

Phylogeny and evolutionary radiation in seasonal rachovine killifishes: biogeographical and taxonomical implications

WILSON J. E. M. COSTA

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(at)acd.ufrj.br

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Abstract

A phylogenetic analysis combining available mitochondrial DNA sequences (total of 3,339 bp) and 161 morphological characters for 22 species of rachovine genera (*Aphyolebias*, *Austrofundulus*, *Gnatholebias*, *Llanolebias*, *Micromoema*, *Moema*, *Neofundulus*, *Pterolebias*, *Rachovia*, *Renova*, *Terranatos*, and *Trigonectes*), and 16 outgroups, supports monophyly of the group containing genera endemic to the Orinoco river basin and adjacent coastal drainages. Results of the present analysis are compared to previous studies. The tree topology indicates that the genera *Moema* and *Rachovia* as presently delimited are paraphyletic; consequently, *Aphyolebias* and *Austrofundulus* are respectively placed in the synonymy of *Moema* and *Rachovia*. This study also indicates that rachovines were in the past geographically restricted to the Amazonas-Paraguay area where diversification in niche exploitation was constrained by competition with sympatric members of older seasonal fish lineages. Rachovines later reached the Orinoco basin and adjacent coastal drainages by dispersal through the Paleo-Amazonas river basin, when major evolutionary radiation taken place.

Key words

Amazon Forest, Amazonas river, Orinoco river, Phylogeny, Biogeography.

Introduction

Independent molecular phylogenies (HRBEK & LARSON, 1999; MURPHY *et al.*, 1999) using mitochondrial DNA (mt-DNA) sequences have corroborated a group of seasonal killifishes, endemic to central and northern parts of South America, including the present genera *Aphyolebias* COSTA, 1998, *Austrofundulus* MYERS, 1932, *Gnatholebias* COSTA, 1998, *Llanolebias* HRBEK & TAPHORN, 2008, *Micromoema* COSTA, 1998, *Moema* COSTA, 1989, *Neofundulus* MYERS, 1924, *Pterolebias* GARMAN, 1895, *Rachovia* MYERS, 1927, *Renova* THOMERSON & TAPHORN, 1995, *Terranatos* TAPHORN & THOMERSON, 1978, and *Trigonectes* MYERS, 1925. This group is herein referred as tribe Rachovini, a name erected in COSTA (1990a) having

nomenclatural priority over other suprageneric names involving the group.

The Rachovini, as herein delimited, was not recognised in former phylogenetic analyses, which used only a few informative morphological characters for species of this group (PARENTI, 1981; COSTA, 1990b, 1998). All species of this group are seasonal fishes, uniquely found in temporary pools formed during the rainy seasons, a unique life style occurring both in American and African aplocheiloid killifishes (*e.g.*, PARENTI, 1981; COSTA, 1998). As already noted by COSTA (1998), some morphological features have independently arisen in different seasonal fish lineages, generating some ambiguity in

phylogenetic analyses. Consequently, some rachovines were formerly considered to be more closely related to members of other seasonal fish lineages than to most rachovines, but subsequent morphological phylogenies directed to part of rachovines (COSTA, 2005) or to another rivulid clade (COSTA, 2011a) have reported characters potentially diagnostic for the tribe. On the other hand, molecular phylogenetic studies have indicated conflicting relationships among rachovine genera (HRBEK & LARSON, 1999; MURPHY *et al.*, 1999). These studies used slightly different set of terminal taxa and distinct gene samples of the mitochondrial genome. As a result of incongruent phylogenies, relationships among some rachovine genera are still poorly understood. The primary objective of the present study is to concatenate mt-DNA available in those studies in a single analysis, combining them to morphological taxa in order of to search the best phylogenetic tree using a total evidence approach, which is the basis for a discussion on evolutionary radiation and biogeography of rachovines.

Material and methods

Terminal taxa were species with mt-DNA sequences available in GenBank, including 22 rachovine in-group taxa, *Aphyolebias peruensis* (MYERS, 1954), *Austrofundulus guajira* HRBEK, TAPHORN & THOMERSON, 2005, *Austrofundulus limnaeus* SCHULTZ, 1949, *Austrofundulus transilis* MYERS, 1932, *Gnatholebias hoignei* (THOMERSON, 1974), *Gnatholebias zonatus* (MYERS, 1935), *Llanolebias stellifer* (THOMERSON & TURNER, 1973), *Micromoema xiphophora* (THOMERSON & TAPHORN, 1992), *Moema piriiana* COSTA, 1989, *Moema staecki* (SEEGERS, 1987), *Neofundulus ornatipinnis* MYERS, 1935, *Neofundulus paraguayensis* (EIGENMANN & KENNEDY, 1903), *Rachovia brevis* (REGAN, 1912), *Pterolebias longipinnis* GARMAN, 1895, *Pterolebias phasianus* COSTA, 1988, *Rachovia maculipinnis* (RADDA, 1964), *Rachovia pyropunctata* TAPHORN & THOMERSON, 1978, *Renova oscari* THOMERSON & TAPHORN, 1995, *Terranatos dolichopterus* (WEITZMAN & WOURMS, 1967), *Trigonectes aplocheiloides* HUBER, 1995, *Trigonectes balzanii* (PERUGIA, 1891), and *Trigonectes rubromarginatus* COSTA, 1990, and 16 out-group taxa, *Anablepsoides stagnatus* (EIGENMANN, 1909), *Atlantirivulus luelingi* (SEEGERS, 1984), *Atlantirivulus santensis* (KÖHLER, 1906), *Cynodonichthys tenuis* (MEEK, 1904), *Cynodonichthys weberi* (HUBER, 1992), *Fundulopanchax gardneri* (BOULENGER, 1911), *Kryptolebias marmoratus* (POEY, 1880), *Laimosemion strigatus* (REGAN, 1912), *Maratecoara formosa* COSTA & BRASIL, 1995, *Maratecoara lacortei* (LAZARA, 1991), *Melanorivulus punctatus* (BOULENGER, 1895), *Nematolebias whitei* (MYERS, 1942), *Pituna poranga* COSTA, 1989, *Papiliolebias bitteri* (COSTA, 1989), *Plesiolebias aruana* (LAZARA,

