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ADVANCES IN CICHLID RESEARCH III

Phylogenomics of pike cichlids (Cichlidae: *Crenicichla*) of the *C. mandelburgeri* species complex: rapid ecological speciation in the Iguazú River and high endemism in the Middle Paraná basin

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Abstract The *Crenicichla mandelburgeri* species complex from the Middle Paraná basin is a diverse group of cichlid species and contains all known ecomorphs found within the entire genus *Crenicichla*. Here, we study the phylogenetic relationships within the *C. mandelburgeri* species complex using ddRAD sequencing with focus on its two candidate species flocks endemic to the Iguazú and Urugua-í Rivers, and on two putative sympatric species in the Piray Guazú River. These species flocks include four and three syntopic species, respectively, which are strongly adapted to different trophic niches and include derived ecomorphs of *Crenicichla* (molluscivores, a periphyton grazer, and a crevice-feeding thick-lipped invertivore). Our phylogenomic analyses strongly support monophyly and rapid diversification of the Iguazú species flock, but reveal more complex evolutionary histories in the Urugua-í and Piray Guazú tributaries. Most species in the Middle Paraná, including one species in the Urugua-í and both species in the Piray Guazú show cytonuclear discordance, and in both of these tributaries, we also found hybridization in one of the resident species. Population-level analyses reveal complete isolation of the Iguazú species and coupled with their dramatic ecological diversity, this radiation exemplifies characteristics of a species flock that arose via ecological speciation.

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Introduction

Cichlids are prime examples of very diverse, sympatric, and endemic species assemblages that are composed of rapidly adapting closely related species. Such monophyletic species assemblages are termed species flocks (Greenwood, 1984; Mayr, 1984; Salzburger & Meyer, 2004) and for some time it was believed that cichlid species flocks were limited to lacustrine habitats such as the East African Rift Valley lakes (e.g., Kocher, 2004; Salzburger & Meyer, 2004; Seehausen, 2015), Cameroonian and Ugandan crater lakes (Schliewen et al., 2001; Machado-Schiaffino et al., 2015), Tanzanian-Kenyan alkaline lakes (Seegers & Tichy, 1999; Ford et al., 2015), or Neotropical crater lakes (Barluenga et al., 2006; Geiger et al., 2010; Elmer et al., 2014). Contrary to this initial evidence, cichlid species flocks also appear in riverine habitats. Such is the case again in Africa, e.g., in the mighty Lower Congo rapids (Schwarzer et al., 2011) and in the Neotropics in Middle America (Říčan et al., 2016), but the most striking case has been found in South America in the genus *Crenicichla*. Here, in two large La Plata basin tributaries, the first case of parallel riverine species flocks has been found (Piálek et al., 2012; Burress et al., 2018a). These two species flocks (contained in the *C. missioneira* Lucena & Kullander, 1992 and *C. mandelburgeri* Kullander, 2009 species complexes) are endemic to the Uruguay and the Middle Paraná River basins, respectively (Lucena & Kullander, 1992; Lucena, 2007; Piálek et al., 2012; Burress et al., 2018a).

Crenicichla is a large genus widespread throughout South America east of the Andes with a rather uniform morphology (elongated body and a large head; i.e., their name 'Pike Cichlid') and predatory-piscivorous ecology. The subtropical regions of southeastern South America have a comparatively high *Crenicichla* diversity because of the two complexes (plus about 15 other species in the surrounding geographic area). The two species complexes, together with all remaining subtropical lineages, form a clade (Kullander et al., 2010; Piálek et al., 2012; Burress et al., 2018a, b) classified as the *C. lacustris* species group, which is one of five traditional groups within *Crenicichla* (Ploeg, 1991; Stawikowski & Werner, 2004; Piálek et al., 2012) that are supported as clades in molecular phylogenies (which also place *Teleocichla* within *Crenicichla*; Piálek et al., 2012; Burress et al., 2018a, b).

The subtropical *C. lacustris* group is the only largely allopatric group within the genus while the other species groups are largely sympatric in their distributions centered in the Amazon and Orinoco River drainages. The diversity of the *C. lacustris* group is centered on a southern area of the geological formation known as the Brazilian shield (which together with the Guiana shield forms the geological core of the South American continent), more specifically on its southern subgroup called the Paraná formation. This area is unique in South America by being composed of and having exposed at its surface volcanic flood basalts, which are the direct results of the rifting between South America and Africa (Fodor et al., 1989; Bryan et al., 2010). The Paraná flood basalts are the reason why this southern region of the Brazilian shield has the largest number and highest concentration of waterfalls (and rapids) in South America of which the most remarkable are the Iguazú Falls positioned close to where the Iguazú River flows into the Paraná River. The two river basins (the Uruguay and the Middle Paraná) with the endemic *C. missioneira* and *C. mandelburgeri* species complexes occur in the center of the Paraná flood basalts region.

The Uruguay and Middle Paraná *Crenicichla* species complexes have the largest diversity of ecomorphs in the genus, including several that are otherwise rare (molluscivores) or not otherwise found within *Crenicichla* (periphyton grazers, thick-lipped crevice-feeders, and open-water piscivores) and the two species complexes thus strongly depart from the ancestral predatory-piscivorous ecomorphology of the genus (Piálek et al., 2012; Burress et al., 2018a, b). The Uruguay River drainage *C. missioneira* complex includes eight endemic highly diversified species (Lucena & Kullander, 1992; Lucena, 2007; Burress et al., 2018a). The Middle Paraná River drainage (including the Iguazú) *C. mandelburgeri* complex is presently known to include 10 endemic and equally diversified described species and several more putative species (Piálek et al., 2012).

Within the *C. missioneira* complex, five species (the ecomorphologically most distinct) can be found in

sympatry and syntopy throughout almost the whole basin and form a species flock (*C. missioneira*, *C. minuano* Lucena & Kullander, 1992, *C. tendybaguassu* Lucena & Kullander, 1992, *C. celidochilus* Casciotta, 1987 and *C. hadrostigma* Lucena, 2007; Lucena & Kullander, 1992; Lucena, 2007; Piálek et al., 2012, Burress et al., 2013, 2018a, b). The situation in the *C. mandelburgeri* species complex (Fig. 1) is different and geographically more structured and the whole spectrum of ecomorphs that are replicates to those in the Uruguay *C. missioneira* species flock is found only in the Lower Iguazú River above the Iguazú Falls where they live in sympatry and syntopy and thus form a candidate species flock (Piálek et al., 2012, 2015). The Iguazú species include a piscivorous ecomorph (*C. iguassuensis* Haseman, 1911), a molluscivore (*C. tesay* Casciotta & Almirón, 2009), a gregarious periphyton grazer (*C. tapii* Piálek, Dragová, Casciotta, Almirón & Říčan, 2015), and a thick-lipped crevice-feeding specialized invertivore (*C. tuca* Piálek, Dragová, Casciotta, Almirón & O. Říčan, 2015) (Piálek et al., 2015). The remaining species of the *C. mandelburgeri* species complex are found in the Middle Paraná and its remaining

