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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
	CytBI-1F	F-A	CGATTCTTCGCATTCCACTTCCT					
	CytBI-3R	R-A	GGGGTAAAGTTGTCTGGGTCTCC					
	CytBI-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.

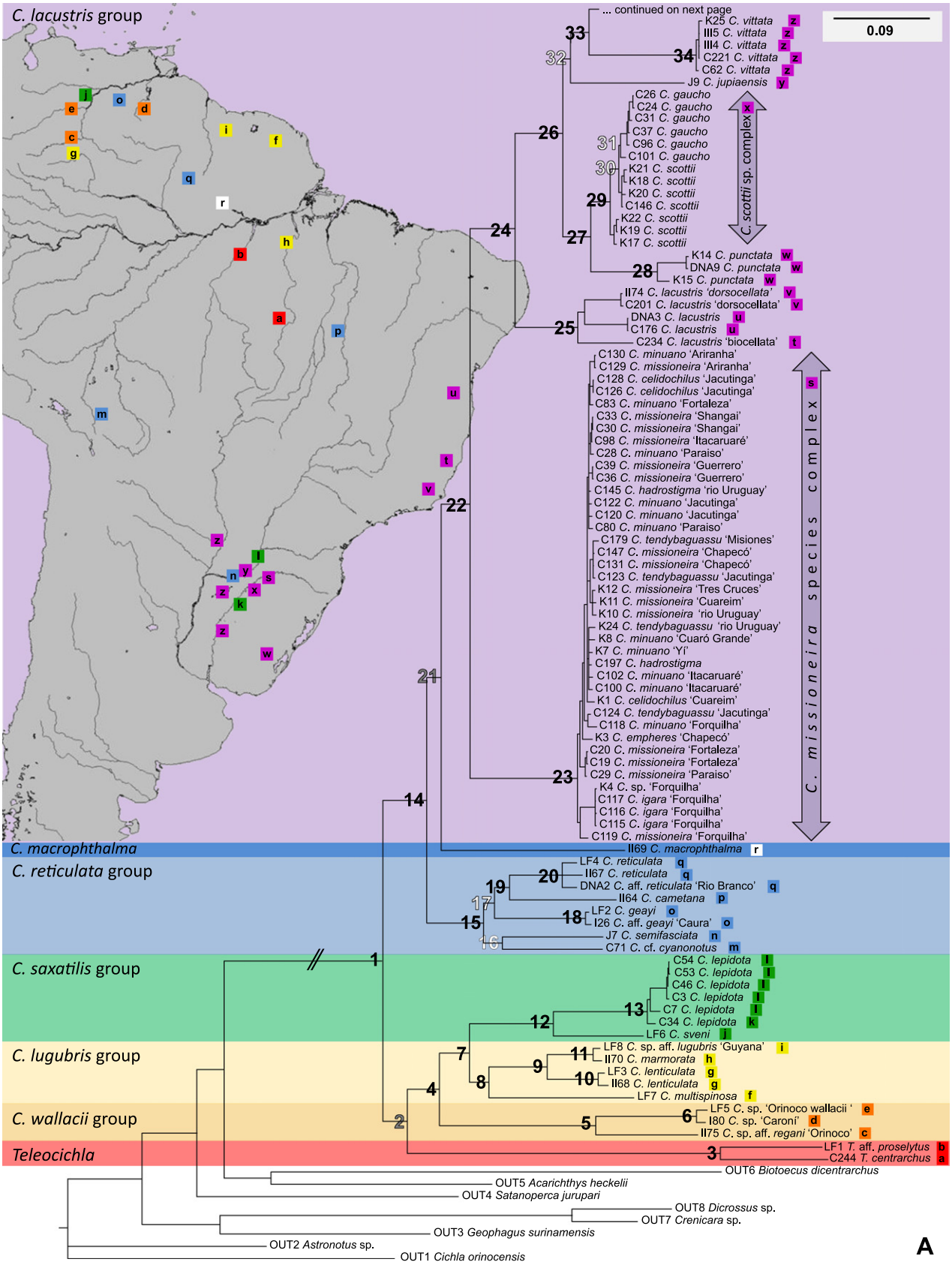

Fig. 1. Phylogenetic relationships of *Crenicichla* inferred from BA analysis of the combined dataset. Nodes with black numbers are well supported (PP ⩾ 0.95), gray numbers indicate nodes well supported in the dataset with reduced or removed 16S locus, white numbers indicate nodes with PP < 0.95. Specimens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