1991), and *Rivulus cylindraceus* POEY, 1860. A complete list of material examined appears in COSTA (2012). Fragments of the following mt-DNA genes were analysed: cytochrome b (cytb), cytochrome oxidase I (cox1), 12S ribosomal RNA (12S) and 16S ribosomal RNA (16s) first analysed by MURPHY *et al.* (1999), and, transfer RNAs for valine, glutamine, methionine, tryptophan, alanine, asparagines, cysteine, and tyrosine, and NADH dehydrogenase subunit II (ND2), first analysed by HRBEK & LARSON (1999); protocols for extraction, amplification and sequencing, tests for differential saturation, and GenBank accession numbers are available in HRBEK & LARSON (1999) and MURPHY *et al.* (1999). Sequences were aligned using Clustal-W (CHENNA *et al.*, 2003) and subsequently optimized manually; regions of sequences showing high degree of ambiguity for alignment, making homology of sites questionable, were deleted from the final aligned sequence, which had a total of 3,339 bp. Morphological characters were obtained from recent phylogenetic studies involving rachovines (COSTA, 1998, 2005, 2011a), besides new characters derived from the comparative analysis of brain morphology and osteological and myological structures. Morphological characters with a derived character state occurring in a single terminal taxon were excluded. The analysis includes a total of 161 characters, listed in Appendix 1, where character statements are formatted following SERENO (2007); complete descriptions of morphological traits will appear elsewhere (COSTA, in prep.) Osteological preparations (c&s) were made according to TAYLOR & VAN DYKE (1985). Terminology for osteological structures followed COSTA (2006); for frontal squamation, HOEDEMAN (1958); for cephalic neuromast series, COSTA (2001); for brain morphology, EASTMAN & LANOO (2003); and for striated muscles, WINTERBOTTOM (1974). Distribution of character states of morphological characters among terminal taxa appears in Appendix 2. The phylogenetic analysis was carried out with maximum parsimony (MP), performed with TNT 1.1 (GOLOBOFF *et al.*, 2008), 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. Character states of all morphological characters were treated as unordered and genes were analysed giving equal weight to all sites. Molecular and morphological data were analysed together and separately using MP; molecular data alone were also analysed with Maximum Likelihood (ML) methods using MEGA 5 (TAMURA *et al.*, 2011). Independent analyses comprising gene partitions, each encompassing individual genes or group of contiguous gene sequences were performed to assess the phylogenetic content of each genetic marker. Habitat preferences were inferred from field observation made during 16 collecting trips between 1986 and 2013 in all the main areas within the geographical range of rachovines.