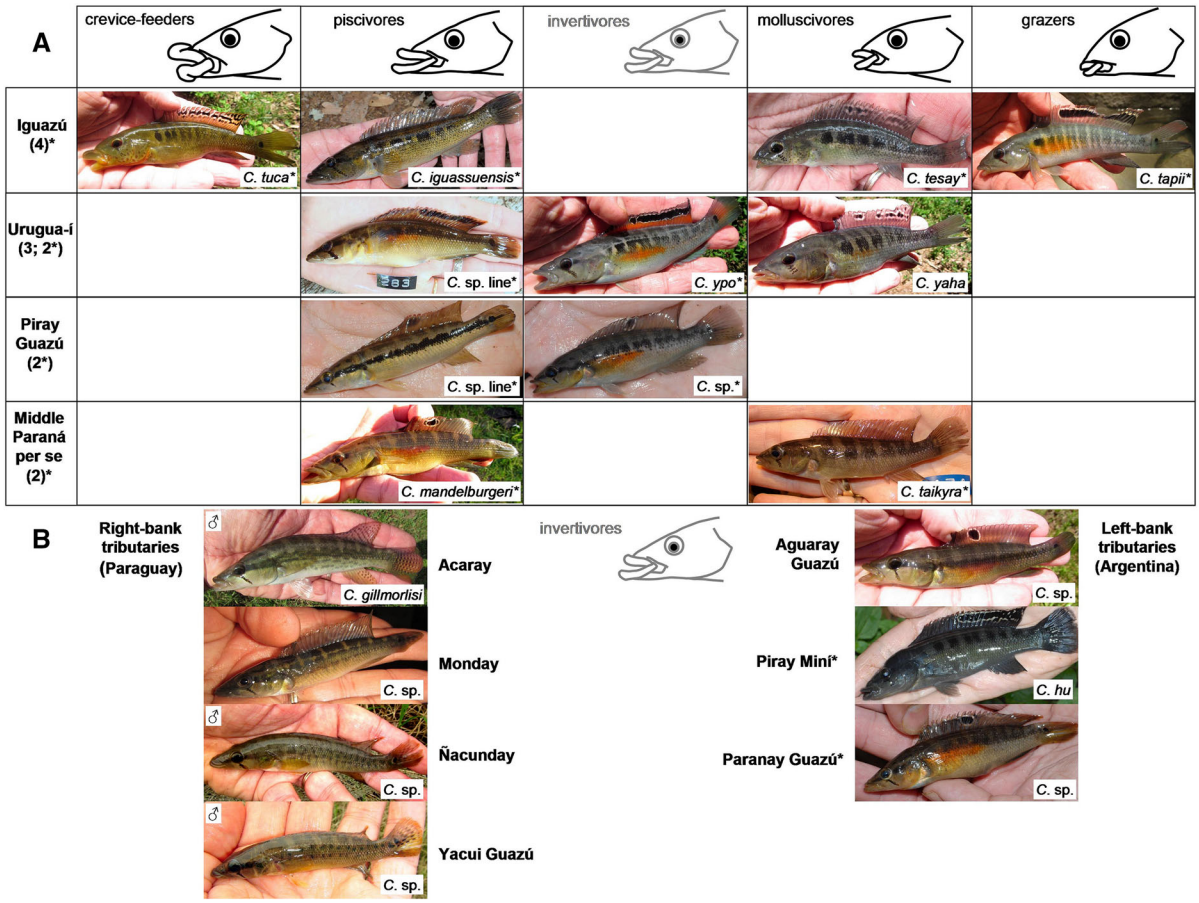


Fig. 1 Species diversity, distribution patterns, and ecomorphological classification of the *Crenicichla mandelburgeri* species complex in the Middle Paraná basin. Horizontal rows of species correspond to tributaries, vertical columns of species correspond to ecomorphs. Tributaries are arranged from north to south in both **A** and **B**. All described and putative undescribed species are shown. Species studied in the present work are marked by an asterisk. **A** Multi-species sympatry and endemism in Middle Paraná tributaries. All these endemics differ in their ecomorphology due to sympatry and syntopy. The crevice-feeder and grazer ecomorphologies are only found in the Iguazú within the Paraná basin. Numbers in parentheses show numbers of species co-occurring within a given tributary of the Middle Paraná. **B** Single-species endemism in Middle Paraná tributaries. All single-species endemics are generalized invertivores. All specimens except where indicated show females (for males see Fig. S1)

tributaries and most of them appear to be generalistic invertivores or piscivores (Kullander, 2009; Casciotta et al., 2010, 2013; Piálek et al., 2010, 2012; Kullander & Lucena, 2013; Burress et al., 2018a) and all but two are endemic to single tributaries (Fig. 1). Apart from the Iguazú with four species, three sympatric and syntopic species (*C.* sp. Urugua-í line, *C. ypo* Casciotta, Almirón, Piálek, Gómez & Říčan, 2010 and *C. yaha* Casciotta, Almirón & Gómez, 2006; a generalistic invertivore, a piscivore, and a molluscivore; respectively) are also found in the Urugua-í tributary, which is thus a second likely candidate for a species flock within the Middle Paraná basin. Within the Middle Paraná there are two partially sympatric species representing different ecomorphs (the invertivorous–piscivorous *C. mandelburgeri* and the molluscivorous *C. taikyra* Casciotta, Almirón, Aichino, Gómez, Piálek & Říčan, 2013). The sympatric distribution of these two species is likely the result of immigration rather than in situ speciation (Casciotta et al., 2013), contrary to the isolated Iguazú and Urugua-í tributaries. The last tributary where there is more than one type of *Crenicichla* in sympatry and syntopy within the Middle Paraná is the Piray Guazú tributary where two undescribed forms occur together (Piálek et al., 2012).

The *Crenicichla* species in the Iguazú are thus the most ecologically and morphologically diverse assemblage in the *C. mandelburgeri* species complex (Piálek et al., 2015). Piálek et al. (2012), in the first study of the relationships within the whole subtropical *C. lacustris* species group, focused on testing of the monophyly of the Iguazú *Crenicichla* species. Their multilocus phylogenetic analysis was dominated by mtDNA and it rejected the monophyly of the Iguazú species. Since only four markers were used, three of which were mitochondrial (cytochrome b, ND2, 16S) and the one nuclear (S7 intron 1) had limited resolution, the question about the relationships of the Iguazú species remained only partially answered.

The recently introduced genotyping methods based on next-generation sequencing (NGS) technologies offered a new opportunity to study the *Crenicichla* species complexes at a genomic scale and in particular to test the monophyly of candidate species flocks within the Middle Paraná and Uruguay species complexes. Burress et al. (2018a, b) have demonstrated the great promise of the NGS methods for the study of rapid diversification in *Crenicichla* and in the Uruguay *C. missioneira* species complex in particular. Using the NGS-based ddRAD method (Peterson et al., 2012), Burress et al. (2018a) have confirmed the phylogenetic independence of the Middle Paraná *C. mandelburgeri* and the Uruguay *C. missioneira* species complexes postulated by Piálek et al. (2012) and also have demonstrated parallel morphological and ecological evolution between the two species complexes.

Here, we use the ddRAD method to study in detail diversification within the *C. mandelburgeri* species complex in the Middle Paraná. Our goals are (1) to test the monophyly of the two proposed species flocks in the Iguazú and Urugua-í Rivers and thus form a baseline for future studies focusing on their postulated parallel evolution, (2) to test the species delimitation and genetic isolation of the recently described/revised Iguazú species (Piálek et al., 2015) and of the several putative undescribed species in the complex (*C.* sp. Urugua-í line, *C.* sp. Piray Guazú, *C.* sp. Piray Guazú line, and *C.* sp. Paranay Guazú), and (3) to unravel the biogeographical history of the group within the area. We also discuss some looming threats to the conservation of these species and the area.

Materials and methods

Taxon sampling

Our study included 307 individuals representing the majority of described and putative species diversity of the *Crenicichla mandelburgeri* species complex (sensu Piálek et al., 2012). Figures 1 and S1 show all presently known species and forms of *Crenicichla* from the *C. mandelburgeri* group (Fig. 1 shows females, males are shown in Fig. S1). Several of the putatively new taxa plus two described species (*C. gillmorlisi* Kullander & Lucena, 2013 and *C. yaha*) were not available at the time of our study. The sampling in the present study (Table S1) thus includes all four species from the Iguazú River (*C. iguassuensis*, *C. tesay*, *C. tapii*, *C. tuca*), two out of three species from the Urugua-í tributary (*C. ypo*, *C.* sp. Urugua-í line), two putative species from the Piray Guazú tributary (*C.* sp. Piray Guazú, *C.* sp. Piray Guazú line), two species from the Middle Paraná River (*C. mandelburgeri*, *C. taikyra*), and the single tributary endemics *C. hu* Piálek, Říčan, Casciotta & Almirón,

2010 and *C.* sp. Paranay Guazú. The taxon sampling is thus the same as in the previously largest study of Piálek et al. (2012) at the species level but importantly includes a much denser specimen sampling (also including new localities/populations) both for the previously used mtDNA markers and for the novel nDNA ddRAD analysis. For the ddRAD analysis, 232 ingroup specimens were genotyped and 186 specimens were sequenced for two mitochondrial markers (Table S1, Supplementary material). Rooting of phylogenetic trees was done by *C. vittata* Heckel, 1840 after a previous check of the root position in analyses augmented by several successive outgroups based on Piálek et al. (2012; *C. vittata*, *C. jupiaensis* Britski & Luengo, 1968, *C. jaguarensis* Haseman, 1911, *C. gaucho* Lucena & Kullander, 1992, *C. scottii* (Eingenmann, 1907), *C. lacustris* (Castelnau, 1855), *C. punctata* Hensel, 1870, *C. missioneira* species complex, *C. macrophthalma* Heckel, 1840 and *C. reticulata* (Heckel, 1840); not shown here). The samples were obtained during faunistic field work in the Misiones Province, Argentina between 2007 and 2014; 12 additional specimens were acquired from the aquarium trade.

Since the time of the first molecular phylogenetic analysis (Piálek et al., 2012) of the species group, the taxonomy somewhat changed and became clarified (Piálek et al., 2015; Říčan et al., 2017). Two new species, *C. tuca* and *C. tapii*, were described (formerly studied as *C.* sp. 'Iguazú big lips 2' and *C.* aff. *yaha* 'Iguazú 1', respectively), populations referred to as *C.* aff. *yaha* 'Iguazú 2' in Piálek et al. (2012) were reclassified as *C. tesay*, and those of *C. tesay* reclassified as *C. iguassuensis* (the latter with tentative taxonomic status given the absence of diagnostic coloration-pattern characters in the preserved type specimens). Following its recent rediscovery (Říčan et al., 2017), *Crenicichla yaha* has been found to be a distinct species from *C. tesay*. The changes in nomenclature are evident from a comparison between Fig. 1 in Piálek et al. (2012) and the mtDNA phylogenetic tree presented in this study.

mtDNA phylogenetic analyses

The mtDNA dataset in this study has an extended specimen sampling compared to Piálek et al. (2012), most importantly in the most interesting Iguazú species, and includes two mitochondrial markers (cytochrome b, cyt b; NADH dehydrogenase subunit 2, ND2) (GenBank Accession Nos. given in Table S1). All sequences were edited and aligned in Genious v7.1.7 (https://www.geneious.com, Kearse et al., 2012) and analyzed in RaxML v8.2.4 (Stamatakis 2014) under substitution model inferred in jModelTest v0.1.1 (Posada, 2008) according to AIC (Akaike Information Criterion).

ddRADseq library preparation and marker generation

The Double Digest Restriction-site Associated DNA Sequencing method (ddRADseq; Peterson et al., 2012) was used to acquire a sufficient amount of nuclear markers without prior knowledge of a reference genome. 300 ng of genomic DNA from each individual (extracted by standard column chromatography-based kits) was digested with two restriction enzymes, SphI and MluCI (New England BioLabs, NEB) in one 30 µl reaction (0.25 µl of SphI-HF 20 kU/ml, 2.0 µl of MluCI 10 kU/ml, 3.0 µl of NEBuffer 4; enzymes concentrations in NEB CELU units) for 3 h at 37°C. Digestion products were purified with AMPure XP beads (Beckman Coulter) with a ratio of beads/product volume set to 1.5, eluted into 40 µl of 1× TE buffer, and quantified. P1 and P2 "flex" adapters (Peterson et al., 2012) were ligated in a 40 µl reaction with 100 ng of the purified digestion product (0.1 µl of NEB T4 DNA Ligase 400 kU/ml, 4 µl of 10× T4 buffer, 1.6 µl of P1 adapter 0.5 µM, 0.12 µl of P2 adapter 100 µM) performed in a thermal cycler (ligation 23°C, 30 min; ligase deactivation 65°C, 10 min; slow cooling 1°C/45 s). The total volume of each 48 ligation products differing in adapter barcode were pooled together (into a "sublibrary") and cleaned with AMPure XP beads (1.5 ratio) in a two-step procedure enabling final elution into 30 µl volume (1× TE buffer); the order of samples was randomized between and within sublibraries. Automated size selection of a fraction of 276–324 bp separately from each sublibrary was performed on Pippin Prep system (Sage Science) with CSD2010 kit. PCR amplification with primers bearing the multiplexing indices and Illumina flow cell annealing regions was done in several 50 µl reactions (for each sublibrary) containing 10 ng of separated DNA, 1 µl of each primer 25 µM, 1 µl of Herculase II Fusion DNA Polymerase (Agilent), 10 µl of 5 × Herculase II

Reaction Buffer, and 0.5 µl of dNTPs 100 mM: 98°C, 2 min; 10× [98°C, 30 s; 65°C, 30 s; 72°C, 1 min]; 72°C, 5 min; hold on 10°C. PCR products were purified on AMPure XP beads and combined in equimolar ratios to compose a final library. Sequencing was performed on an Illumina HiSeq 2500 (1+0.4 lanes, 125 cycles P/E, v4 kit) in the EMBL Genomic Core Facility, Heidelberg, Germany.