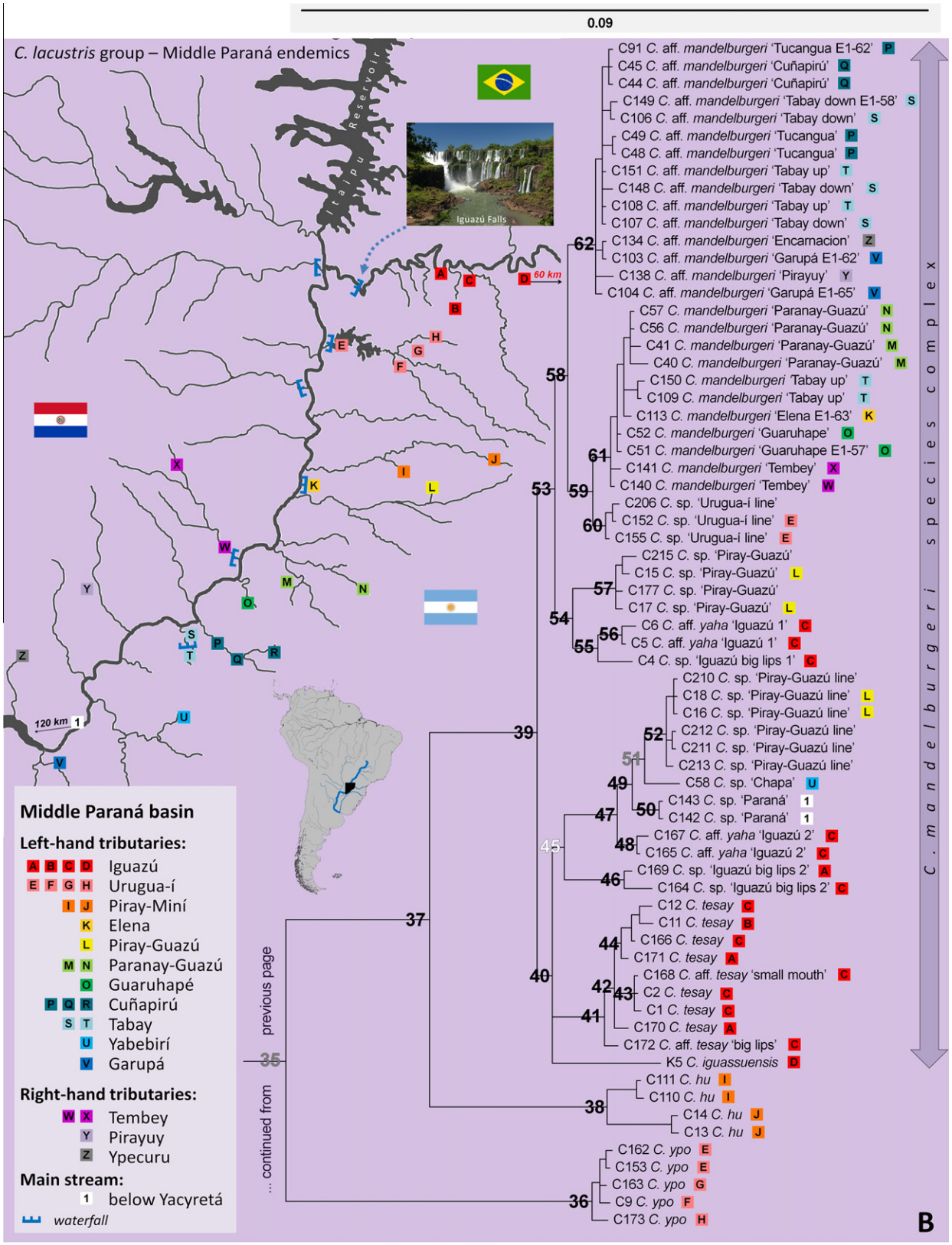

Fig. 1 (*continued*)

