Molecular phylogeny and biogeography of the South American savanna killifish genus *Melanorivulus* (Teleostei: Aplocheilidae)

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Abstract

This study comprises the first molecular phylogeny of *Melanorivulus*, a genus of small killifishes inhabiting shallow streams draining South American savannas, using segments of the mitochondrial genes 16S and ND2 and the intron 1 of the nuclear S7 gene, total of 2,138 bp, for 26 taxa. Monophyly of the genus is highly supported and some clades previously diagnosed on the basis of colour patterns are corroborated. A biogeographical analysis using event-based methods indicated that the most recent common ancestor of *Melanorivulus* occupied a region comprising the savannas of the eastern Amazon and the ecotone Amazon-Cerrado, and the present day distribution has been shaped by a series of dispersal and vicariance events through areas today including the upland Cerrado and the lowland Pantanal. The presence of a broad stripe of dense rain forest today separating the savannas of the eastern Amazon, inhabited by *M. schuncki*, from the savannas located south of the Amazon, from where a clade comprising all other species of the genus is endemic, is regarded as evidence of possible geographical expansion of *Melanorivulus* lineages through savanna areas during past cooler and drier periods, when South American grasslands and savannas expanded and rain forests were restricted to small areas.

Key words

Amazon, Cerrado, Chaco, Event-based methods, Pantanal.

Introduction

The South American savannas comprise diverse biomes with high occurrence of endemic species, including the Cerrado that has been listed among the most important and threatened biodiversity hotspots in the world (Myers et al., 2000). With great occurrence of endemic taxa, biogeographical relationships of organisms inhabiting these savannas are still poorly known (e.g., Silva & Bates, 2002), as well as biological inventories in past decades have neglected some habitats, making biodiversity underestimated until recent years. This is the case of the killifish genus *Melanorivulus* Costa, 2006, with most species only living in shallow marginal parts of small streams draining South America savannas (Costa, 1995, 2006; Oliveira et al., 2012), habitats that were poorly sampled in fish collections until recently. As a consequence, only two of the about 35 valid species of *Melanorivulus* were first described before 1989, in spite of the huge area occupied by this genus, between the Oiapoque river basin in northern Brazil, about 4º N, and the Uruguay river basin in northern Argentina, about 27º S, and between the Paraguay river basin in eastern Bolivia, about 60º W, and the coastal plains of north-eastern Brazil, about 37º W (e.g., Costa, 1995; Bragança et al., 2012; Costa et al., 2015). After 1994, intensive field studies directed to *Melanorivulus* habitats took place, generating several taxonomic studies (Costa, 1995, 2003a–b, 2005, 2006,
Material and methods

Taxon sampling. Nineteen described and two still undescribed species of Melanorivulus were analysed in this study. This taxon sample represents all the main generic lineages previously described in morphological studies (Costa, 2007a,b, 2008a, 2010, 2012a; Costa & De Luca, 2010) and covers the entire geographical range of the genus. Outgroups comprise three representatives of all other genera of the melanorivuline clade as defined by Costa (2011), Anablepsoides gamae Costa, Bragança & Amorim, 2013, Atlantirivulus janeiroensis Costa, 1991, and Cynodonichthys tenuis Meek, 1904, besides one species of the basal rivuline genus Laimosemion, L. stri-gatus (Regan, 1912), and one of the basal rivulid genus Kryptolebias, K. brasiliensis (Valenciennes, 1821). A list of species and the respective GenBank accession numbers appear in Table 1.

DNA sequencing. DNeasy Blood & Tissue Kit (Qiagen) was used to extract DNA from muscle tissue of the caudal peduncle of specimens fixed and conserved in absolute ethanol. Using PCR (polymerase chain reaction), portions of two mitochondrial loci were amplified, the ribosomal gene 16s with the primers 16sar-L, 16sbr-H (Palumbi et al., 2002) and R16sn (5’-GGA TGT CCT GAT CCA ACA TCG AGG TCG TA-3’), herein described, and the gene NADH dehydrogenase subunit 2 (ND2) with the primers described in Hirbek & Larson (1999) and the primer R5859 (Costa & Amorim, 2014); besides one nuclear locus, the intron 1 of the nuclear ribosomal protein S7 (S7) gene, with the primers STRPEx1F and STRPEx2R (Chow & Hazama, 1998). PCR was performed in 15 μl reaction mixtures containing 5 × Green Go Taq Reaction Buffer (Promega), 3.6 mM MgCl2, 1 μM of each primer, 50 ng of total genomic DNA, 0.2 mM of each dNTP and 1U of Taq polymerase. The thermocycling profile was: (1) 1 cycle of 4 minutes at 94 °C; (2) 35 cycles of 1 minute at 92 °C, 1 minute at 49-60 °C (varying according to the primer and the sample) and 1 minute at 72 °C; and (3) 1 cycle of 4 minutes at 72 °C. In all PCR reactions, negative controls without DNA were used to check contaminations. Amplified PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Sequencing reactions were made using the BigDye Terminator Cycle Sequencing Mix (Applied Biosystems). Cycle sequencing reactions were performed in 10 μl reaction volumes containing 1 μl BigDye 2.5X, 1.55 μl sequencing buffer 5X (Applied Biosystems), 2 μl of the amplified products (10–40 ng), and 2 μl primer. The thermocycling profile was: (1) 35 cycles of 10 seconds at 96 °C, 5 seconds at 54 °C and 4 minutes at 60 °C. The sequencing reactions were purified and denatured and the samples were run on an ABI 3130 Genetic Analyzer. Sequences were edited using MEGA 6 (Tamura et al., 2013).