Barcode sorting and quality filtering of raw reads were performed in *process_radtags* (Stacks v1.19; Catchen et al., 2011) and reviewed in FastQC v0.10.1 (Andrews, 2010). Two different strategies were used for assembling of the obtained RAD sequences: a de novo assembly, and assembly based on a reference genome. In the de novo approach, *denovo_map* pipeline of Stacks v1.35 was used to find homologous loci between individuals and call for SNPs to build a data matrix (run with default parameters). Alternatively, RAD tags were first aligned onto the genome of *Oreochromis niloticus* (Linnaeus, 1758) GCA_000188235.1 (http://www.ensembl.org) using Bowtie 2 assembler (v2.2.4; Langmead & Salzberg, 2012) and followingly processed in the *ref_map* pipeline implemented in Stacks v1.35. In both approaches, SNP variants calling was processed in *population* component of Stacks v1.35 with several different sets of parameters of which following were the most important: a minimum number of individuals with present locus (in population dataset also percentual number of individuals with present locus in a population), and a minimum stack (locus) depth for each individual.

RAD-based phylogenetic analyses

Phylogenetic inference

Maximum-likelihood analysis (ML) was performed in RaxML v8.2.4 (Stamatakis 2014) using a general time-reversible model with gamma-distributed rate variation (GTR+G) as the most complex applicable model for the concatenated SNP matrix (Jones et al., 2013; Takahashi et al., 2014; Takahashi & Moreno, 2015). We inferred phylogenies both for the whole Middle Paraná clade (*Crenicichla mandelburgeri* sp. complex sensu Piálek et al., 2012 including *C. hu* and *C. ypo*; 232 individuals; rooted with *C. vittata*) and for the Iguazú species only (including allopatric *C. taikyra*; 111 individuals; rooted with *C. ypo*). In the latter case, narrowing the sampling enabled us to generate substantially more variable sites under slightly more stringent conditions (30,782 and 57,139 SNPs, based on variant calling with 70 and 75% minimum locus presence, respectively; minimum stack depth, 10; *ref_map* pipeline). We used 100 bootstrap replicates to evaluate statistical branch supports of ML trees.

MP bootstrap and jackknife

We further tested consistency of phylogenetic signals obtained from different subsets of loci by bootstrapping and jackknifing of maximum-parsimony (MP) trees. This dataset of 14,678 SNPs was concatenated from fixed (homozygous) sites only (*denovo_map* pipeline; min. presence of a locus, 50%; min. stack depth, 3) and phylogeny was inferred in PAUP* v4.0b10 (Swofford, 2003). For the jackknife analysis, we chose very stringent conditions (90% of the dataset was deleted in each of the 200 replicates). For the MP analysis, only homozygous sites were extracted from the SNP dataset to avoid ambiguous handling in maximum-parsimony phylogenetic software; such treatment inherently reduces the influence of ancestral polymorphism and overestimates the divergence between these two species (Lischer et al., 2014), while in ML-based analyses of variable sites, the genetic proximity of the geographically distant species remains recognized.

Species-tree inference

Species trees were reconstructed under the coalescent model using SVDquartets (SVDQ; Chifman & Kubatko, 2014). The SVDQ method does not rely on prior inference of individual gene trees but it analyzes quartets of species in a coalescent framework using singular value decomposition of the matrix of site pattern frequencies and then assembles a species tree from the quartets using a supertree method. The SVDQ analysis was run as implemented in PAUP* v4.0b10 (Swofford, 2003) in the 'species tree' mode. SVDQ datasets were obtained from the reference-mapping pipeline *ref_map* (minimum presence of a locus, 60 and 75%; stacks depth, 7) and included in one analysis 3,890 fixed sites and in the other 73,603 variable sites, respectively. We sampled 1 million quartets assembled with QFM algorithm, and

performed 100 bootstrap replicates of the data to assess branch support.

Population genomic analyses

We estimated population structure and individual ancestries using Admixture (Alexander et al., 2009), fineRADstructure (Malinsky et al., 2018), and TreeMix (Pickrell & Pritchard, 2012).

Admixture

The sampling for the Admixture analyses included 232 individuals divided into 37 populations (defined by sampled drainages; Table S1). The genotype dataset was generated in Stacks (*ref_map*; minimum presence of a locus, 60%; minimum presence in a population, 60%, min. stack depth, 10) and LD-pruned in PLINK package v1.07 (Purcell et al., 2007; filtering with '–indep-pairwise 50 10 0.1' setting) to obtain a subset of 25,173 variants in approximate linkage equilibrium (unlinked sites). To infer a population structure, the Admixture v1.3.0 program was run 30 times with postulated number of ancestral populations K in the range of 5–15; optimal K was chosen based on the lowest cross-validation error (cv = 10).

We have also analyzed a dedicated dataset including only species from the Iguazú clade. The sampling included 111 individuals (the same as in the ML analysis) divided here into 17 populations (defined by localities; *C. iguassuensis*, 33 specimens/6 populations; *C. taikyra*, 7/1; *C. tapii*, 25/2; *C. tesay*, 41/5; *C. tuca*, 5/3; Table S1). This dataset included 11,809 SNPs (min. presence of a locus, 70%; min. presence in a population, 60%; min. stack depth, 10; first SNP of each locus recorded).

FineRADstructure

To infer population structure via shared ancestry we further used the program fineRADstructure v0.3.1, a modified version of the fineSTRUCTURE package (Lawson et al., 2012) specifically adopted for RADseq data that does not require information about location of loci on chromosomes or phased haplotypes. SNP matrix generated in Stacks (*ref_map*; min. presence, 75%; min. stack depth, 7) was quality-filtered by only allowing 10% missing data per SNP across all individuals, resulting in a matrix comprising 15,898 SNPs and a reduced number of individuals (from 232 to 166). The RAD loci were first reordered according to linkage disequilibrium in sampleLD.R script and then the co-ancestry matrix was calculated in RADpainter (both scripts implemented in the fineRADstructure package). As a next step, individuals were assigned to populations and a tree was built in fineSTRUCTURE v1.0 with 3,000,000 (plus the same number for a burnin) and 300,000 of MCMC iterations, respectively. To visualize the results, we used the fineSTRUCTURE GUI program.

TreeMix

To infer historical relationships between populations considering secondary gene flow, we used TreeMix. This method enables to evaluate introgression and to visualize it as "migration edges" between phylogenetic branches in the population graph. The number of migration events was gradually increased until they explain more than 99.8% of the variance in the SNP data (Pickrell & Pritchard, 2012); the explained variance was counted according to Card (2015). The genotype output for TreeMix included 37 populations and 13,766 SNPs as a result of the *denovo map* pipeline (forward reads; min. presence of a locus, 60%; min. presence in a population, 60%; min. stack depth, 10; Table S1).

All newly generated sequence data were deposited in GenBank (Table S1). Most of the data processing and phylogenetic analyses were performed using the National Grid Infrastructure MetaCentrum (www.metacentrum.cz) and the CERIT Scientific Cloud computing and storage facilities (www.cerit.cz).

Results

mtDNA phylogeny

The concatenated dataset of two mitochondrial genes (cytb and ND2) representing 189 individuals (including three specimens of *C. vittata* as an outgroup) contained 2,163 characters of which 346 were parsimony-informative. ML-based phylogenetic analysis using the GTR+G nucleotide-substitution model confirmed the results of the previously best-sampled analysis (Piálek et al., 2012) in regard to relationships and phylogenetic status of all species under a

substantially extended specimen sampling (Fig. 2). The Iguazú species group is not monophyletic in the mtDNA analyses as in Piálek et al. (2012). All four of the Iguazú species are here supported as monophyletic under the much denser specimen sampling. The Urugua-í species are also not found as a monophyletic group as in Piálek et al. (2012).

ddRAD phylogenomics

ddRAD-tags processing

Two Illumina paired-end sequencing lanes (the second one with 40% capacity used for this project) comprising 240 individuals resulted in 457.7 million 125 bp. sequenced fragments of which 442.4 million (96.7%) passed through the procedure of barcode sorting and raw reads quality filtering. Discarded reads contained ambiguous barcodes, 1.8 M; low quality reads, 4.1 M; ambiguous restriction sites, 8.8 M; reads containing adapter sequence, 0.6 M. One sample was represented in average by 1,843,175 ± 622,096 SE retained reads.