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

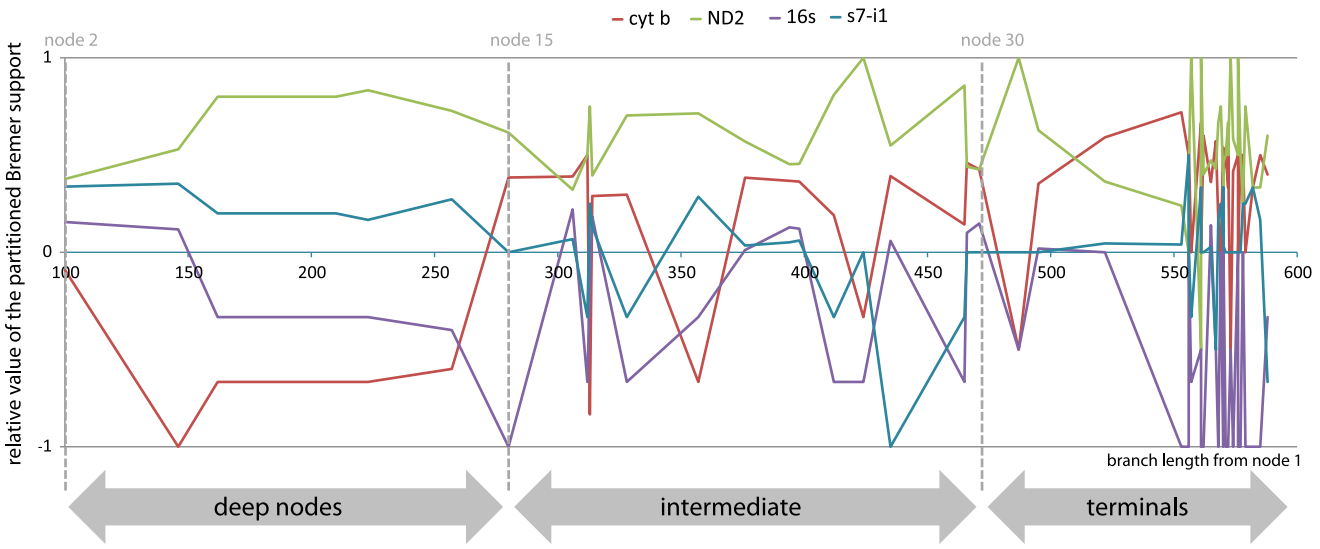

Fig. 2. Dependence of relative PBS values on the cumulative branch length from the tree root for all loci. The division between deep, intermediate and terminal nodes is arbitrarily assigned to nodes 15 and 30 in the phylogeny (Fig. 1).

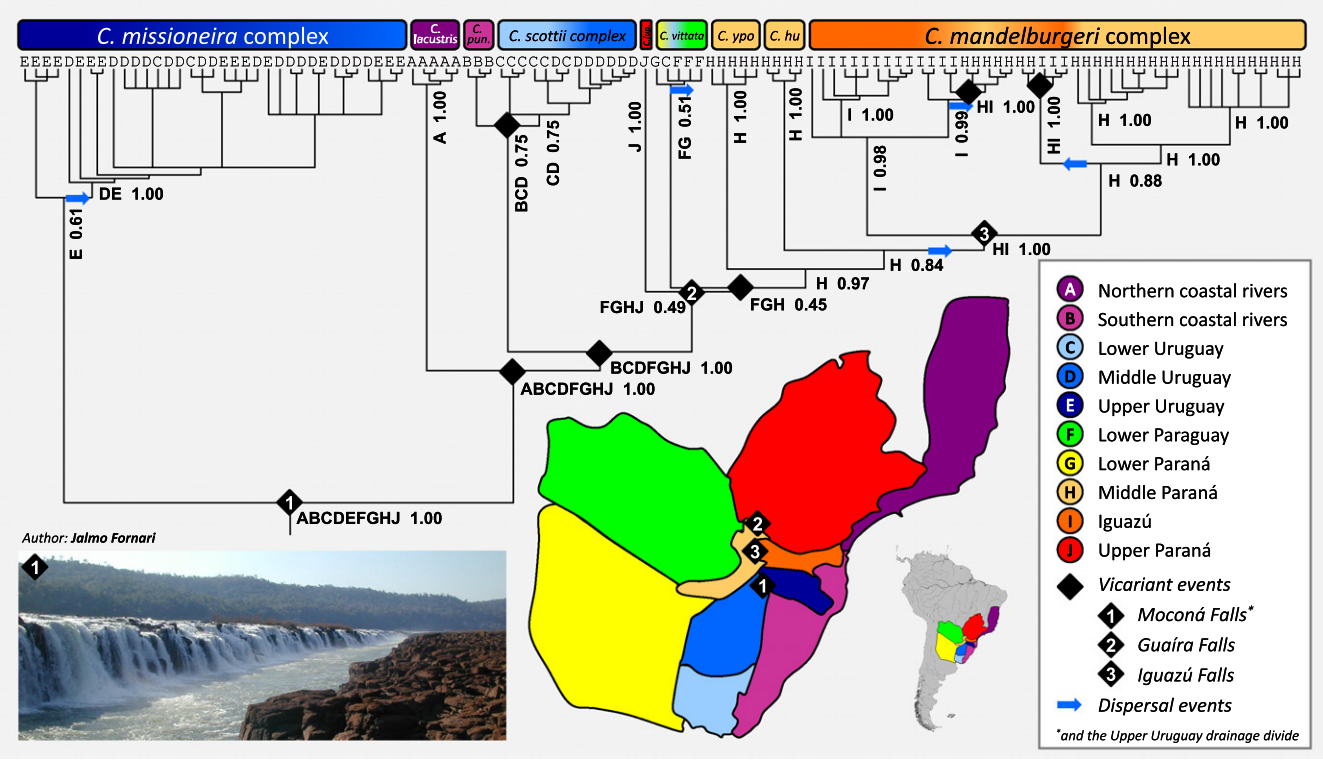

Fig. 3. Biogeographical reconstruction of ancestral areas (RASP analysis; see text).