Phylogenetic analysis. The edited sequences were aligned using ClustalW as implemented in MEGA 6, and each alignment was checked by eye using Bioedit 7.1 (Hall, 1999). To check for major discordance among individual gene trees, maximum likelihood trees were generated for each gene alignment, using MEGA 6 (Tamura et al., 2013). Since separate analyses did not result in conflicting trees, data were concatenated, with the whole dataset having 2,138 characters. The phylogenetic analysis of the concatenated dataset was conducted through a Bayesian inference using the program MrBayes v3.2.5 (Ronquist et al., 2012), assuming the best fit substitution models for each loci, considering each position of the ND2 gene separately. The Akaike Information Criterion (AIC) was used to select the best-fit model of nucleotide substitution for each data partition, as implemented by jModelTest 2.1.7 (Darriba et al., 2012), which indicated GTR+I+G for the 16s partition and the first and second codon positions of the ND2 partitions, Trn+G for the third codon position of the ND2 partition, and HKY+G for the S7 partition. The Bayesian analysis was conducted using two Markov chain Monte Carlo (MCMC) runs of two chains each for 1 million generations, a sampling frequency of 100. The final consensus tree and Bayesian
posterior probabilities (PP) were generated with the remaining tree samples after discarding the first 25% of samples as burn-in. The dataset was also analysed using Maximum Parsimony methods performed with TNT 1.1 (Goloboff et al., 2008), when the search for most parsimonious trees was conducted using the ‘traditional’ search and setting random taxon-addition replicates to 10, tree bisection-reconnection branch swapping, multi-trees in effect, collapsing branches of zero-length, characters equally weighted, and a maximum of 1,000 trees saved in each replicate. Branch support was assessed by bootstrap analysis, using a heuristic search with 1,000 replicates and the same settings used in the MP search.

**Biogeographical analysis.** Five areas were defined according to the occurrence of Melanorivulus in major phytogeographical regions: (A) the eastern Amazon savanna (i.e., savannas of Amapá and Marajó); (B) the ecotone Amazon-Cerrado; (C) the Cerrado; (D) the Pantanal-Chaco; (E) the Caatinga-coastal Restinga. Biogeographical event-based methods were used to infer possible past biogeographical scenarios of Melanorivulus diversification without aprioristic assumptions about areas relationships (Ronquist, 1997). Two different analytical approaches, both implemented in program RASP 3.02 (Yu et al., 2011), were examined: the parsimony-based DIVA (Ronquist, 1997), modified by Nylander et al. (2008), using S-DIVA (Yu et al., 2010), and the likelihood-based DEC model (Rie et al., 2005; Rie & Smith, 2008), using Lagrange (Rie & Smith, 2008).

**Results**

**Phylogeny.** The Bayesian Analysis (BA) generated a tree with most included clades receiving high support (posterior probabilities above 0.95 %; Fig. 1). The Maximum Parsimony analysis (MPA) generated three equally most parsimonious trees (not depicted), with a resulting consensus strict tree congruent with the tree generated by the BA, but showing low resolution at two different nodes (see bootstrap values for MPA in Fig. 1). These nodes include the uncertain position of M. violaceus and M. dapazi, which appear, respectively, as sister group of M. pindorama and the clade comprising M. atlanticus and M. jalapensis. Since the MPA tree had low resolution and the two clades supported only in the BA are in accordance with previous morphological studies (see Discussion below), only the tree resulting from the latter analysis was considered for the biogeographical reconstruction.

**Biogeography.** Both geographical analyses generated similar results and for this reason only the tree generated by the likelihood-based DEC model is depicted in Fig. 2.

<table>
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<th>Species</th>
<th>Catalog number</th>
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Fig. 1. Phylogenetic relationship tree generated by a Bayesian analysis of molecular data, total of 2,138 bp, comprising segments of the mitochondrial genes 16S and ND2, and the nuclear S7 for 21 species of Melanorivulus and five outgroups. Numbers above the node are posterior probabilities of the Bayesian analysis higher than 75%, below are bootstrap percentages higher than 50% of the Maximum Parsimony analysis.

Fig. 2. Biogeographical analysis of the killifish genus Melanorivulus: tree generated by the likelihood-based DEC model (A) and areas of endemism used in this study (B). Letters on nodes of the tree (A) are areas of endemism delimited in the map (B) and listed in the text. The specimen illustrated is Melanorivulus rutilicaudus, male.
The analysis consistently indicates that the most recent common ancestor of *Melanorivulus* probably occupied a region comprising the eastern Amazon savanna and the ecotone Amazon-Cerrado (areas A and B), and that the present day distribution is a result of a series of dispersal and vicariance events during the evolutionary history of the genus.