Phylogenomics of the putative species flocks

Iguazú The genomic nDNA ddRAD data strongly support the monophyly of the Iguazú *Crenicichla* species (*C. iguassuensis*, *C. tapii*, *C. tesay*, *C. tuca*) plus the allopatric *C. taikyra*, contrary to the analysis of mtDNA (Fig. 2). Bootstrap branch support (BBS) for the Iguazú clade in ML analysis (Fig. 3) is 100%, bootstrap and jackknife supports in the MP analysis (Fig. S2) are also 100%, and bootstrap supports in the coalescent SVDQ species-trees (Fig. S3) are also 100% in both analyses (fixed and variable sites).

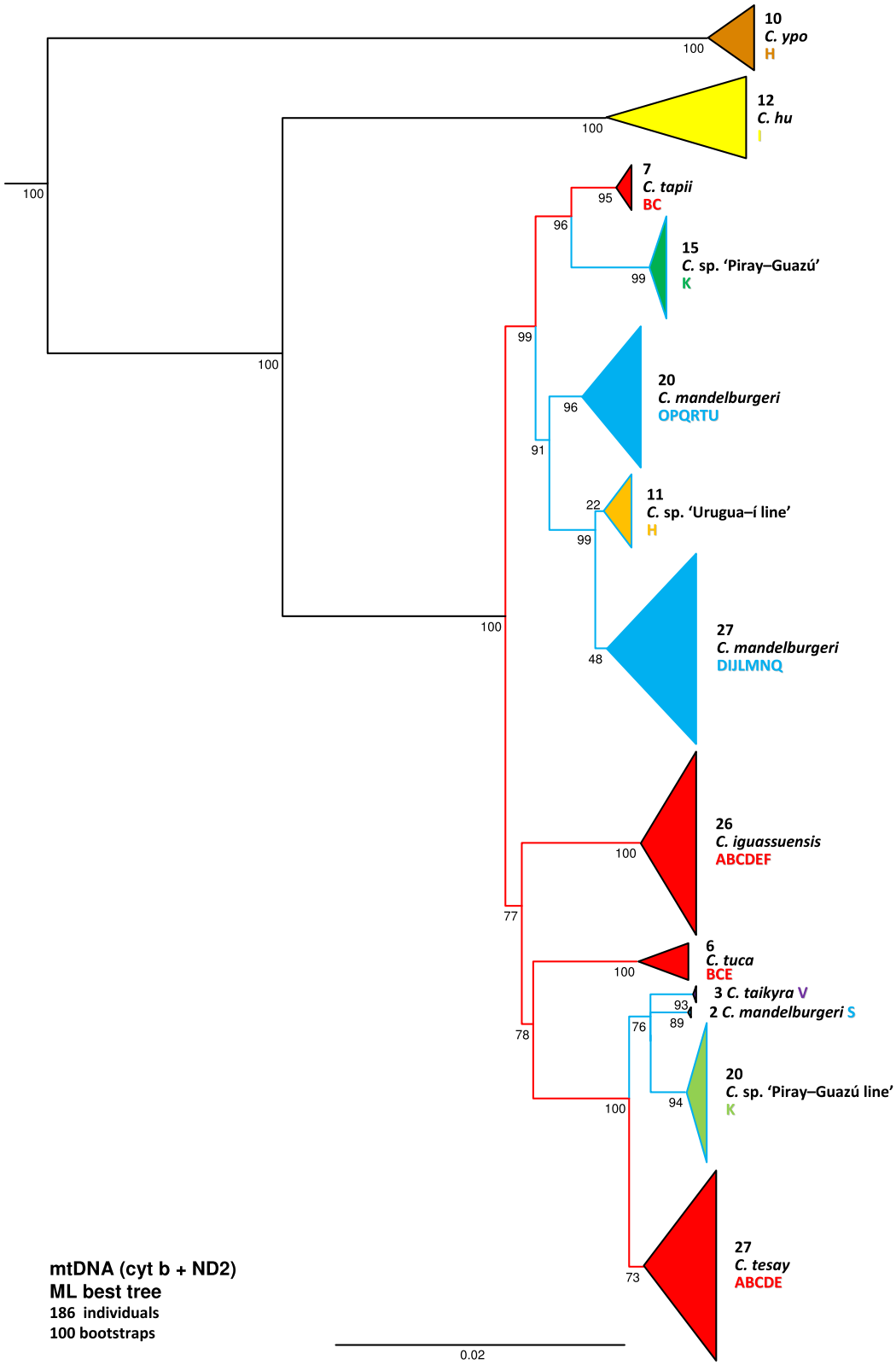
Population genomic analyses (fineRADstructure, Admixture, TreeMix) did not reveal any gene flow between the Iguazú species and the species in the Middle Paraná basin (Figs. 4, S4, S5) and the fineRADstructure analysis (Fig. 4) also found strong co-ancestry for the Iguazú clade (including *C. taikyra*). The Iguazú clade is thus based on population analyses completely isolated from the Paraná species.

The Iguazú clade is based on the ML analyses (Figs. 3, 5) composed of two species pairs, with *C. tapii* a sister species of *C. tuca* (BBS 95% and 100%, respectively), and *C. iguassuensis* possibly of *C. tesay* (49% and 94%, respectively). Based on the population-based ML tree (Fig. S5), *C. iguassuensis* is not a sister group of *C. tesay*, but rather an early diverging species in the Iguazú clade. Based on species-tree analyses (Fig. S3), only *C. tapii* and *C. tuca* are sister species (BBS 60% and 93%, respectively), while *C. iguassuensis* is their sister group (BBS 54% and 95%, respectively), and *C. tesay* is a basal species (BBS 94% and 100%, respectively), in variable data analysis a sister species of *C. taikyra* (BBS only 62%). The Iguazú species are separated from each other by very short internodes suggesting rapid divergence (Figs. 3, 5, S2, S5). The interspecific internodes are even shorter than between populations within *C. mandelburgeri*.

Crenicichla taikyra is the only species in the Iguazú clade found outside the Iguazú basin far below the Iguazú Falls in the lowermost Middle Paraná (Fig. 3). Phylogenetically and biogeographically, *C. taikyra* is clearly derived from the Iguazú clade and Iguazú River, being either the sister species of the Iguazú clade (MP jackknife analysis in Fig. S2, species-tree analysis with variable data in Fig. S3a), the sister species of *C. tesay* (species-tree analysis with fixed data in Fig. S3b), or an inner group of *C. tesay* (ML analyses in Figs. 3, 5, S5). Both *C. tesay* and *C. taikyra* are molluscivorous species (Casciotta et al., 2013; Piálek et al., 2015; Říčan et al., 2017), and are also found as close relatives (together with *C.* sp. 'Piray–Guazú line' and *C. mandelburgeri* from the Chapa River) in the mitochondrial phylogeny (Fig. 2). *Crenicichla taikyra* based on admixture and migration analyses (Figs. 4, S4, S5) and based on comparisons of mtDNA and nDNA topologies (Fig. 2 vs. Figs. 3, 5, S2, S3, S5) contributed genetic material from the Iguazú River into the Paraná River. The weak gene flow between *C. taikyra* and most of the Paraná species was found in the fineRADstructure analysis (Fig. 4), and more specifically between *C. taikyra* and *C. mandelburgeri* in the TreeMix analysis (Fig. S5).

Urugua-í The two sampled species in the putative Urugua-í species flock (the generalistic predator *C.*

Fig. 2 MtDNA maximum-likelihood phylogeny of the *C. mandelburgeri* complex based on two mitochondrial markers (cytochrome b, ND2) with bootstrap supports. Red branches show the Iguazú clade that is monophyletic in the ddRAD analyses (Figs. 3, 4, S2). Blue branches show the Middle Paraná species that have cytonuclear discordance ▶


10
C. ypo
H
12
C. hu
I
7
C. tapii
BC
15
C. sp. 'Piray–Guazú'
K
20
C. mandelburgeri
OPQRTU
11
C. sp. 'Urugua–í line'
H
27
C. mandelburgeri
DIJLMNQ
26
C. iguassuensis
ABCDEF
6
C. tuca
BCE
3 C. taikyra V
2 C. mandelburgeri S
20
C. sp. 'Piray–Guazú line'
K
27
C. tesay
ABCDE
100
100
100
95
96
99
99
96
91
22
99
100
48
100
77
100
78
100
93
89
76
94
100
73
mtDNA (cyt b + ND2)
ML best tree
186 individuals
100 bootstraps
0.02

