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

WILSON J. E. M. COSTA, PEDRO F. AMORIM & RAISA C. RIZZIERI

Laboratory of Systematics and Evolution of Teleost Fishes, Institute of Biology, Federal University of Rio de Janeiro, Caixa Postal 68049, CEP 21944-970, Rio de Janeiro, Brasil; wcosta@acd.ufrj.br

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 4° N, and the Uruguay river basin in northern Argentina, about 27° S, and between the Paraguay river basin in eastern Bolivia, about 60° W, and the coastal plains of north-eastern Brazil, about 37° 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,

2007a–c, 2008a–d, 2009, 2010, 2012a–b; Costa & Brasil, 2008; Costa & De Luca, 2010; Costa *et al*., 2014), where data on distribution, habitats and tentative delimitation of species groups were first available. Due to the elaborated colour patterns in males, some species have became popular aquarium fishes, commonly appearing in Aquarium fish websites. However, phylogenetic relationships among included species are still unknown.

Species of *Melanorivulus* are small, reaching between about 25 and 50 mm of total length as adult maximum size (Costa, 2007b, 2010). Like species of the closely related genera *Anablepsoides* and *Atlantirivulus*, species of *Melanorivulus* typically inhabits shallow marginal areas close to streams, about 5 to 30 cm deep (Costa, 1995, 2006). However, differently from species of those two genera that are found in dense rain forests, species of *Melanorivulus* inhabit savanna-like environments (Costa, 2007b, 2011; Oliveira *et al*., 2012). Most species are endemic to the Cerrado savanna of central Brazil and the adjacent Cerrado-Amazon ecotone (Costa, 1995, 2005, 2012a–b). Exceptions are *M. schuncki*, endemic to the lowland savannas of Amapá and Marajó in northern Brazil (Bragança *et al*., 2012); *M. punctatus*, endemic to an area encompassing the northeastern Chaco and the adjacent Pantanal in Bolivia, Brazil, Paraguay and Argentina (Costa, 1995; Schindler & Etzel, 2008); and the clade comprising *M. atlanticus* and *M. decoratus*, occurring in savanna enclaves within the semi-arid Caatinga and coastal plains of northeastern Brazil (Costa, 2010; Costa *et al*., 2015). The objective of this paper is to provide the first molecular phylogeny for *Melanorivulus*, using the resulting phylogenetic tree for searching informative historical patterns of biogeographical distribution.

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. strigatus* (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 Hrbek & 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 S7RPEX1F and S7RPEX2R (Chow & Hazama, 1998). PCR was performed in 15 μl reaction mixtures containing 5 × Green GoTaq 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

Table 1. List of specimens, and respective catalogue numbers (fish collection of the Institute of Biology, Federal University of Rio de Janeiro), and GenBank accession numbers.

Species	Catalog number	GenBank (16s;ND2;S7)		
Kryptolebias brasiliensis	UFRJ 8807	KP290115	------------	KP721754
Atlantirivulus janeiroensis	UFRJ 8793	KP721707	KP721732	------------
Anablepsoides gamae	UFRJ 8841	KP721708	KP721733	KP721755
Laimosemion strigatus	UFRJ 7980	KP721709	KP721734	KP721756
Cynodonichthys tenuis	UFRJ 8103	KP721710	KP721735	KP721757
Melanorivulus violaceus	UFRJ 9412	KP721711	KP721736	KP721758
Melanorivulus pindorama	UFRJ 8274	KP721712	KP721737	KP721759
Melanorivulus planaltinus	UFRJ 9170	KP721713	KP721738	KP721760
Melanorivulus kayopo	UFRJ 9172	KP721714	KP721739	------------
Melanorivulus rutilicaudus	UFRJ 9174	KP721715	KP721740	------------
Melanorivulus litteratus	UFRJ 9177	KP721716	KP721741	KP721761
Melanorivulus salmonicaudus	UFRJ 9283	KP721717	KP721742	KP721762
Melanorivulus crixas	UFRJ 9284	KP721718	KP721743	KP721763
Melanorivulus jalapensis	UFRJ 9338	KP721719	KP721744	KP721764
Melanorivulus schunki	UFRJ 8015	KP721720	KP721745	KP721765
Melanorivulus megaroni	UFRJ 9415	KP721721	KP721775	KP72176
Melanorivulus kayabi	UFRJ 9417	KP721722	KP721746	KP721767
Melanorivulus rubroreticulatus	UFRJ 9557	KP721723	KP721747	KP721768
Melanorivulus karaja	UFRJ 9670	KP721724	KP721748	KP721769
Melanorivulus sp. 1	UFRJ 9674	KP721725	KP721749	KP721770
Melanorivulus sp. 2	UFRJ 9860	KP721726	------------	KP721771
Melanorivulus atlanticus	UFRJ 10003	KP721727	KP721750	------------
Melanorivulus punctatus	UFRJ 10032	KP721728	KP72175	KP721772
Melanorivulus dapazi	UFRJ 9771	KP721729	KP721752	KF311310
Melanorivulus egens	UFRJ 9184	KP721730	KP721753	KP721773
Melanorivulus zygonectes	UFRJ 9684	KP721731	------------	KP721774