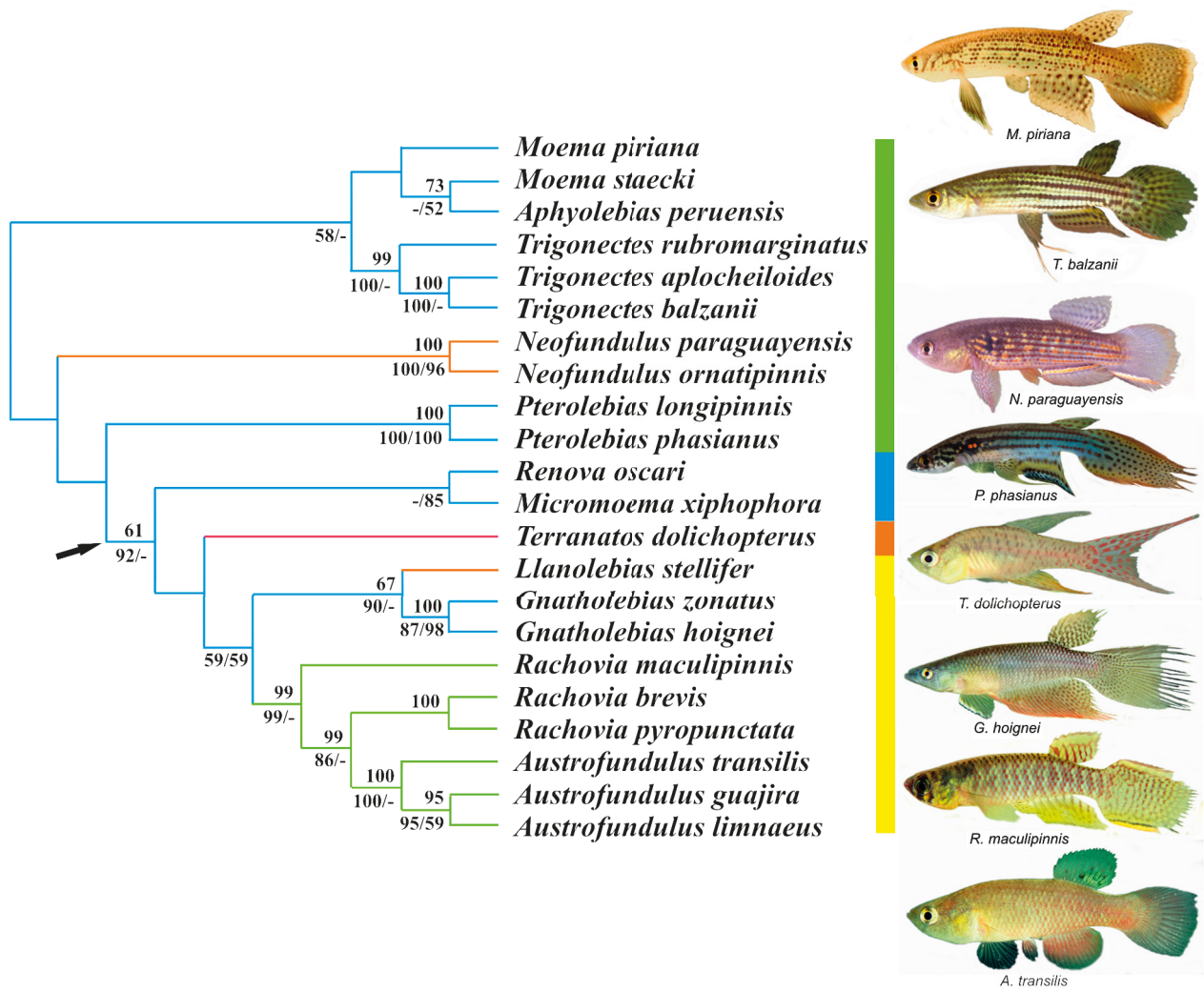


Fig. 1. Maximum Parsimony (MP) tree of relationships among 22 species of rachovine killifishes (outgroups not depicted) combining mitochondrial DNA (mt-DNA) sequences (3,339 bp) and 161 morphological characters (tree length 12,728). Numbers near branch are bootstrap values above 50 %; above values for the combined analysis, below values for the Maximum Likelihood analysis of mt-DNA alone, followed by values for the MP analysis of morphological characters alone. Arrow indicates dispersal event from the Amazonas-Paraguay area to the Orinoco-Maracaibo-Magdalena area. Colours of horizontal lines of the tree indicate evolution of habitat preference: blue, surface dweller; green, mid-water dweller; red, bottom dweller; orange, shallow water dweller. Colour of the vertical bar indicate geographical distribution: green, Amazonas-Paraguay basins; blue, savannahs of the upper Orinoco; yellow, Llanos of Middle and Lower Orinoco and adjacent river basins; orange, taxon occurring in the savannahs of the upper Orinoco, Llanos of Middle and Lower Orinoco and adjacent river basins.

Results and Discussion

Phylogenetic trees. The MP analysis of the complete data set, combining mt-DNA sequences and morphological characters generated a single tree (Fig. 1). Another similar tree was derived from the MP and ML analyses of mt-DNA sequences alone (not depicted), but ML analysis finding higher bootstrap for certain nodes (Fig. 1). The analysis of morphological characters alone generated six most equally parsimonious trees, for which the consensus tree (not depicted) differed from the total evi-

dence tree by *Te. dolichocheilus* appearing as the sister group of a clade including all species of *Rachovia* and *Austrofundulus*; species of *Neofundulus* forming a clade sister to the clade containing all species of *Trigonectes*; the clade comprising *Neofundulus* and *Trigonectes* as the sister group of the clade comprising the other rachovine genera.

All the trees herein generated are highly concordant with that tree obtained by MURPHY *et al.* (1999: Fig. 3). In these phylogenetic trees the genera endemic to the Orinoco river basin appear as a monophyletic group, sister to *Pterolebias*. The combined tree is also similar to that obtained in MURPHY *et al.* (1999: Fig. 3) by

Trigonectes being sister to a clade including *Aphyolebias* and *Moema*, and all the three genera forming a clade sister to the clade comprising *Neofundulus*, *Pterolebias* and Orinoco basin endemics (Fig. 1). This tree topology, however, conflicts with that generated by the analysis of HRBEK & LARSON (1999: fig. 6), in which the genera endemic to the Orinoco river basin do not form a clade, *Renova* being more closely related to *Pterolebias* than to the other Orinoco basin endemics; *Trigonectes* is the sister group of *Neofundulus*; and, *Aphyolebias* and *Moema* are not closely related genera.