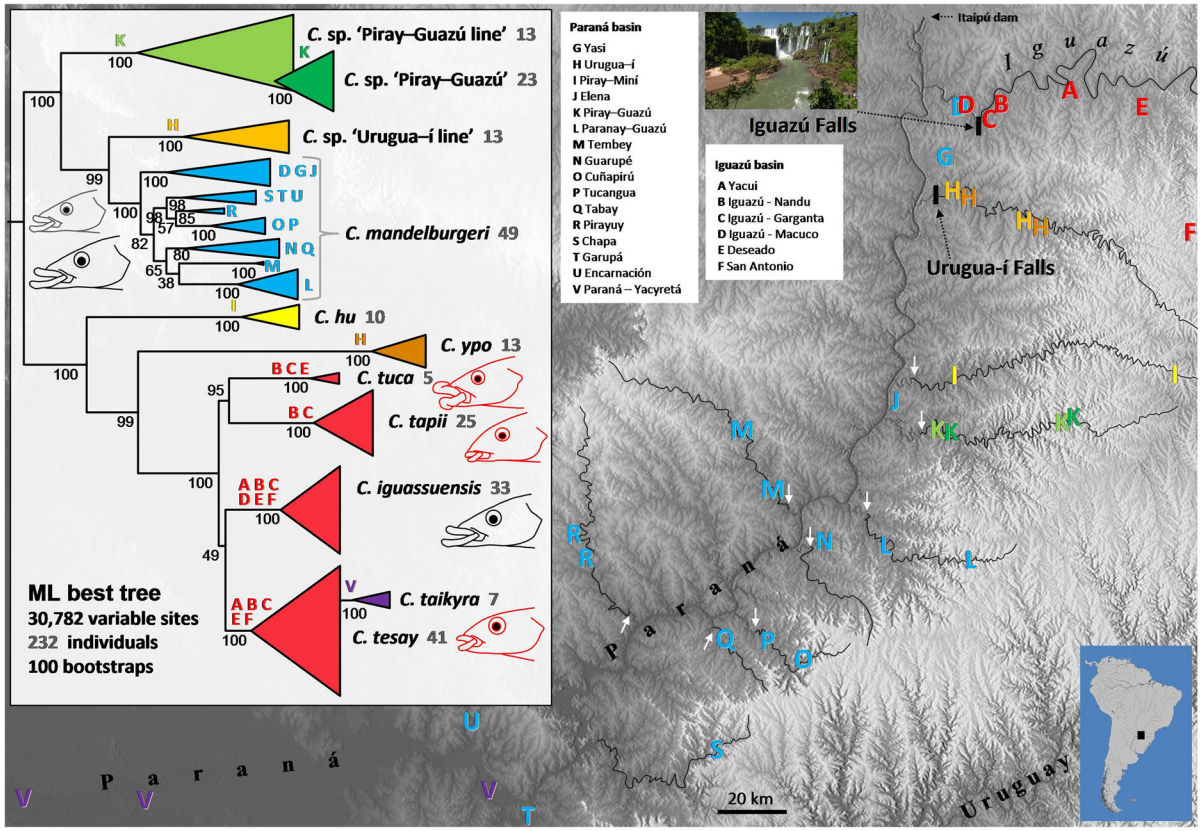


Fig. 3 SNP-based ddRAD maximum-likelihood phylogeny of the *C. mandelburgeri* complex in Middle Paraná basin. Node support is represented by bootstrap values; gray numbers behind taxa show numbers of analyzed specimens; capital letters show localities/populations; non-ancestral ecomorphs (head drawings) are shown in red. Black vertical bars show waterfalls separating tributaries (Iguazú Falls pictured); white arrows show important sections of rapids on tributaries (note correspondence between barriers and endemism/or molecular lineages)

ypo and the piscivorous *C.* sp. Urugua-í line) were found to share strong co-ancestry (Figs. 4, S2, S3a, S4), but *C.* sp. Urugua-í line also shares co-ancestry with *C. mandelburgeri* and is thus either a species of hybrid origin or with strong secondary introgression from *C. mandelburgeri*. The secondary co-ancestry of *C.* sp. Urugua-í line with *C. mandelburgeri* is evident in TreeMix analysis (Fig. S5) which shows a strong migration edge from *C. mandelburgeri* into the *C.* sp. Urugua-í line. Admixture analysis (Fig. S4) shows a 50/50 co-ancestry between *C. ypo* and *C. mandelburgeri*, and fineRADstructure analysis (Fig. 4) shows dominant co-ancestry with *C. ypo* and secondary co-ancestry with *C. mandelburgeri*. These results coincide with the cytonuclear discordance where in mtDNA phylogeny *C.* sp. Urugua-í line is found as a clade within *C. mandelburgeri*.

Piray Guazú The Piray Guazú species pair includes two undescribed putative species (*C.* sp. Piray Guazú, *C.* sp. Piray Guazú line) with a similar ecomorphological dichotomy as the two aforementioned Urugua-í species. This is the only other tributary of the Middle Paraná except the Iguazú and the Urugua-í where more than one endemic species of *Crenicichla* are found (Fig. 1). Based on the mtDNA phylogeny, the two putative species are not closely related (Fig. 2). Based on all nDNA ddRAD analyses they form a strongly supported clade (BBS 100% in all analyses; Figs. 3, S2, S3, S5), but *C.* sp. Piray Guazú line is paraphyletic to *C.* sp. Piray Guazú in ML and MP analyses (Figs. 3 and S2). All phylogenomic analyses additionally reveal that the two putative species are the sister group of *C. mandelburgeri*.

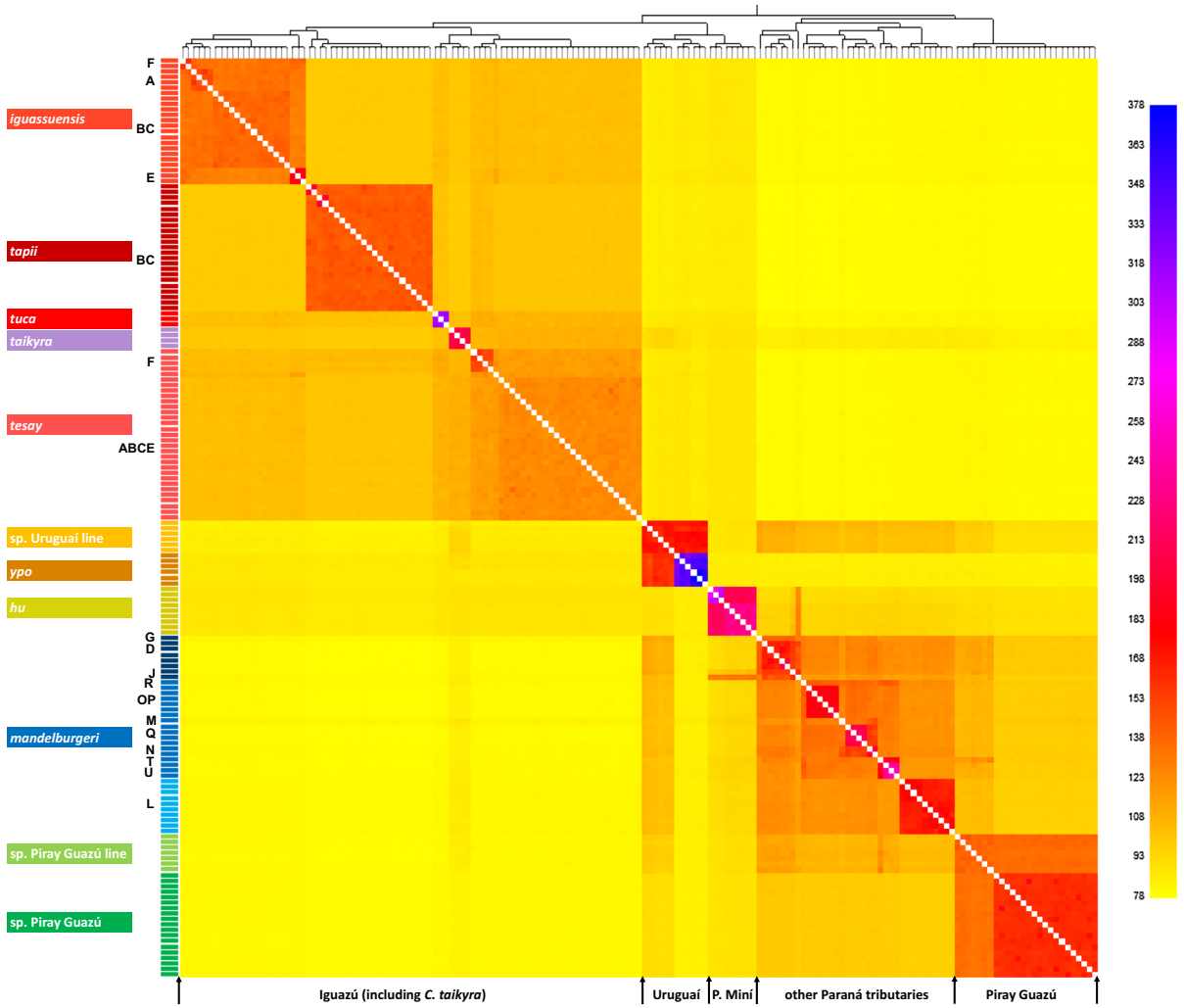


Fig. 4 FineRADstructure co-ancestry matrix with the tree showing inferred relationships between samples. Each tip and label corresponds to an individual, with labels colored according to the species. Populations are marked with capital letters (see Fig. 3 for the legend). The highest levels of co-ancestry are indicated in blue and purple. The lowest levels of co-ancestry sharing are indicated by yellow

Population analyses show that *C.* sp. Piray Guazú line (unlike C. sp. Piray Guazú) has secondary admixture from *C. mandelburgeri* (fineRADstructure, Fig. 4; Admixture analysis, Fig. S4; TreeMix analysis, Fig. S5). The evolutionary history of *C.* sp. Piray Guazú line is thus the same as in *C.* sp. Urugua-í line, i.e., original ancestry with the native species and secondary admixture from *C. mandelburgeri*, except that the secondary admixture is likely younger in *C.* sp. Piray Guazú line than in *C.* sp. Urugua-í line (see Admixture analysis, Fig. S4).