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, multitrees 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 likeli-

hood-based DEC model (Ree *et al.*, 2005; Ree & Smith, 2008), using Lagrange (Ree & 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.
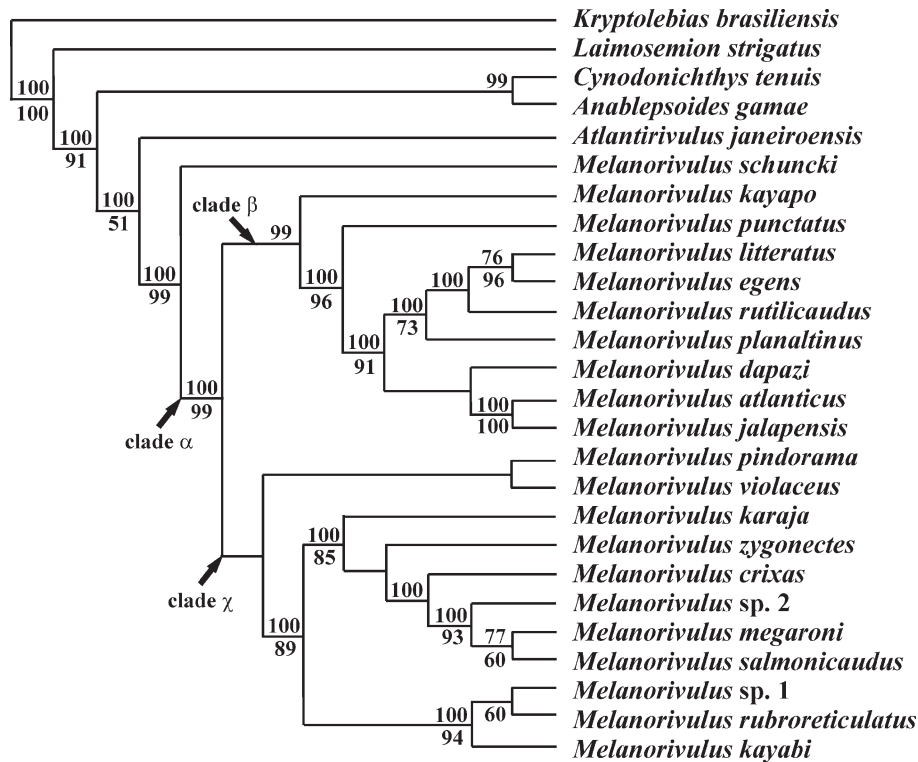
Fig. 1. Phylogenetic relationship tree generated by a Bayesian analysis of molecular data, total of 2,138 bp, comprising segments of the mitochondrial genes 16S and ND2, and the nuclear S7 for 21 species of *Melanorivulus* and five outgroups. Numbers above the node are posterior probabilities of the Bayesian analysis higher than 75%, below are bootstrap percentages higher than 50% of the Maximum Parsimony analysis.
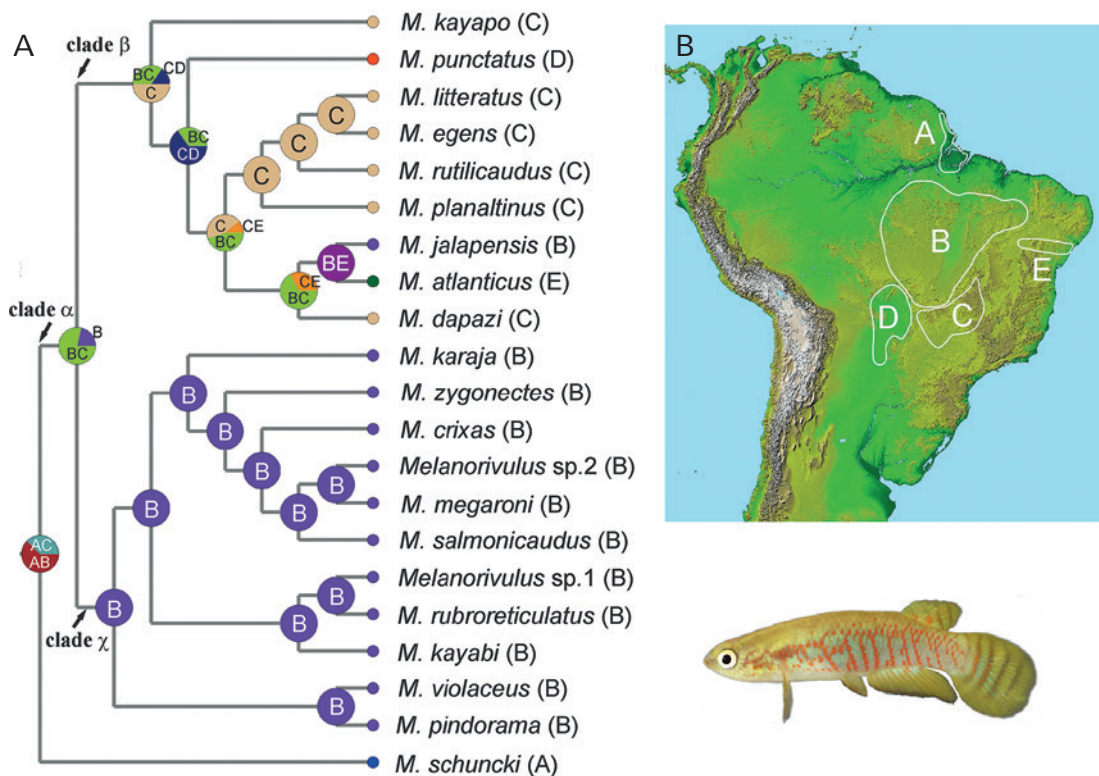