The trees herein obtained mainly differ from previous analyses by supporting the monotypic genera *Micromoema* and *Renova* as sister taxa, in contrast to *Micromoema* being the sister group of *Austrofundulus* and *Rachovia* in previous analyses, and *Renova* sister to *Pterolebias* in HRBEK & LARSON (1999) or to a clade comprising the remaining genera endemic to the Orinoco river basin and adjacent coastal plains in MURPHY *et al.* (1999). The sister group relationship between *Micromoema* and *Renova* is here independently supported by molecular and morphological analyses. This clade is morphologically supported by the relative position of the first two proximal radials of the dorsal fin, which strongly diverge ventrally (character state 78.1), and the presence of a yellowish white or pale yellow stripe with a broad dark red upper margin on the ventral portion of the caudal fin in males (character 145.1). Moreover, morphological characters neither support sister group relationships between *Micromoema* and the clade *Austrofundulus* plus *Rachovia*, neither between *Renova* and *Pterolebias*. It is interesting to note that when analyzing genes separately, *Renova* and *Micromoema* appear as sister groups both in the *cox1* and 16S analyses; both *Micromoema* and *Renova* are members of a clade also containing *Terranatos* in the ND2 analysis; and both *Micromoema* and *Renova* are not part of a clade including members of the genera *Austrofundulus*, *Gnatholebias*, *Rachovia* and *Terranatos* in the *cytb* analysis (*Llanolebias* not included). Contrastingly, a clade comprising only *Pt. longipinnis* and *Re. oscar* is highly supported in the 12S analysis (bootstrap 100%), which also indicates *Pt. phasianus* as the sister group of a major clade, including *Pt. longipinnis*, *Re. oscar*, and species of *Austrofundulus*, *Gnatholebias*, *Llanolebias*, *Micromoema*, *Rachovia*, and *Terranatos*, thus greatly differing from all other analyses in which both species of *Pterolebias* form a well corroborated monophyletic group, corroborating morphology. The origin of this conflicting phylogenetic signal is not herein investigated.

On the other hand, the sister group relationships between *Gnatholebias* and *Llanolebias* first proposed by HRBEK & LARSON (1999), is here also supported by morphological characters not previously reported. In both genera there is a prominent projection on the lateral border of the middle portion of the parasphenoid (character state 64.1); the middle portion of the coracoid has a peculiar circular groove and the adjacent scapula is dorsally projected (character state 88.1); the tip of the pelvic fin

in males bears a single filamentous ray (character state 105.1); the pelvic fins are medially fused (character state 106.1); and there is a white stripe with ventral narrow black margin on the ventral portion of the pectoral fin in males (character state 148.1). Thus the clade comprising *Gnatholebias* and *Llanolebias* is here supported by both molecular and morphological data, thus contrasting with former morphological studies corroborating sister group relationships between *Gnatholebias* and *Pterolebias* (COSTA, 1998, 2005). Species of *Gnatholebias* and *Pterolebias* share some morphological features, such as large caudal fin with posterior filaments, anal fin broad and with scales on its basal portion, and long pelvic fin with numerous rays and united basis. However, this study indicates that some of these conditions are not homologous by having some important structural differences, thus being independently derived, whereas others are not unique for these genera or are better explained by constituting homoplastic conditions (see list of characters and their distribution among taxa in Appendices 1–2).

This study consistently indicates that two genera, *Rachovia* and *Moema*, are paraphyletic. *Rachovia* was first described by MYERS (1927) to include *Rivulus brev* REGAN, 1912. Subsequently, MYERS (1932) described *Austrofundulus transilis* as a new genus and species, considered closely related to *Neofundulus*, *Cynolebias* STEINDACHNER, 1877, and *Cynopoeilus* REGAN, 1912, without comparing the new genus with *Rachovia*. WEITZMAN & WOURMS (1967) demonstrated that *Rachovia* and *Austrofundulus* were not distinguishable by current diagnostic characters, but TAPHORN & THOMERSON (1978) considered both genera as valid, tentatively distinguishing them by modal values of broadly overlapped morphometric and meristic characters. COSTA (1990a–b) noted that available diagnostic characters were not useful to distinguish those genera, considering *Austrofundulus* as a junior synonym of *Rachovia*, but this synonymy was not broadly accepted (COSTA, 1998). More recently, HRBEK *et al.* (2007) revised *Austrofundulus*, considering it as a valid genus by forming a morphologically distinct unit, and again, *Austrofundulus* and *Rachovia* were distinguished by modal values of broadly overlapped characters. In addition, the two phylogenetic trees presented in that paper were incongruent with this proposal, with *Rachovia* appearing as paraphyletic in the maximum likelihood tree (HRBEK *et al.*, 2007: Fig. 2) and both *Austrofundulus* and *Rachovia* as paraphyletic in the maximum parsimony tree (HRBEK *et al.*, 2007: fig. 3). Since those genera cannot be objectively diagnosed neither by morphological nor molecular data, *Austrofundulus* should be considered as a synonym of *Rachovia*, which has chronological priority. *Rachovia*, herein delimited as a more inclusive taxon, is strongly supported (bootstrap value 99 %; Fig. 1) and unambiguously diagnosed by the presence of a foramen on the hyomandibula, near the limit with the metapterygoid (COSTA, 2011a: ch. 26; character state 30.1 of the present analysis); an adipose ridge on the pre-dorsal region in old males (PARENTI, 1981; character state 101.1); and a distinctive projection in the posterolateral