Biogeography and cytonuclear discordance

The ddRAD phylogenetic topologies (Figs. 3, S2, S3, S5) confirmed the biogeographic reconstruction of Piálek et al. (2012) based predominantly on the mtDNA data (see Fig. 2) in the point that the Iguazú has been colonized from the Middle Paraná. The ddRAD topologies, however, represent a different and more intuitive overall biogeography of the *C. mandelburgeri* species complex. It is composed of two geographical clades, one including the southern species (*C. mandelburgeri*, *C.* sp. Piray Guazú, *C.* sp. Piray Guazú line), and one including the Iguazú

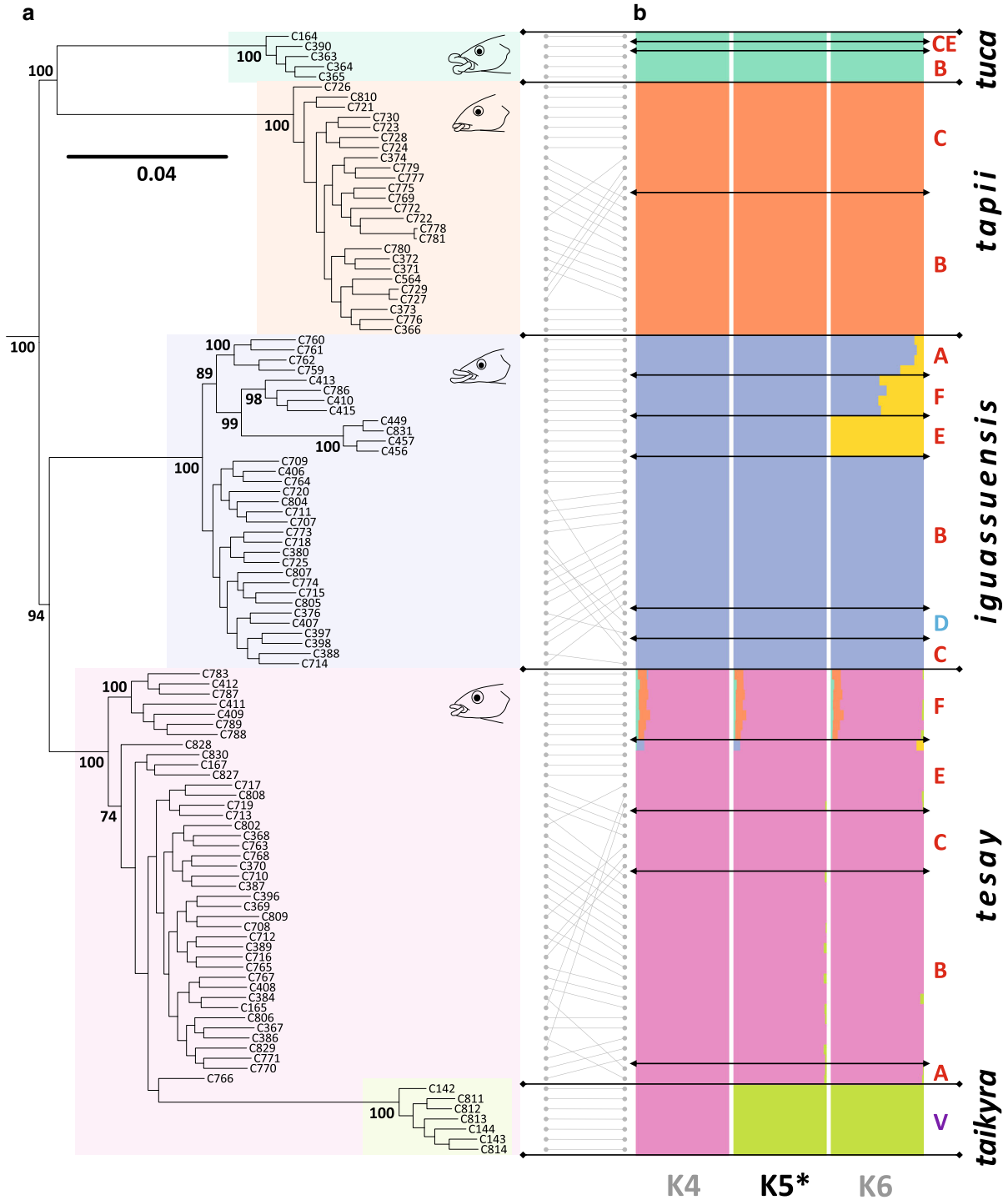


Fig. 5 Species relationships inside the Lower Iguazú flock: **a** ML tree with bootstrap supports; **b** inference of population structure based on the Admixture analysis. Optimal number of ancestral populations is marked with an asterisk. Gray lines connect the corresponding specimens between both analyses

and the northern species (*C. ypo*, *C.* sp. Urugua-í line, *C. hu*). The southern nDNA clade plus *C.* sp. Urugua-í line are in the mtDNA phylogeny (Fig. 2) nested non-monophyletically within the Iguazú clade showing thus a marked cytonuclear discordance.

The fineRADstructure analysis (Fig. 4) offers additional results regarding the biogeography of the *C. mandelburgeri* group. The Iguazú clade and the southern Paraná clade each form a distinct genetic cluster (Fig. 4). In between these two clades, both geographically and genetically are the three species most closely related to the Iguazú clade (*C. ypo*. *C.* sp. Urugua-í line, *C. hu*). *Crenicichla ypo* has slightly stronger co-ancestry with the Iguazú clade than with the southern Paraná clade, which coincides with its most often reconstructed sister-group relationship to the Iguazú clade (Figs. 3, S2–S4). *Crenicichla hu* has a weak but completely symmetrical co-ancestry with both the Iguazú and the southern Paraná clusters, and is thus likely genetically closest to the common ancestor of both clades. This hypothesis is supported by its position in the species-tree analyses (Fig. S3). Lastly, the southern Paraná clade and also the nominal *C. mandelburgeri* based on fineRADstructure (Fig. 4) and Admixture (Fig. S4) analyses and MP jackknife analysis (Fig. S2) contain strong and sharply delineated genetic structure, that together with morphological distinctiveness, hint at notable putative species diversity (*C.* sp. Piray Guazú, *C.* sp. Piray Guazú line, *C.* sp. Paranay Guazú = *C. mandelburgeri* L, *C. mandelburgeri* DGJ, OPR, QM, UT).

Genetic differentiation and species boundaries in the Iguazú species flock

In order to better ascertain the phylogenetic relationships and the degree of admixture between species in the Iguazú clade, we have analyzed restricted de novo assembled datasets solely including the Iguazú species (Fig. 5). Results of ML phylogenetic analysis found *Crenicichla tapii* and *C. tuca*, and also *C. iguassuensis* and *C. tesay* (including *C. taikyra*), as sister taxa (BBS 100 and 94, respectively; Fig. 5a). The Admixture analysis revealed an optimum of five ancestral populations that correspond to the described species (Fig. 5b, K5). Structuring the estimated individual ancestries into suboptimal numbers of clusters led to the inclusion of *C. taikyra* into *C. tesay* (K4). At K6, the *C. iguassuensis* specimens from the Deseado tributary form an additional ancestral cluster (to a smaller degree also present in the San Antonio and Yacui population ancestries) which is also reflected in a more distinct genetic cluster of these populations in the fineRADstructure analysis matrix (Fig. 4).

Admixture analysis (Fig. 5b) and fineRADstructure analysis (Fig. 4) both find the most genetically isolated species to be the thick-lipped *C. tuca*, the grazing *C. tapii* and the piscivorous *C. iguassuensis*. Neither Admixture nor fineRADstructure analyses did find any admixture between these species. The only species with admixture is the molluscivorous *C. tesay* (Fig. 5b) that is also revealed as the least distinct species in the fineRADstructure (Fig. 4). Both analyses show less admixture in populations A–E of *C. tesay* (from or close to the main river) while population F (from the most distant San Antonio tributary) has more admixture with the other Iguazú species.

Discussion

Phylogenomics of the *C. mandelburgeri* species complex

The development of restriction site-associated sequencing (RADseq) has fueled important advances in ecological, evolutionary, and conservation genomics studies (Andrews et al., 2016) including those focused on cichlid diversifications (O'Quin et al., 2012; Keller et al., 2013; Takahashi et al., 2013; Wagner et al., 2013; Malinsky et al., 2015; Fruciano et al., 2016; Kautt et al., 2016; Říčan et al., 2016; Burress et al., 2018a) and the method is considered one of the most important scientific breakthroughs in the past decade (Andrews et al., 2016).

RADseq technologies have some inherent artifacts and biases (Andrews et al., 2016; Verdu et al., 2016) and in order to avoid these we used conscientious and stringent quality sorting of obtained tags and generated various datasets under different sets of parameters that were analyzed by philosophically diverse approaches (Rubin et al., 2012, Brawand et al., 2014; Takahashi et al., 2014; Takahashi & Moreno 2015). Changes of parameters (e.g., depth of coverage and locus scoring rate) and hence matrix sizes did not strongly affect the resulting topologies and always revealed a monophyletic Iguazú clade (including *C. taikyra*) with 100% support. The sum of bootstrap

values of the inferred tree tended to grow with dataset size. We also performed *denovo* assembly in parallel with assembly based on reference-genome mapping (both with various sets of parameters), mapping on unmasked/masked genome (to reduce influence of repetitive regions) or LD-pruning of SNPs; all analyses with identical result. Also, MP-based jackknifing (i.e., random selection of small datasets from the whole pool of fixed SNPs) under stringent conditions for the tree topology resolution (only 10% of characters retained) did not result in any evolutionary alternative hypothesis to the Iguazú clade (100% support in 200 replicates). Population genomic analyses found the Iguazú clade (except *C. taikyra*) to be completely genetically isolated from the Paraná species.

Cytonuclear discordance

The previously employed mtDNA markers (Piálek et al., 2012) and our extended specimen sampling in the present study have resulted in a phylogeny of the *C. mandelburgeri* species complex that is in conflict with the nDNA (RADseq) topologies based on various approaches to the analysis of the ddRAD data (Fig. 2 vs. Figs. 3, 4, 5, S2, S3, S5). The mtDNA and nDNA topologies are, except for the conflict, similar in that the Iguazú clade has in both topologies very short interspecific branch-lengths while all other species in the Paraná and all outgroup species have much longer interspecific branches. The conflict is that the Iguazú clade is monophyletic in nDNA but includes most species from the Middle Paraná (except *C. hu*, *C. ypo*) in the mtDNA topology. The Lower and Upper Paraná species and the outgroup species have the same phylogenetic position and comparable interspecific branch-lengths in both the mtDNA and nDNA topologies.

