rio Uruguay drainage, and *C. igara* and *C. jurubi*, which are sympatric in the upper Rio Uruguay. Two species from the upper Rio Uruguay, viz. *C. empheres* and *Crenicichla hadrostigma*, were added by Lucena (2007). An additional species included in the present analysis (*Crenicichla* sp. 'Forquilha'), from the Rio Forquilha in the upper Rio Uruguay drainage, remains undescribed.

Lucena and Kullander (1992) were unable to associate *C. celidochilus* with any particular other species of *Crenicichla*, and emphasized numerous autapomorphies, including a unique form of neoteny occurring in *C. celidochilus* only. The species was then known only from the upper Rio Uruguay drainage, and the type locality in the upper Rio Cuareim drainage. The distribution is herewith extended to the Rio Yí, a tributary of the Rio Negro, draining to the lower Rio Uruguay. The present analysis shows that *C. celidochilus* is a member of the C. *missioneira* group, which then has nine known species. Specimens of *C. igara*, *C. jurubi*, or *C. hadrostigma*, were not available for molecular analysis.

The six species of the *C. missioneira* group examined are morphologically distinct, but turn out genetically to be very similar to each other. This applies particularly to *C. missioneira*, *C. minuano*, *C. tendybaguassu,* and *C. celidochilus,* which occur syntopic in different combinations in the middle Rio Uruguay basin (Lucena and Kullander 1992). The two species from the upper Uruguay, *C. empheres* and *C.* 'Forquilha' are morphologically similar to *C. minuano* but not found together with other species of the group. The positions of *C. missioneira* and *C. celidochilus*, although differing only in a few bases, are well resolved, and in a trichotomy with *C. minuano* + *C. tendybaguassu* (Fig. 3), or more unresolved with one *C. minuano* and one *C. tendybaguassu* (Fig. 2). The specimen of *C. minuano* from Artigas is a juvenile, and the option remains that it represents a juvenile *C. tendybaguassu* or a cryptic species, and it may be that *C. minuano* and *C. tendybaguassu* are the same species. In as much as haplotypes are distinct, and genetic variability within the *C. missioneira* group is very limited, we cannot adopt the last option. Cytochrome *b* is clearly too conservative to provide complete resolution within the *C. missioneira* group. The possibility that the trophic specializations distinguishing the sympatric *C. minuano* and *C. missioneira* could represent intraspecific plasticity is refuted both because the morphological differences between *C. celidochilus* and the remaining species are very strong, yet they differ by only a few bases; because haplotypes are species specific, and because the best-sampled component, *C. missioneira,* is recovered as monophyletic with several haplotypes.

The contrast in genetic and morphological variation may be compared with that within the monomorphic *C. lepidota* and *C. scottii*, of which larger samples from a wider geographical area were analyzed. Within-species nucleotide variation in *C. scottii* (up to 1.2%) is thus comparable with variation within the *C. missioneira* group, and within-species variation in *C. lepidota* (up to 2%) is greater than within the *C. missioneira* group.

*Crenicichla lepidota* is a widespread species, with type locality in the Rio Guaporé drainage, and since reported from the Paraguay, lower Paraná and Uruguay drainages, and coastal drainages of Uruguay and Rio Grande do Sul. In this analysis, there is some geographical variability, and one population from Caaguazú is the sister group of the remainder with a 2% nucleotide divergence. The Caaguazú sample represents the Acaray population distinguished by Kullander (1982), but not recognized as morphologically distinct from other Paraguayan populations of *C. lepidota*. The variability in the present sample of *C. lepidota* is minor (collapsing in both trees) and not clearly structured geographically, and considered to suggest *C. lepidota* being a single species. The extent of geographical sampling, or sample size, is apparently correlated with intraspecific variability also in *C. scottii*. Also in this species, it is difficult to make out a clear geographical structure. The two major clades within *C. scottii* include specimens from both the Rio Uruguay in the north, and

southern coastal rivers. Morphological variation was not examined in detail, but a preliminary screening did not discover any structured variation correlated with haplotypes.