region of the telencephalon, which is medially coalesced (character state 154.1). *Rachovia* comprises 11 species: *Ra. brevis* (REGAN, 1912), *Ra. guajira* (HRBEK, TAPHORN & THOMERSON, 2005), *Ra. hummelincki* DE BEAUFORT, 1940, *Ra. leohoignei* (HRBEK, TAPHORN & THOMERSON, 2005), *Ra. leoni* (HRBEK, TAPHORN & THOMERSON, 2005), *Ra. limnaeus* (SCHULTZ, 1949), *Ra. maculipinnis* (RADDA, 1964), *Ra. myersi* (DAHL, 1958), *Ra. pyropunctata* TAPHORN & THOMERSON, 1978, *Ra. rupununi* (HRBEK, TAPHORN & THOMERSON, 2005), and *Ra. transilis* (MYERS, 1932).

Moema was first proposed as a genus to include *M. piriana* COSTA, 1989, the type species, and *M. portugali* COSTA, 1989, both endemic to the Brazilian Amazon, then diagnosed by a pointed pectoral fin and dentary with a ventro-proximal process (COSTA, 1989). *Aphyolebias* was described nine years later (COSTA, 1998), diagnosed by the metapterygoid being dorsally constricted, presence of posterior dorsal and ventral extensions on the caudal fin in males, a dark brown to black humeral spot, and a narrow black margin on the ventral margin of the pectoral fin in males, then comprising *A. peruensis* (MYERS, 1952), the type species, and other three species endemic to the western Amazon river basin in Peru and Bolivia. As a result of the quick increasing of known species, nine in *Moema* and eight in *Aphyolebias*, in more recent years the known geographical range of both genera was greatly overlapped and generic diagnostic features were not applicable to some recently described taxa, making generic limits unclear. The present study shows that the clade containing species of *Moema* and *Aphyolebias* is morphologically diagnosable by the unique opercle shape, which has its ventro-posterior part expanded and the its dorsal portion distinctively longer than the ventral portion (character state 33.1), and by a pectoral fin with distal portion narrow, forming a long pointed tip. However, each genus cannot be properly distinguished, besides the analysis supporting *Mo. staecki* as being more closely related to *A. peruensis* than to *Mo. piriana*. Consequently, *Aphyolebias* is here considered as a synonym of *Moema*, which then comprises 17 valid species: *M. apurinan* COSTA, 2004, *M. boticarioi* (COSTA, 2004), *M. claudiae* (COSTA, 2003), *M. hellneri* COSTA, 2003, *M. heterostigma* COSTA, 2003, *M. manuensis* (COSTA, 2003), *M. nudifrontata* COSTA, 2003, *M. obliquus* (COSTA, SARMIENTO & BARRERA, 1996), *M. ortegai* COSTA, 2003, *M. pepotei* COSTA, 1992, *M. peruensis* (MYERS, 1954), *M. piriana* COSTA, 1989, *M. portugali* COSTA, 1989, *M. rubrocaudatus* (SEEGERS, 1984), *M. schleseri* (COSTA, 2003), *M. staecki* (SEEGERS, 1987), and *M. wischmanni* (SEEGERS, 1983). Another nominal species often appearing as a valid species in aquarium fish internet sites is *Moema quiii* HUBER, 2003, which was first published in the December 2013 edition of the Freshwater and Marine Aquarium and is a synonym of *M. ortegai*, first published on 24 November 2003.

Biogeography. HRBEK & LARSON (1999) concluded that their phylogenetic hypothesis of Neotropical killifishes