Fig. 2. Biogeographical analysis of the killifish genus *Melanorivulus*: tree generated by the likelihood-based DEC model (A) and areas of endemism used in this study (B). Letters on nodes of the tree (A) are areas of endemism delimited in the map (B) and listed in the text. The specimen illustrated 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 α, then restricted to the Amazon-Cerrado ecotone area. The ancestor of the clade α 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 β in the upland Cerrado, from the ancestor of the clade clade χ in the Amazon-Cerrado ecotone area (Fig. 2).

All descendents of the clade χ were confined to the Amazon-Cerrado ecotone area through successive splits. On the other hand, further sporadic dispersals occurred in lineages of the clade β 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 monophyly 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 α in Fig. 1) is in agreement with data presented by COSTA & DE LUCA (2010), where clade α 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 α contains two well-supported inclusive clades, clade β and clade χ. Among lineages contained into the clade χ, the analysis also strongly corroborates the *Melanorivulus zygonectes* group as delimited by COSTA (2007e), 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 β (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 β. 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 in, 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; ROSSETI *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*.

Acknowledgements

References

Bragança, P.H.N., Amorim, P.F. & Costa, W.J.E.M. (2012): Geographic distribution, habitat, colour pattern variability and synonymy of the Amazon killifish *Melanorivulus schuncki* (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **23**: 51–55 .

Chow, S. & Hazama, K. (1998): Universal PCR primers for S7 ribosomal protein gene introns in fish. – Molecular Ecology, **7**: 1255–1256.

Costa, W.J.E.M. (1991): Redescrição do gênero *Rivulus* (Cyprinodontiformes, Rivulidae), com notas sobre *R. stellifer* e *R. compactus* e a descrição de duas novas espécies do Brazil central. – Revista Brasileira de Biologia, **51**: 327–333.

Costa, W.J.E.M. (1995): Revision of the *Rivulus punctatus* species-complex (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **6**: 207–226.

Costa, W.J.E.M. (2003a): A new species of the genus *Rivulus* Poey, 1860 from the Parnaiba river basin, northeastern Brazil (Teleostei, Cyprinodontiformes, Rivulidae). – Boletim do Museu Nacional, **511**: 1–7.

Costa, W.J.E.M. (2003b): *Rivulus paracatuensis* (Cyprinodontiformes: Rivulidae): a new rivuline species from the Rio São Francisco basin, Brazil. – Aqua Journal of Ichthyology and Aquatic Biology, **7**: 39–44.

Costa, W.J.E.M. (2005): Seven new species of the killifish genus *Rivulus* (Cyprinodontiformes: Rivulidae) from the Paraná, Paraguay and upper Araguaia river basins, central Brazil. – Neotropical Ichthyology, **3**: 69–82.

Costa, W.J.E.M. (2006): *Rivulus kayapo* n. sp. (Teleostei: Cyprinodontiformes: Rivulidae): a new killifish from the serra dos Caiapós, upper rio Araguaia basin, Brazil. – Zootaxa, **1368**: 49–56.