The analysis support a vicariance event at the base of the *Melanorivulus* crown clade separating the lineage containing *M. schuncki* in the eastern Amazon savanna from the ancestor of the clade \( \alpha \), then restricted to the Amazon-Cerrado ecotone area. The ancestor of the clade \( \alpha \) first expanded its distribution from the Amazon-Cerrado ecotone towards the neighbouring upland Cerrado, which was followed by a vicariance event separating the ancestor of the clade comprising of the clade \( \beta \) in the upland Cerrado, from the ancestor of the clade clade \( \chi \) in the Amazon-Cerrado ecotone area (Fig. 2).

All descendants of the clade \( \chi \) were confined to the Amazon-Cerrado ecotone area through successive splits. On the other hand, further sporadic dispersals occurred in lineages of the clade \( \beta \) from the upland Cerrado to neighbouring biomes. Later, *M. punctatus* colonized the Pantanal-Chaco area and lineages of the clade comprising *M. jalapensis* and *M. atlanticus* dispersed to the Amazon-Cerrado ecotone, subsequently reaching areas to East, including the distant coastal Restinga of northeastern Brazil.

**Discussion**

**Phylogeny.** The phylogenetic analyses corroborated morphphyly of *Melanorivulus* and the resulting topologies are consistent with previous taxonomical studies in recovering species groups based mainly on colour patterns. The well-supported position of *M. schuncki* as the sister group of a clade including all other congeners (clade \( \alpha \) in Fig. 1) is in agreement with data presented by COSTA & De Luca (2010), where clade \( \alpha \) is diagnosed by the presence of black pigmentation along the anterior margin of the pelvic fin in females and dark brown oblique bars on post-orbital region.

The clade \( \alpha \) contains two well-supported inclusive clades, clade \( \beta \) and clade \( \chi \). Among lineages contained into the clade \( \chi \), the analysis also strongly corroborates the *Melanorivulus zygonectes* group as delimited by COSTA (2007), diagnosed by the presence of read chevron-like marks on the body side, which have the vertex placed on the ventral portion of the flank. The BA found low values of posterior probabilities (< 75%) for the proposed sister group relationships between *M. pindorama* and *M. violaceus*, whereas this clade was not recovered in the MPA. However, *M. pindorama* and *M. violaceus* share a unique colour pattern in males, consisting of a row of brown blotches on the flank (COSTA, 1991, 2012a), thus congruent with the topology generated by the BA. Among species of the clade \( \beta \) (Fig. 1), the clade comprising *M. atlanticus* and *M. jalapensis* is concordant with previous taxonomic studies, in which a clade comprising those species and *M. decoratus* (not available for the molecular analysis) has been diagnosed by all included species having five branchiostegal rays instead of 6 as in other congeners (COSTA, 2010; COSTA et al., 2015). On the other hand, the position of *M. dapazi* as the sister group of the clade comprising *M. atlanticus* and *M. jalapensis* is weakly supported in the BA, whereas in the MPA, the position of this species is uncertain within the clade \( \beta \). However, *M. dapazi*, *M. atlanticus* and *M. jalapensis* share the presence of a dark orange stripe on the anterior margin of the pelvic fin and distal margin of the anal fin and, narrow oblique red bars over a broad grey stripe on the flank in males (COSTA, 2005, 2010; COSTA et al., 2015), thus corroborating the BA topology.

**Biogeography.** It is possible that events of geographical expansion and dispersal of *Melanorivulus* lineages among savanna areas are related to past cooler and drier periods, when South American grasslands and savannas expanded and rain forests were restricted to small areas. In the Late Miocene, for example, an intense global cooling resulted in a sharp shift in the vegetation of South America, with dense rain forests being replaced by open formations (e.g., Latorre et al., 1997), giving origin to the modern Cerrado vegetation (Keeley & Rundel, 2005; Graham, 2011). A similar geographical expansion during periods of intense aridity has been postulated for the African savanna killifish genus *Nothobranchius* (Dorn et al., 2014).

The savannas of the eastern Amazon inhabited by *M. schuncki* is presently separated by a stripe of dense rain forest from the ecotone Amazon-Cerrado in the south (Fig. 2), but compelling evidence of a drastic reduction of rain forests and their substitution for patches of open vegetation in cooler and drier periods in the central-southern Amazon has been documented for the Pleistocene (Pennington et al., 2000; Rossetti et al., 2004). On the other hand, some studies focusing on animals associated with savannas and other open vegetation formations have reported an increasing species diversification in periods of global cooling, which may be better explained by greater temporal availability of ecological opportunities and subsequent niche diversification (e.g., Delsuc et al., 2004; Gamble et al., 2008). However, further studies are necessary to accurately erect hypotheses correlating phylogenetic splits in *Melanorivulus* with major palaeogeographical events responsible for past climate changes. The present absence of rivulid killifishes and closely related taxa in fossil records prevents the development of accurate hypotheses of diversification timing in *Melanorivulus*. 
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References


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