was highly concordant with the geological history of South America on the basis of a schematic distribution of taxa among geological formations and main South American biogeographical provinces, which were plotted directly on a phylogenetic tree (HRBEK & LARSON, 1999: Fig.7). According to this scheme, plesiolebiasines (present genera *Maratecoara*, *Papiliolebias*, *Plesiolebias*, and *Pituna*) are endemic to the old geological formation of the Brazilian Shield, whereas rachovines are endemic to new geological formations, with the genera *Trigonectes* and *Neofundulus* being endemic to the Chaco and Pantanal, the species *Mo. peruensis*, *Mo. staecki*, *Pt. longipinnis*, *Pt. phasianus*, and *Re. oscari* to the Amazonian savannah, and *Ra. limnaeus*, *Ra. maculipinnis*, *Mi. xiphophora*, *G. zonatus*, *L. stellifer*, and *Te. dolichopterus* to the Orinoco Llanos. However, this scheme is equivocal in many aspects, not serving to illustrate South American killifish biogeographical patterns. Equivocal generalizations include: plesiolebiasines do not occur in core areas of the Brazilian Shield, being endemic to an area comprising southern Amazonian river tributaries, and the Chaco and Pantanal areas of the Paraguay river basin (e.g., COSTA, 1998); the genera *Trigonectes* and *Neofundulus*, besides occurring in the Chaco and Pantanal, are also present in southern Amazonian river tributaries (COSTA, 1988a, 1990c), having geographical distribution nearly equal to plesiolebiasines; species of *Pterolebias* are not restricted to the Amazon, but also have geographical distribution similar to plesiolebiasines, *Trigonectes* and *Neofundulus* (COSTA, 1988b, 2005); species of the genus *Moema* are never found in savannahs, they are always found in dense rain forests of the Amazonas river basin (e.g. COSTA, 1989), besides one species of *Moema* being endemic to the Pantanal area of the Paraguay river basin (COSTA, 2003); *Mi. xiphophora* does not occur in the Orinoco Llanos, but both it and *Re. oscari* are endemic to a savannah area in the upper section of the Orinoco river basin (THOMERSON & TAPHORN, 1992, 1995); *Te. dolichopterus* is not restricted to the Orinoco Llanos, being also recorded to the upper Orinoco savannah (THOMERSON & TAPHORN, 1995); *Ra. maculipinnis* and *G. zonatus* are not endemic to the Orinoco Llanos, since they have been also reported to occur in the Unare river basin, a coastal drainage of Venezuela (THOMERSON *et al.*, 1990); and *Ra. limnaeus* does not occur in the Orinoco Llanos, but is endemic to Maracaibo lake basin (TAPHORN & THOMERSON, 1978; HRBEK *et al.*, 2005). These equivocal geographical assignments made inaccurate the discussion on biogeography of Neotropical killifishes provided by HRBEK & LARSON (1999). MURPHY *et al.* (1999) did not provide biogeographical explanations about the distribution patterns of the clades supported in their analysis.

The geographical distribution of the rachovine clade, comprising the Amazonas, Paraguay, Orinoco and Magdalena river basins, and smaller adjacent river basins of northern South America, is coincident with the geographical limits of the Paleo-Amazonas river basin (COSTA, 2013). This paleogeographical river basin was

fully developed at the late Oligocene, about 25 Ma, as a result of the formation of the western Amazonian Corridor originated by the uplift of northern Andes, but was broken by the uplift of the Vaupes Arch definitively separating the Orinoco and Amazonas river basins, and by the uplift of the Eastern Cordillera, splitting the Magdalena and Llanos in separate basins, during the Late Miocene, about 11 Ma (HOORN *et al.*, 1995, 2010). However, the occurrence of three successive basal rachovine lineages endemic to the area involving the Amazonas-Paraguay area parsimoniously indicates that rachovines were formerly geographically restricted to this area, later dispersing to northern areas (*i.e.* Orinoco, Magdalena, and smaller adjacent river basins; Fig. 1). This geo-dispersal event would be favoured by the main flow course of the Paleo-Amazonas river, running from central-western South America to north, with its mouth situated between the present day Maracaibo lake and the lower Orinoco river (HOORN *et al.*, 2010).

Evolutionary radiation. Former phylogenetic studies had indicated that the annual life style in the subfamilies Cynolebiasinae and Rivulinae were independent evolutionary events (COSTA, 1998), and most recent phylogenetic analyses support annualism in the Rivulinae as independently acquired in rachovines and plesiolebiasines, with annualism in plesiolebiasines being older than annualism in rachovines, and the origin of annualism in rachovines corresponding to the most recent event among the three annual South American killifish lineages (*e.g.*, COSTA, 2011b; Fig. 1). Rachovines inhabit temporary pools in a broad array of tropical habitats, including savannahs, rain forests and semi-arid areas, often exhibiting strong diversification in body form associated to specific habitat preferences. Typical surface dwellers are slender bodied with dorsal profile nearly straight, whereas mid-water dwellers often are deep-bodied with convex dorsal profile; in addition, surface dwellers have the dorsal fin situated posteriorly on the body and simple cephalic latero-sensory system, whereas mid-water dwellers often are deep-bodied, with elaborated cephalic latero-sensory system, corresponding to a general trend recorded both in South American and African seasonal killifishes (COSTA, 1998).

Most rachovine genera (*Gnatholebias*, *Pterolebias*, *Moema*, *Renova*, *Trigonectes*) comprise species that live in the central portions of the pools, close to the water surface (*e.g.*, THOMERSON, 1974; COSTA, 2005; pers. observ.). Interestingly, this habitat preference, which is parsimoniously interpreted as the primitive condition of habitat preference for rachovines (Fig. 1), is never shared by representatives of other seasonal killifish groups (cynolebiasines and plesiolebiasines) that are almost always found sympatric to those rachovines in biotopes of the Amazonas and Paraguay river basins, which is the area here considered to be the centre of origin of rachovines (see above). Species of the genera *Micromoema*, *Neofundulus*, and *Llanolebias* are also commonly found close the water surface, but in very shallow parts of pools

with not more than 10 cm deep, often in isolated shallow small pools (THOMERSON & TURNER, 1973; THOMERSON & TAPHORN, 1992, 1995; pers. observ.), making a clear shift from the plesiomorphic rachovine condition for habitat preference. This specialized habitat preference found in species of *Micromoema*, *Neofundulus*, and *Llanolebias* is parsimoniously interpreted as occurring independently in each genus (Fig. 1). Interesting to note that in the Amazonas and Paraguay river basins, species of *Neofundulus* are never found sympatric to the plesiolebiasine genus *Pituna*, which exhibits the same habitat preference (COSTA, 2011a).