The species in the *C. missioneira* group clade (Figs 2–3) are arranged in a pectinate sequence starting with smaller invertebrate feeders, terminated by a trichotomy including large piscivores. Considering the usual piscivore morphology of *Crenicichla*, this seems to be an unlikely evolutionary scenario. Given the minimal genetic differences, a more reasonable hypothesis is of a species flock, and the seemingly resolved tree in Figs 2–3 should rather be considered as equivalent of a polytomy. This scenario is compatible with a species flock hypothesis. A species flock is a monophyletic group of endemic, closely related species (Greenwood 1984; Salzburger and Meyer 2004), often with a large number of species (Ribbink 1984). In fishes, species flocks are commonly marked by divergence in trophic structures among the constituent species. Species flocks of fishes with major adaptive evolution along intrinsic developmental trajectories, without much genetic differentiation and thus considered of young age have been described for, e.g. Mexican cyprinodonts (Humphries 1984), cichlids in Lake Victoria (Verheyen et al. 2003; Salzburger and Meyer 2004), Lake Malawi (Salzburger and Meyer 2004; Won et al. 2006), Lake Barombi Mbo (Schliewen et al. 1994) and Nicaraguan lakes (Barluenga et al. 2006). There are also cichlid species flocks with more genetic variation and apparently situations with several co-occurring radiating clades as is the case in Lake Tanganyika (Salzburger and Meyer 2004). All these species flocks are endemic to particular lakes with special environmental characteristics, constituting closed or semiclosed systems. The Uruguay River has a largely endemic fish fauna, including also many endemic non-cichlids (Lucena and Kullander 1992). Although open to the Rio de la Plata, it remains hydrographically relatively distinct from the Rio Paraná drainage, and characterized by numerous rapids in the main river as well as in the tributaries. The rapids provide dispersal barriers, but also habitat for specialized rheophilic species. Argentinian swamps between the Uruguay and Paraná rivers, however, provide a dispersal route which explains the marginal presence of otherwise mainly Paraguayan species such as *C. vittata* and *Apistogramma commbrae* (Lucena and Kullander 1992), but are apparently limiting for the lotic Uruguayan species. The many endemic fish species in the Rio Uruguay drainage, and often more than one endemic species within the same genus (Lucena and Kullander 1992), suggest that the Rio Uruguay drainage is largely a closed system comparable with a lake, probably because of its numerous falls and rapids, providing both dispersal barriers, and specialized environments for rheophilic species.

Based on the present analysis, the *C. missioneira* group meets the three criteria set by Salzburger and Meyer (2004) for a species flock, namely: (1) geographical circumscription, (2) high level of endemicity, and (3) close phyletic relationship. Yet, the taxon sampling is unfortunately not complete. From the upper Rio Uruguay are known also *C. hadrostigma*, *C. igara*, and *C. jurubi*, which are assigned to the *C. missioneira* group on morphological criteria, and inclusion of molecular data from those taxa will improve on the phylogenetic analysis and the understanding of geographic separation of upper and middle Uruguayan species (Lucena and Kullander 1992). Study of faster evolving genes or other genetic markers will also be in place to obtain a more resolved within-group phylogeny and to check for introgression and lineage sorting. Only one specimen of the rare *C. tendybaguassu* was available for analysis, and should be supplemented with more specimens. Lucena and Kullander (1992) described a collection of *Crenicichla* from the Rio Forquilha in the upper Rio Uruguay, with five syntopic specimens resembling and except for a special shared colouration being identical with respectively *C. minuano*, *C. missioneira*, *C. tendybaguassu*, *C. jurubi* and *C. celidochilus*. One species from the Rio Forquilha, resembling *C. minuano*, was included in this study (*C.* sp. 'Forquilha'). It comes out as sister species to other *C. missioneira* group species (Figs 2–3). Comparing the Forquilha forms with the same or similar species from elsewhere in the Uruguay drainage will add considerably in understanding species differentiation within the Rio Uruguay drainage.