Costa, W.J.E.M. (2007a): Five new species of the aplocheiloid killifish genus *Rivulus*, subgenus *Melanorivulus*, from the middle Araguaia river basin, central Brazil (Teleostei: Cyprinodontiformes: Rivulidae). – Aqua International Journal of Ichthyology, **13**: 55–68.

Costa, W.J.E.M. (2007b): *Rivulus illuminatus*, a new killifish from the serra dos Caiapós, upper rio Paraná basin, Brazil (Teleostei: Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **18**: 193–198.

Costa, W.J.E.M. (2007c): A new species of *Rivulus* from the Claro river drainage, upper Paraná river basin, central Brazil, with redescription of *R. pinima* and *R. vittatus* (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **18**: 313–323.

Costa, W.J.E.M. (2008a): *Rivulus kayabi*, a new killifish from the Tapajós river basin, southern Brazilian Amazon (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **18**: 345–350.

Costa, W.J.E.M. (2008b): *Rivulus bororo* and *R. paresi*, two new killifishes from the upper Paraguay River basin, Brazil (Teleostei: Rivulidae). – Ichthyological Exploration of Freshwaters, **18**: 351–357.

Costa, W.J.E.M. (2008c): *Rivulus formosensis*, a new aplocheiloid killifish from the upper Corrente River drainage, upper Paraná River basin, central Brazil. – Ichthyological Exploration of Freshwaters, **19**: 85–90.

Costa, W.J.E.M. (2008d): *Rivulus giarettai*, a new killifish from the Araguari River drainage, upper Paraná River basin, Brazil (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **19**: 91–95.

Costa, W.J.E.M. (2009): *Rivulus megaroni*, a new killifish from the Xingu River drainage, southern Brazilian Amazon (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **20**: 365–370.

Costa, W.J.E.M. (2010): *Rivulus jalapensis*, a new killifish from the Tocantins River basin, central Brazil (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **21**: 193–198.

Costa, W.J.E.M. (2011): Phylogenetic position and taxonomic status of *Anablepsoides*, *Atlantirivulus*, *Cynodonichthys*, *Laimosemion* and *Melanorivulus* (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **22**: 233–249.

Costa, W.J.E.M. (2012a): *Melanorivulus pindorama*, a new killifish from the Tocantins River drainage, central Brazilian Cerrado (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **23**: 57–61.

Costa, W.J.E.M. (2012b): Two new species of *Melanorivulus* from the Caiapós hill, upper Araguaia river basin, Brazil (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **23**: 211–218.

Costa, W.J.E.M. (2013): A new killifish of the genus *Melanorivulus* from the upper Paraná river basin, Brazil (Teleostei: Cypronodontiformes). – Vertebrate Zoology, **63**: 277–281.

Costa, W.J.E.M. & Amorim, P.F. (2014): Integrative taxonomy and conservation of seasonal killifishes, Xenurolebias (Teleostei: Rivulidae), and the Brazilian Atlantic Forest. – Systematics and Biodiversity, **12**: 350–365.

Costa, W.J.E.M., Amorim, P.F. & Bragança, P.H.N. (2014): A new miniature killifish of the genus *Melanorivulus* (Cyprinodontiformes: Rivulidae) from the Xingu river drainage, Brazilian Amazon. – Vertebrate Zoology, **64**: 193–197.

Costa, W.J.E.M., Bragança, P.H.N. & Ottoni, F.P. (2015): A new miniature killifish of the genus *Melanorivulus* (Cyprinodontiformes: Rivulidae) from the coastal plains of north-eastern Brazil. – Vertebrate Zoology, **65**: 31–35.

Costa, W.J.E.M. & Brasil, G.C. (2008): A new pelvicless killifish species of the genus *Rivulus*, subgenus *Melanorivulus* (Cyprinodontiformes: Rivulidae), from the upper Tocantins River basin, central Brazil. – Copeia, **2008**: 82–85.

Costa, W.J.E.M. & De Luca, A.C. (2010): *Rivulus schuncki*, a new species of the killifish subgenus *Melanorivulus*, from eastern Brazilian Amazon (Cyprinodontiformes: Rivulidae). – Ichthyological Exploration of Freshwaters, **21**: 289–293.

Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. (2012): jModelTest 2: more models, new heuristics and parallel computing". – Nature Methods, **9**: 772.

Delsuc, F., Vizcaíno, S.F. & Douzery, E.J.P. (2004): Influence of Tertiary paleoenvironmental changes on the diversification of South American mammals: a relaxed molecular clock study within xenarthrans. – BMC Evolutionary Biology, **4**: 11.