Representatives of the two remaining rachovine genera, *Rachovia* and *Terranatos*, are mid-water dwellers, but whereas species of *Rachovia* are usually found swimming in all parts of the pool, usually at middle portions of the water column, *Te. dolichopterus* is found close to the bottom, in areas protected by dense vegetation (NICO & THOMERSON, 1989; pers. observ.). Acquisition of mid-water dweller habits is parsimoniously interpreted as representing two independent evolutionary events among rachovines, one for *Rachovia*, and another for *Terranatos* (Fig. 1). Species of *Rachovia*, all endemic to the middle Orinoco basin and adjacent drainages, occupy a similar niche as that in species of the plesiolebiasine genus *Plesiolebias* and the cynolebiasine genus *Spectrolebias* in the Amazonas and Paraguay river basins (*e.g.*, COSTA, 2011a). *Terranatos dolichopterus*, endemic to the Orinoco river basin, occupy the same niche as that in species of the genus *Maratecoara* in the Amazonas river basin (COSTA, 2011a), besides species of both genera exhibiting a remarkably similar general morphology of body and fins, including long dorsal and anal fins in males, and a deep body with convex dorsal profile contrasting with an almost straight ventral profile.

This study indicates that major adaptations for divergent habitats among rachovines arose after the group reached the Orinoco river basin and adjacent northern areas (Fig. 1). Specialised morphology, such as the deep body, anteriorly positioned dorsal fin and elaborated latero-sensory system, and most divergent habitat preferences, like mid-water or bottom dweller habits, are concentrated in the Llanos and the adjacent coastal plains of northern South America (Fig. 1). The data on rachovine ecology and phylogeny combined to data on ecology of sympatric older annual fish lineages suggest that evolution through a constant slender morphology associated with surface dweller habits among lineages endemic to the Amazon and Paraguay basins was constrained by competition with mid-water plesiolebiasines and cynolebiasines inhabiting the same temporary pools, with rachovines reaching major diversification in niche exploitation only after reaching the savannahs of the middle Orinoco and adjacent coastal basins, where rachovines are the only rivulids inhabiting the seasonal pools.

The phylogenetic analysis also supports three clades comprising sympatric sister species, indicating that sympatric speciation may be common evolutionary events among annual killifishes. However, in all the three cases,

species of the same sister group pair exhibit some degree of ecological disjunction. *Pterolebias longipinnis* and *P. phasianus*, the only valid species of *Pterolebias*, are commonly found sympatric in temporary pools along the huge flooded area of the Brazilian Pantanal, but the former species is usually found in sunny places, whereas *P. phasianus* inhabits shaded areas (COSTA, 2005). The sister monotypic genera *Micromoema* and *Renova* are endemic to the savannah of the upper Orinoco river basin, where *Mi. xiphophora* is found in the shallowest parts of the pool margin and *Re. oscaris* in the deeper central part of the pool (THOMERSON & TAPHORN, 1992, 1995). The genus *Gnatholebias* includes only two species, *G. hoignei* and *G. zonatus*, that are sometimes found sympatric in temporary pools of the Orinoco Llanos (PIÑERO *et al.*, 1991; person. observation). Both species are surface dwellers occurring in the central parts of the pool (NICO & THOMERSON, 1989; person. observation), but the former is found in zones shaded by trees and aquatic vegetation, whereas the latter is found in sunny places (THOMERSON, 1974; NICO & THOMERSON, 1989; person. observation), besides *G. hoignei* preferring to feed on insects, whereas *G. zonatus* mainly feeds on planktonic organisms (PIÑERO *ET AL.*, 1991). In addition, *Llanolebias stellifer*, the only species of its genus, is the sister group of *Gnatholebias*, besides being sympatric to species of the latter genus. *Llanolebias stellifer* is found in extremely shallow, isolated forest pools (THOMERSON & TURNER, 1973) and studies on stomach contents indicated an omnivorous diet with greater occurrence of ostracods (NICO & THOMERSON, 1989).

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Appendix 1.

List of morphological characters.