### Intrageneric hybrids

Two specimens analyzed are of special interest, because the morphological identification is incongruent with the cytochrome *b* identification.

A young specimen, 121.7 mm SL (NRM 52139), from the Rio Cuareim/Quaraí on the border between Uruguay and Brazil agrees morphologically with *C. vittata* particularly in the pointed snout. It is, however, more stout-bodied, and the dark stripe below the eye is broad as in *C. scottii* instead of narrow as in *C. vittata*. Stripes along the abdominal side, typical of *C. scottii*, are absent as in *C. vittata*. The lateral scale count, 65, is closer to the range of *C. scottii* (45–59) than to that of *C. vittata* (78–85). The scale count is significant because it was observed that species of *Crenicichla* have scale counts either up to about 70 or about 80 and higher, with only a few exceptional specimens recorded (Kullander 1998). This specimen has a cytochrome *b* sequence corresponding to *C. scottii*, and clusters with *C. scottii* in phylogenetic analyses (Figs 2–3). Since both *C. vittata* and *C. scottii* occur in the general area of northern Uruguay, we consider this specimen to be a hybrid between *C. scottii* and *C. vittata*, based on the combination of morphological characteristics from both species. A morphological PCA places it closer to *C. vittata* than to *C. scottii* (Fig. 4, Table S2).

This may be the first wild hybrid specimen of South American cichlids to be reported. Hybrids from natural waters have been reported before in the genus *Cichla* (Andrade et al. 2001; Brinn et al. 2004; Teixeira and Oliveira 2005), but are suggested to be based on mistakes in identification of species and sex (Kullander and Ferreira 2006). Oliveira et al. (2006), however, presented evidence of hybridization between *Cichla kelberi* and *Cichla piquiti* in the Rio Paraná, in which both species are introduced. The only other wild hybrid involving two South American fish species is that between *Semaprochilodus insignis* and *Semaprochilodus taeniurus* (Prochilodontidae) reported from the Amazon basin by Ribeiro (1985) based on morphological characters only.

Most collections of *C. vittata* so far have been made in large rivers such as the Rio Uruguay itself. The Rio Cuareim where we sampled is a relatively small river, but open to the Rio Uruguay, and although we did not sample it from the Rio Cuareim *C. vittata* was recorded from the Rio Cuareim by Luengo (1971). A specimen of *C. vittata* is, however, available from a nearby smaller stream, the Arroyo Cuaró, and presence of *C. vittata* in the middle Rio Cuareim seems reasonable. *Crenicichla scottii*, on the other hand, occurs all over Uruguay

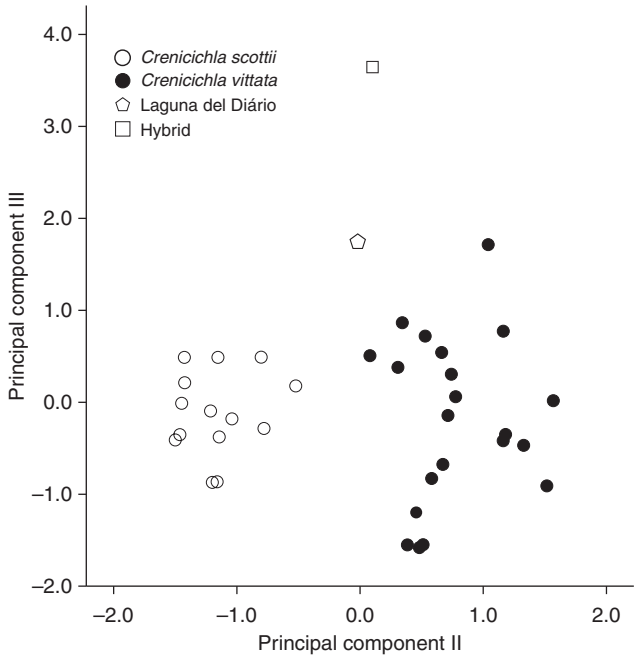


Fig. 4. Plot of scores of principal component 3 against component 2 for pooled sample of scores of morphometric data of *Crenicichla scottii*, *C. vittata* (data as in Lucena and Kullander 1992), putative hybrid specimen from Rio Cuareim (NRM 52139), and specimen from Laguna del Diário (NRM 54361)

west of the Laguna Mirím drainage, and in different types of habitat.