Dorn, A., Musilová, Z., Platzer, M., Reichwald, K. & Cellerino, A. (2014): The strange case of East African annual fishes: aridification correlates with diversification for a savannah aquatic group? – BMC Evolutionary Biology, **14**: 210.

Gamble, T., Simons, A.M., Colli, G.R. & Vitt, L.J. (2008): Tertiary climate change and the diversification of the Amazonian gecko *Gonatodesi* (Sphaerodactylidae, Squamata). – Molecular Phylogenetics and Evolution, **46**: 269–277.

Goloboff, P.A., Farris, J.S. & Nixon, K.C. (2008): TNT, a free program for phylogenetic analysis. – Cladistics, **24**: 774–786.

Graham, A. (2011): The age and diversification of terrestrial new world ecosystems through Cretaceous and Cenozoic time. – American Journal of Botany, **98**: 336–351.

Hall, T.A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. – Nucleotid Acids Symposium Series, **41**: 95–98.

Hrbek, T. & Larson, A. (1999): The evolution of diapause in the killifish family Rivulidae (Atherinomorpha, Cyprinodontiformes): A molecular phylogenetic and biogeographic perspective. – Evolution, **53**: 1200–1216.

Keeley, J.E. & Rundel, P.W. (2005): Fire and the Miocene expansion of C4 grasslands. – Ecology Letters, **8**: 683–690.

Latorre, C., Quade, J. & McIntosh, W.C. (1997): The expansion of C4 grasses and global change in the late Miocene: stable isotope evidence from the Americas. – Earth and Planetary Science Letters, **146**: 83–96.

Myers, N., Mittermeir, R.A., Mittermeir, C.G., da Fonseca, G.A.B. & Kent, J. (2000): Biodiversity hotspots for conservation priorities. – Nature, **403**: 853–858.

Nylander, J.A.A., Olsson, U., Alström, P. & Sanmartin, I. (2008): Accounting for phylogenetic uncertainty in biogeography: a Bayesian approach to dispersal-vicariance analysis of the trushes (Aves: *Turdus*). – Systematic Biology, **57**: 257–268.

Oliveira, L.E., Facure, K.G. & Giaretta, A.A. (2012): Habitat, density, and spatial distribution of Rivulus giarettai (Actinopterygii, Cyprinodontiformes) in southeastern Brazil. – Environmental Biology of Fishes, **93**: 89–94

Palumbi, S., Martin, A., Romano, S., McMillan, W.O., Stice, L. & Grabowski, G. (2002): The simple foll's guide to PCR volume 2.0. Honolulu: University of Hawaii.

Pennington, R.T., Prado, D.E. & Pendry, C.A. (2000): Neotropical seasonally dry forests and Quaternary vegetation changes. – Journal of Biogeography, **27**: 261–273.

Ree, R.H., Moore, B.R., Webb, C.O. & Donoghue, M.J. (2005): A likelihood framework for inferring the evolution of geographic range on phylogenetic trees. – Evolution, **59**: 2299–2311.

Ree, R.H. & Smith, S.A. (2008): Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. – Systematic Biology, **57**: 4–14.

Ronquist, F. (1997): Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. – Systematic Biology, **46**: 195–203.

Ronquist, F., Teslenko, M., Van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A. & Huelsenbeck, J.P. (2012): MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. – Systematic Biology, **61**: 539–542.

Rossetti, D.F., Toledo, P.M., Moraes-Santos, H.M. & Santos, A.E.A. (2004): Reconstructing habitats in central Amazonia using megafauna, sedimentology, radiocarbon, and isotope analyses. – Quaternary Research, **61**: 289–300.

Schindler, I. & Etzel, V. (2008): Re-description and distribution of *Rivulus punctatus* Boulenger, 1895 (Teleostei: Rivulidae) and its habitats in Paraguay. – Vertebrate Zoology, **58**: 33–43.

Silva, J.M.C. & Bates, J.M. (2002): Biogeographic patterns and conservation in the South American Cerrado: a tropical savanna hotspot. – BioScience, **52**: 225–233.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013): MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. – Molecular Biology and Evolution, **30**: 2725–2729.

Yu, Y., Harris, A.J. & He, X.J. (2010): S-DIVA (Statistical Dispersal-Vicariance Analysis): a tool for inferring biogeographic histories. – Molecular Phylogenetics and Evolution, **56**: 848–850.

Yu, Y., Harris, A.J. & He, X.J. (2011): RASP: reconstruct ancestral state in phylogenies v.2.0.1.0, beta 1, build 110304. Available at: http://mhn.scu.edu.cn/soft/blog/RASP.