The conflicting Middle Paraná species do not form a single clade in the mtDNA topology but rather are variously related to the four Iguazú species. Additionally, *C. mandelburgeri*, which is monophyletic in the ddRAD topologies is polyphyletic in the mtDNA topology. Also, the two Piray Guazú putative species, which form a clade in the ddRAD topologies, form two unrelated clades in the mtDNA topology.

This pattern of the cytonuclear discordance could suggest independent introgressions of mtDNA from the Iguazú clade into virtually all the Middle Paraná species except *C. hu* and *C. ypo*. Our analyses of the nDNA ddRAD data using fineRADstructure, Admixture, and TreeMix (Figs. 4 and S4, S5), however, found no introgressions between the individual Iguazú species and Middle Paraná species. We have only found admixture between *C. taikyra* and *C. mandelburgeri* (Figs. 4 and S5). These results and the topology and branch-lengths of the mtDNA phylogeny (Fig. 2) thus do not support recent introgressions and demonstrate that the Iguazú and Paraná species are presently effectively isolated.

The only species that is not monophyletic in the mtDNA phylogeny is *C. mandelburgeri* and together with the distributional pattern of *C. mandelburgeri* (the only widespread species in the Middle Paraná basin), we hypothesize that *C. mandelburgeri* could be the vector that has spread the allochthonous mtDNA variants throughout the Middle Paraná basin. This hypothesis finds support in the TreeMix analysis (Fig. S5), which finds *C. mandelburgeri* as the sole source of all secondary gene flow in the Middle Paraná basin and together with Admixture analysis demonstrates that it has admixed with *C. taikyra*, *C.* sp. Urugua-í line, *C. hu*, and C. sp. Piray Guazú line.

Species flocks in the C. mandelburgeri complex

Three tributaries of the Middle Paraná contain more than a single endemic species or putative species of *Crenicichla* and based on their divergence in morphological and in life-history traits are likely candidates for cases of sympatric speciation within the *C. mandelburgeri* species complex. Four sympatric and syntopic species are found in the Iguazú River, three in the Urugua-í tributary, and two in the Piray Guazú tributary.

Our nDNA ddRAD analyses clearly supported the four endemic species in the Iguazú as a monophyletic group of very closely related species with very little gene flow between each other and as completely genetically isolated from the Paraná species. The Iguazú clade also includes a fifth, allopatric species separated by the Iguazú Falls, *C. taikyra*.

Based on our molecular analyses and the morphological analyses of Burress et al. (2018a), the Iguazú clade of the *C. mandelburgeri* species complex thus represents a species flock that exhibits a parallel pattern of diversification with the unrelated Uruguay species flock of the *C. missioneira* species complex.

Burress et al. (2018a) have demonstrated this parallel evolution within the broad geographical boundaries of the Paraná and Uruguay River basins, but our results demonstrate that the parallelism in the Paraná River basin is geographically structured and is thus not a feature of the Middle Paraná basin as a whole. The Iguazú species flock forms the core of morphological and ecological diversity within the Middle Paraná *C. mandelburgeri* species complex and includes the same four ecomorphs that are also found in the Uruguay River (Piálek et al., 2012, 2015; Burress et al., 2018a). The four closely related Iguazú *Crenicichla* species represent all known derived ecomorphs described in *Crenicichla* (Burress et al., 2018a): the piscivorous *C. iguassuensis* with a long head and large prognathous mouth; the molluscivorous *C. tesay* with a smaller isognathous mouth, a robust lower pharyngeal jaw and molariform pharyngeal teeth; the periphyton grazer *C. tapii* with the smallest head and hypognathous mouth and with a much less robust lower pharyngeal jaw with pointed pharyngeal teeth; and the thick-lipped invertivorous crevice-feeding *C. tuca* with a large hypognathous mouth with hypertrophied fleshy lips.

Our analyses of the evolutionary relationships within the Iguazú clade revealed several topologies differing in the position of the species (Figs. 3, 5, S2, S3, S5). All analyses, however, find *C. tuca* and *C. tapii* as sister species. *Crenicichla iguassuensis* and *C. tesay* are found as sister species in ML concatenated analyses (Figs. 3, 5), but not in MP jackknife analysis (Fig. S2) and also not in species-tree analyses (Fig. S3), where *C. iguassuensis* is always the sister group of the *C. tuca* and *C. tapii* pair, and *C. tesay* is sister to those three species. The allopatric *C. taikyra* was found either as an inner branch of *C. tesay* (Fig. 3), at the base of the Iguazú clade (Fig. S2), or as the sister species of *C. tesay* (Fig. S5). The results are thus fully in agreement with the hypothesis of allopatry-driven speciation of *C. taikyra* from a *C. tesay* ancestral population over the Iguazú Falls (Casciotta et al., 2013).

The evolutionary inference of relationships between the Iguazú River species is to a large degree intuitive and agrees predominantly with coloration patterns (the head morphology being unique in each species): *C. tapii* and *C. tuca* are sister species united by clear coloration-pattern synapomorphies, while *C. iguassuensis* and *C. tesay* share virtually identical coloration (Piálek et al., 2015) that, however, does not include such clear synapomorphies and that based on our phylogenomic results might be the ancestral color pattern for the species flock. The branches separating all four species are very short, which is also manifested in uncertainty and lower bootstrap support of the relationships of the latter two species. This result reflects, in our opinion, the process of recent adaptive radiation where single SNP phylogenies can reflect different genetic mechanisms responsible for the diversification. Such a situation was described in Brawand et al. (2014), who studied in detail the genomic mechanisms underlying the phenotypic evolution in five closely related Victoria Lake cichlid species; their conclusions support the hypothesis that variation in coding-regions is mostly involved in differentiated traits like color, while regulatory variation (which can be less likely recorded in SNP phylogenies) is more important in morphological changes controlled by pleiotropic-gene networks.

For the two other candidate cases (Urugua-í and the Piray Guazú faunas) of sympatric speciation, our nDNA ddRAD analyses provided mixed results. In the case of the Urugua-í, the two analyzed species were not consistently recovered as sister species, but population analyses have shown that they have strong co-ancestry (Figs. 4, S4, S5) with the conflict being due to introgression of *C.* sp. Urugua-í line with *C. mandelburgeri*. In the case of the Piray Guazú, the situation is analogous except that the two putative species were found as a monophyletic group in all ddRAD analyses. The ddRAD data also revealed introgression of *C.* sp. Piray Guazú line with the widespread *C. mandelburgeri* (Fig. 4). Both river faunas, however, are composed of distinct ecomorphs that suggest ecologically based divergence. Based on Casciotta et al. (2010) and Burress et al. (2018a), one ecomorph is a generalistic invertivore (*C. ypo* and *C.* sp. Piray Guazú) and the other a piscivore (*C.* sp. Urugua-í line) or a putative piscivore (*C.* sp. Piray Guazú line; Fig. 1). The similarity in head and body shape and coloration between the two species pairs (especially between the piscivores) is striking (Figs. 1, S1), yet the species in the two tributary basins are clearly unrelated (Figs. 2, 3, 4, S2, S5) and do not show admixture except through *C. mandelburgeri* (Figs. 4, S4, S5). Both of the piscivores have admixture with *C. mandelburgeri* but both are distinct from *C. mandelburgeri* in morphology and coloration (Figs. 1, S1). The test of in situ evolution in the

Urugua-í clearly necessitates the inclusion of the third, recently rediscovered (Říčan et al., 2017) molluscivorous species (*C. yaha*) for which DNA samples were not available at the time of the presented analyses.

Biogeography of the *C. mandelburgeri* species complex

The biogeography of the *C. mandelburgeri* species complex is more complicated and structured than in the *C. missioneira* species complex. The reason for the higher endemism in the Middle Paraná basin as opposed to the Uruguay basin is evident and straightforward. The Uruguay River does not have such a pronounced canyon as the Middle Paraná and thus tributaries of the Uruguay are only separated by rapids (the notable exception is *C. empheres* Lucena, 2007 isolated above a large waterfall). The endemic *Crenicichla* faunas of the Iguazú and Urugua-í are both separated from the Middle Paraná by large waterfalls (the 70–80-m-high Iguazú Falls and the 28-m-high Urugua-í fall). Towards the south the canyon of the Middle Paraná is much less deep and steep, the tributaries are separated only by series of rapids and not significant waterfalls (Fig. 3), but these rapids are longer and steeper than on the Uruguay tributaries. Endemism of *Crenicichla* in Middle Paraná tributaries is thus higher than in the Uruguay because of dispersal barriers. Several of the tributaries in the central portion of the Middle Paraná have endemic species of *Crenicichla* (Piray Miní, Piray Guazú, Paranay Guazú were analyzed in this study; Figs. 3, 1, S1) while in the south only the widespread *C. mandelburgeri* is found.

Parallel diversification patterns in *Crenicichla* species flocks

The *Crenicichla* clade endemic to the Lower Iguazú comprises four species with four distinct ecomorphs differing mainly in morphological traits directly related to trophic function such as mouth, jaw, and tooth morphology (Piálek et al., 2015) plus coloration patterns. Our analyses confirmed their close phylogenetic relationships, monophyly, and genetic isolation from each other and from the Paraná species. Based on our results and the results of Burress et al. (2018a), this group of species fulfills the definition of a species flock (i.e., a monophyletic group of closely related, endemic and diversified species) sensu Salzburger & Meyer (2004) and represents a case of parallel evolution to the Uruguay *Crenicichla* species flock (Burress et al., 2013, 2015, 2018b).