The locality where the putative hybrid specimen was collected was located among low hills of pasture in a series of pools remaining of a stream that had greatly reduced flow during the dry season. We obtained three specimens of *C. celidochilus* and 15 each of *C. lepidota* and *C. scottii* from this locality. A species of *Gymnogeophagus* was observed guarding offspring, but it was not possible to judge if the species of *Crenicichla* were actively reproducing. A restricted habitat like this may be favourable for hybridization, if only few adult specimens are available. At the time of our sampling, however, there were apparently good numbers of adult *Crenicichla* in the pools. Next to *C. punctata*, the species most closely related to *C. scottii* among those for which molecular data are available, is *C. vittata* (Figs 2–3).

A second, adult specimen, 186.4 mm SL (NRM 54361), identified by cytochrome *b* as *C. scottii*, but morphologically similar to *C. vittata*, was sampled from the Laguna del Diário, a small lake situated only 50 m from the sea in Maldonado. *Crenicichla vittata* is not known from the coast of Uruguay east from the mouth of the Rio Uruguay, but *C. scottii* has been recorded from several streams west of the Laguna Mirím drainage, including the Laguna del Diário. The aberrant specimen has a high-scale count (77, versus 45–59 in *C. scottii*), which is close to the lower end of the range in *C. vittata* (78–85), and it lacks the stripes along the abdominal side. The suborbital stripe is similar to that of *C. scottii*, and the snout is not as acute as in *C. vittata*. A morphometric PCA places the specimen closer to *C. vittata*, and similar to the Cuareim hybrid specimen on PC 2 and at least apart from *C. scottii* on PC3 versus PC2 (Fig. 4, Table S2). Although this specimen also may represent hybridization, it must involve some other species than *C. vittata,* and *C. scottii* is the only species of *Crenicichla* known from the Laguna del Diário (two specimens, NRM 55856, collected in 2007).

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## Zusammenfassung

*Phylogenetische Verwandtschaftsverhältnisse der* Crenicichla-*Arten aus dem südlichen Südamerika basierend auf der Analyse des mitochondrialen Cytochrome b Gens*