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

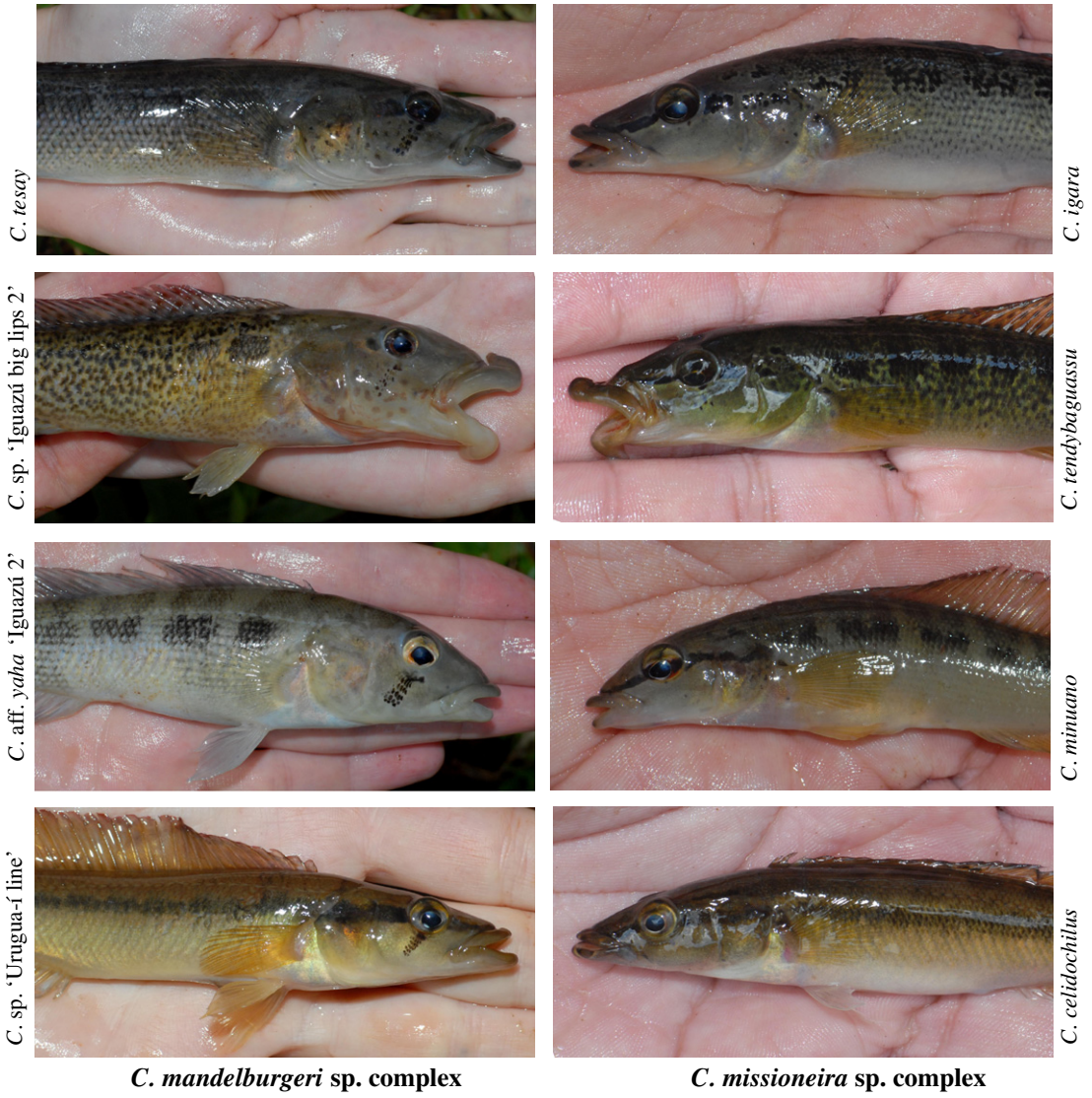

Fig. 4. Overview of several color patterns and eco-morphological variations within *C. mandelburgeri* and *C. missioneira* species flocks (see text).

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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