The Iguazú *Crenicichla* species flock includes the same ecomorphs as are known from the neighboring *C. missioneira* species complex (Lucena & Kullander, 1992; Lucena, 2007) from the Uruguay River basin. The morphologically ancestral ecomorph is represented in the Iguazú species flock by the piscivorous *C. iguassuensis*, and by *C. missioneira*, *C. igara* Lucena & Kullander, 1992, and *C. celidochilus* in the Uruguay River species flock (Burress et al., 2018a). The novel ecomorphs include the small-mouthed isognathous grazers (*C. tapii* vs. *C. hadrostigma*; respectively), the small-mouthed isognathous molluscivores with molariform pharyngeal teeth and robust lower pharyngeal jaws (*C. tesay*, *C. taikyra* vs. *C. minuano*, *C. jurubi* Lucena & Kullander, 1992), and the large-mouthed thick-lipped invertivores (*C. tuca* vs. *C. tendybaguassu*; Piálek et al., 2015).

All presently known novel ecomorphs within *Crenicichla* are present in these two species flocks and reveal striking resemblance not only in the construction of mouth, lower pharyngeal jaw, and teeth, but also in their overall appearance, including coloration patterns (e.g., rectangular blotches in benthic piscivores and molluscivores, double bars, and prominent spot behind the head in gregarious periphyton grazers, a lateral stripe in open-water piscivores) displaying thus an astonishing and detailed example of parallel diversification patterns in two unrelated species complexes.

The reasons for such pronounced, peculiar, and parallel diversification of *Crenicichla* in the two neighboring river basins remain to be studied, but two sets of factors are supposed to be responsible for such diversity in general (Wagner et al., 2012) and specifically in *Crenicichla* (Burress et al., 2018a). The first (intrinsic) factor seems to be the diversification potential of the genus *Crenicichla*, which is the most species-rich genus within Neotropical Cichlidae. *Crenicichla* in itself was a phylogenetic puzzle and it still is the most vigorously debated genus in terms of its phylogenetic position among South American cichlids. Morphological and molecular phylogenies are in complete disagreement regarding the phylogenetic placement of the genus (Kullander, 1998; Farias et al., 1999, 2000, 2001; Smith et al., 2008; López-Fernández et al., 2010). Present knowledge suggests

that *Crenicichla*, despite being a predatory-piscivorous group of cichlids with elongated bodies, is in fact nested deeply within the geophagine cichlids (as revealed solely by molecular phylogenies, e.g., Farias et al., 1999, 2001; López-Fernández et al., 2010; Ilves et al., 2017), which have much more generalized postcranial and completely different head morphologies because most are not piscivorous but are benthic feeders that often feed by sifting substrate. *Crenicichla* is thus a highly derived genus morphologically, one of the most derived among Neotropical cichlid genera. Several *Crenicichla* species, all of which are limited to the discussed two species flocks, are the only ones (except the dwarfed species of the *C. wallacii* Regan, 1905 and *Teleocichla* species groups) which have reverted from the piscivorous morphology to other ecomorphs (e.g., molluscivores, thick-lipped invertivores, and periphyton grazers).

The second (extrinsic) speciation factor appears to be a combination of geological diversity, partial isolation, and ecological opportunity. As stressed in most studies of species flock formation in cichlids, in order to sympatrically or microallopatrically diversify, these fishes need to have a set of available niches into which to specialize (Sturmbauer et al., 2001). This condition is clearly fulfilled equally well in complex lakes as well as in complex riverine habitats. Ecologically complex riverine habitats, however, are not present everywhere; *Crenicichla* is thus known to develop closely related species complexes only in a tiny fraction of the South American landscape being absent from most of the low-lying sandy and muddy rivers. The species complexes are limited only to the large rapid-rich rivers of the Amazon (e.g., the Xingu complexes in *Crenicichla* and in *Teleocichla*) and to the uplands of the La Plata basin, more specifically to the Middle Paraná/Iguazú and Uruguay area. What makes these drainages so special is that they have, as a result of their geological history, the largest number and highest concentration of rapids and waterfalls in South America. The regions have thousands of rapids and waterfalls ranging from those on the smallest tributaries to huge falls on mighty rivers and the most famous of these are the Iguazú Falls. The complex geomorphology of the rivers thus could promote speciation, both by providing diversity of habitats and by diminishing the diversity of competing groups of fishes (such as Characiforms) providing ecological opportunities. The two *Crenicichla* species flocks in the Iguazú and Uruguay indeed seem to have taken much of the ecological space for themselves since the biomass and abundance of specimens of *Crenicichla* often surpasses those of characoids or siluroids (Říčan et al., pers. obs. based on extensive field work), which is not different from the situation in Africa´s Great Lakes but very unlike other faunal assemblages in large river basins, e.g., the Amazon or Congo, where other fish groups dominate.

Threats and conservation

The most imminent threat to the Middle Paraná *Crenicichla* species continued survival is the relentless push towards more hydroelectric dam constructions. Hydroelectric dams have already strongly altered the landscape and ecology of the Middle Paraná basin and have extremely diminished natural habitats in the whole Upper Paraná (Agostinho et al., 2007; Langeani et al., 2007; Júlio Júnior et al., 2009) and Iguazú basins (Baumgartner et al., 2012) in Brazil. The dams are additionally stocked by non-native fish species (and inadvertently by non-native aquatic invertebrates and algae) raised throughout the countryside on exotic-fish farms and these non-native fish species now dominate ichthyofaunas in some parts of the Middle Paraná basin (e.g., the Itaipu reservoir; Langeani et al., 2007). In other parts of the basin, non-native fishes are also strongly and rapidly increasing in abundance in and around other dams (e.g., the Uruguaí in Argentina, Acaray in Paraguay) including the best known protected area in the zone, the Iguazú National Parks system in Argentina and Brazil, where many non-native fishes have arrived from the upstream dams in Brazil and become established (Casciotta et al., 2016).

Of particular interest to the Middle Paraná *Crenicichla* species group is the species flock in the Iguazú. Based on surveys of their occurence and abundance in the relatively pristine condition of Iguazú National park (Piálek et al., 2015; Casciotta et al., 2016), the two species with unique ecomorphology (*C. tuca, C. tapii*) within the whole Middle Paraná species group are found as viable populations only in the Iguazú main stream which offers the best habitats appropriate for them. In tributaries, especially small ones, these two species are almost completely absent (Piálek et al., 2015). Also, as our results here suggest, in tributaries, the two species are found only as introgressed alleles

in the two widespread species (in the here used specimen sampling within *C. tesay*, Fig. 5). Most specimens reported in the literature of *Crenicichla* in the Iguazú basin in Brazil come from the dams (Baumgartner et al., 2012; Frota et al., 2016) and many of these are morphologically intermediate specimens combining characters of the four species (cf. Varella, 2011; Piálek et al., 2015). These intermediate specimens are likely hybrids (pending molecular studies) resulting from erosion of the species in the artificial conditions (deep, stratified, stagnant and murky water) in the reservoirs and also in suboptimal ecological conditions of small tributaries (as supported here by our analyses; Fig. 5b, locality F). The originally shallow, rapid-rich, and clear-water Lower Iguazú mainstream, so crucial for the survival of the Iguazú species flock, has been reduced (after the completion of the latest Baixo Iguaçu dam) from 600 to 160 km (i.e., confined to the stretch of river combining the Brazilian and Argentinean National Parks) due to flooding by the continuous cascade of hydroelectric dams (Baumgartner et al., 2012). Additionally, the dams have flooded previously existing important barriers within the mainstream of the Iguazú River (Baumgartner et al., 2012) which may have additionally contributed to mixing of previously isolated fish populations and species (Júlio Júnior et al., 2009) including those of the *Crenicichla* species flock.

Several new potentially disastrous operations have been proposed for construction in the Middle Paraná basin per se. One of these seeks to divert water through a tunnel from the highly endemic Iguazú basin into the highly endemic Urugua-í basin (the area of the other candidate *Crenicichla* species flock) in Misiones, Argentina to increase production of electricity in the Urugua-í dam, which has not been lucrative immediately from its start of operation in 1989. Another such project on a much larger scale proposes to actually inundate and thus destroy the whole remaining free-flowing stretch of the Middle Paraná and its isolated highly endemic tributaries (between the Yacyretá and Itaipú, i.e., the whole length of the occurence of the Paraná *Crenicichla* species group; Fig. 3). This project seeks to make a hydroway out of the Paraná River by making it navigable (through a series of locks in the dams) all the way between Buenos Aires and São Paulo. This would completely obliterate the whole species group by transforming the area into a continuation of the Itaipú dam. These and other planned projects seriously threaten the survival of biodiversity in Misiones, Argentina and in the whole emerging Middle Paraná endemism hotspot (Říčan et al., 2018) including the fascinating *C. mandelburgeri* species group.

Conclusions

The *C. mandelburgeri* species complex from the Middle Paraná basin shows parallel evolution of ecomorphs to the unrelated *C. missioneira* species complex from the directly neighboring Uruguay basin. The Paraná *C. mandelburgeri* species complex shows virtually complete single tributary endemism with sympatry of resident species found in the Iguazú (4 species), Urugua-í (3 species), and Piray Guazú (2 putative species) tributaries and only one widespread species in the Middle Paraná basin (*C. mandelburgeri*). The four species making-up the Iguazú species flock consist of trophic specialists and represent a striking case of parallel evolution to four species of the Uruguay *Crenicichla missioneira* species flock. The fifth species representing a fifth ecomorph (a pelagic piscivore) in the Uruguay species flock is so far unknown from the Iguazú species flock (but is found in the Urugua-í; *C.* sp. Urugua-í line). Our phylogenomic nDNA analyses obtained by ddRAD sequencing strongly supported the monophyly of the Iguazú species flock, the monophyly of the individual species, and the genetic isolation of the species from each other and from the Paraná species. The two studied endemic species pairs in the Urugua-í and Piray Guazú tributaries were found to share common ancestry in each tributary. One species in each tributary (*C.* sp. Urugua-í line, *C.* sp. Piray Guazú line), however, has strong introgression from the widespread *C. mandelburgeri*. One (*C.* sp. Urugua-í line) or both (Piray Guazú) species also show cytonuclear discordance and the discordance actually involves most of the Middle Paraná except *C. hu* and *C. ypo*.

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