Eine phylogenetische Analyse mit Hilfe von Bayesianischer Inferenz, Likelihood und Parsimonie-Methoden wurde an Hand von 60 kompletten mitochondrialen Cytochrome *b*-Sequenzen von 21 Arten von *Crenicichla* durchgeführt, die alle Arten aus Uruguay (*C. celidochilus*, *C. lepidota C. minuano*, *C. missioneira*, *C. punctata, C. scottii, C. vittata,*), außerdem *C. compressiceps*, *C. empheres, C. geayi, C. iguassuensis*, *C. macrophthalma, C. menezesi, C. notophthalmus*, *C. regani*, *C.* cf. *regani*, *C. semifasciata, C. sveni, C. tendybaguassu,* sowie zwei nicht identifizierte Arten und zwei Arten der Gattung *Teleocichla* umfassen. Die Bayesianische Analyse ergab eine Trichotomie mit drei Gruppen: (1) Die *C. missioneira*- Artengruppe (*C. celidochilus, C. empheres*, *C. minuano, C. missioneira*, *C. tendybaguassu,* und eine noch unbeschriebene Art; (2) eine Gruppe von im Süden verbreiteten Arten (*C. iguassuensis, C. punctata, C. scottii, C. vittata*), und (3) eine recht heterogene Gruppe, die die Typusart *C. macrophthalma*, Mitglieder der *C. reticulata* Artengruppe (*C. geayi*, *C. semifasciata*), Mitglieder der *C. wallacii*- Artengruppe (*C. compressiceps, C. notophthalmus*, *C. regani, C.* cf. *regani)*, Mitglieder der *C. saxatilis*-Artengruppe (*C. lepidota*, *C. menezesi*, *C. sveni*, *C.* sp.), sowie die zwei *Teleocichla*-Arten beinhaltete. Die Parsimonie-Analyse mit Anwendung von Jackknifing erbrachte eine Quadritomie mit (1) *C. macrophthalma*, (2) *Teleocichla,* (3) den Arten der *saxatilis* + *wallacii*-Artengruppen, und (4) dem Rest, der *C. geayi* and *C. semifasciata* enthielt, die als Schwestergruppe zur *C. missioneira*-Artengruppe plus den übrigen im Süden verbreiteten Arten errechnet wurde. Die Variation in der DNA-Sequenz innerhalb der *C. missioneira*-Artengruppe ist erstaunlich niedrig trotz doch recht beträchtlicher morphologischer Unterschiede, was die Hypothese unterstützt, dass es sich um einen endemischen Artenschwarm des Uruguay-Flusses handelt. *Crenicichla celidochilus* war bisher nicht mit der *C. missioneira*-Artengruppe in Verbindung gebracht worden. Ebenso war man bisher nicht von einer näheren Verwandtschaft von *Crenicichla vittata* zu den Arten *C. scottii*, *C. iguassuensis* oder *C. punctata* ausgegangen. Bisher vorgeschlagene Artengruppen innerhalb der artenreichen Gattung *Crenicichla* (mit mehr als 90 Arten) wurden teilweise bestätigt. *Crenicichla lepidota, C. sveni, C. menezesi* und *C.* sp. gehören zur *C. saxatilis*-Artengruppe. Die kleinwüchsigen Arten, die die *C. wallacii*-Artengruppe repräsentieren, sowie auch *Teleocichla* waren durch lange Äste gekennzeichnet, wobei sich die Stellung von *Teleocichla* in den beiden Stammbäumen, die durch Bayesianische oder Parsimonie-Analysen erzielt wurden, stark unterschied. Dies heißt nicht, dass *Teleocichla* keine gültige Gattung darstellt, sondern dass mehrere monophyletische Gruppen innerhalb von *Crenicichla* einen wissenschaftlichen Namen bekommen sollten. Cytochrom *b* besitzt

offenbar nicht das nötige Auflösungspotential, um die Verwandtschaftsverhältnisse zwischen den größeren Gruppen zu klären. Ein mögliches Hybridexemplar mit einer Mischung von morphologischen Merkmalen von *C. vittata* wie auch *C. scottii*, jedoch mit der Cytochrom *b*-Sequenz von *C. scottii* befand sich unter den Exemplaren, die im Rio Quaraí/Cuareim gesammelt worden waren. Ein weiteres mögliches Hybridtier mit einzigartigen morphologischen Merkmalen, aber einer Cytochrom *b*-Sequenz, die mit der von *C. scottii* übereinstimmte, befand sich unter den Exemplaren von Maldonado, obwohl von dort bis auf *C. scottii* keine weitere *Crenicichla*-Art bekannt ist.

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## Supporting Information

Additonal Supporting Information may be found in the online version of this article:

**Table S1.** Specimens sequenced ordered alphabetically by species name. Species group names are as concluded from the present study; species here referred to the southern group include *C. vittata* previously in the *C. lugubris* group, C. *scottii* previously in the *C. scottii* group, *C. iguassuensis* and *C. punctata* in the *C. lacustris* group.

**Table S2.** Character loadings on the first three morphometric principal components in pooled sample of *Crenicichla scottii*, *C. vittata* (same data as in Lucena and Kullander 1992), putative hybrid specimen from Rio Cuareim (NRM 52139), and specimen from Laguna del Diário (NRM 54361).

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