Osteology

— *Jaws, jaw suspensorium and opercular series*

- [1] Autopalatine, ventral extremity, extent relative to dorsal portion of quadrate: (0) contacting; (1) not contacting (Costa, 1998: ch.23).
- [2] Autopalatine, anterior margin, shape: (0) slight concavity; (1) distinctive notch (modified from Costa, 1998: ch.30).
- [3] Autopalatine, dorsal portion, prominent bony flange: (0) absent; (1) present (Costa, 1998: ch.24).
- [4] Autopalatine, posterior margin, bony flange: (0) absent; (1) present (Costa, 2011a: ch.4).
- [5] Autopalatine, dorsomedial process: (0) absent; (1) present (modified from Costa, 1998: ch.28).
- [6] Autopalatine, dorsomedial process, extent relative to anterior margin of autopalatine: (0) not surpassing; (1) surpassing (Costa, 2011a: ch.6).
- [7] Entopterygoid, anterior portion, extent relative to autopalatine: (0) overlapping; (1) not overlapping (Costa, 1998: ch.35).
- [8] Entopterygoid, posterior portion, extent relative to metapterygoid: (0) contacting; (1) not contacting (modified from Costa, 1990b).
- [9] Metapterygoid, general shape: (0) about rectangular, dorsal and ventral portions wide, approximately equal in width; (1) triangular, dorsal portion strongly constricted, ventral portion wide; (?) metapterygoid rudimentary (Costa, 1998: ch. 37).
- [10] Quadrate, anterior border, angle formed with posterior process: (0) 130–160; (1) 90–110 (modified from Costa, 1990b, 1998: ch39).
- [11] Quadrate, posterior process, length relative to quadrate length without process: (0) equal or shorter; (1) longer (modified from Costa, 1995).
- [12] Jaws, length relative to jaw suspensorium: (0) longer; (1) shorter (modified from Costa, 1989 and Costa, 1998: ch.1).
- [13] Maxilla, main axis, torsion: (0) not twisted; (1) slightly twisted (modified from Parenti, 1981).
- [14] Maxilla, ventral process, anterior expansion: (0) absent; (1) present (modified from Parenti, 1981).
- [15] Maxilla, subdistal portion, anterior border, projection supporting ligament: (0) present; (1) absent (Costa, 2011b: ch.12).
- [16] Maxilla, distal part, abrupt widening: (0) absent; (1) present (Costa, 2011b: ch.13).
- [17] Maxilla, dorsal process, distal expansion: (0) absent; (1) present (Costa, 2011a: ch.12).
- [18] Premaxilla, ascending process, shape: (0) sub-triangular; (1) sub-rectangular (modified from Costa, 2005: ch.11).
- [19] Premaxilla (in males), teeth, outer row, shape and orientation: (0) slightly curved, directed inside mouth; (1) strongly curved, directed laterally, outside mouth (modified from Costa, 1998: ch.22).
- [20] Rostral cartilage, shape: (0) approximately triangular; (1) approximately circular; (2) approximately hexagonal to rectangular, always longer than wide (modified from Costa, 1998: ch.11).
- [21] Rostral cartilage, posterior region, abrupt narrowing: (0) absent; (1) present (Costa, 2004: ch.4).
- [22] Dentary (in adult males), teeth, outer row, shape and orientation: (0) slightly curved, tip posteriorly directed; (1) strongly curved, tip postero-laterally directed; (2) slightly curved, tip anteriorly directed (modified from Costa, 1998: ch.21–22).
- [23] Dentary, coronoid process, shape: (0) robust; (1) slender (Costa, 1998: ch.13).
- [24] Dentary, posteroventral process, shape: (0) pointed, narrow; (1) truncate, broad (modified from Costa, 1998: ch.14).
- [25] Angulo-articular, ventral process, shape: (0) wide, about rectangular; (1) narrow, about triangular (modified from Costa, 1998: ch.17).
- [26] Angulo-articular, ventral process, development: (0) well-developed; (1) rudimentary (modified from Costa, 1998: ch.16).
- [27] Angulo-articular, ventral process, axis orientation: (0) straight; (1) curved (Costa, 1998: ch.20).
- [28] Angulo-articular, ventral process, tip shape, direction and extent relative to dentary: (0) anteroventrally directed, not reaching dentary, slightly pointed; (1) anteroventrally directed, terminating in long sharp point, reaching dentary; (2) anteriorly directed, pointed, surpassing or in close proximity to vertical through posterior limit of dentary (modified from Costa, 2011b: ch.24).
- [29] Symplectic, dorsal margin, expansion towards entopterygoid: (0) absent; (1) present; (?) rudimentary expansion, often absent (Costa, 2011: ch.24).
- [30] Hyomandibula, area adjacent to metapterygoid, foramen: (0) absent; (1) present (Costa, 2011b: ch.26).
- [31] Preopercle, shape: (0) robust, L-shaped, with a well developed anteromedian rim; (1) thin, C-shaped, with a reduced anteromedian rim (Costa, 1990b).
- [32] Preopercle, dorsal arm, shape: (0) blunt; (1) pointed (Costa, 1990b).
- [33] Preopercle, dorsal arm, length relative to anteroventral arm: (0) approximately equal or shorter; (1) distinctively longer (modified from Costa, 1989).
- [34] Opercle, ventro-posterior part, expansion: (0) absent; (1) present (Costa, 1998: ch.48). Remarks: all taxa exhibiting the apomorphic condition also have the dorsal portion of the opercle distinctively longer than the ventral portion (vs. about equal in length in the remaining taxa), thus considered as a dependent condition.

— *Hyoid and branchial arches*

- [35] Interhyal, ossification: (0) ossified; (1) cartilaginous (Parenti, 1981).
- [36] Interhyal, development: (0) well-developed; (1) rudimentary or absent (Costa, 2005: ch.28).
- [37] Basihyal, length relative to longitudinal length of three basibranchials: (0) shorter; (1) longer (modified from Costa, 1998: ch.54).
- [38] Basihyal, shape, anterior width relative to length: (0) triangular, more than 50 %; (1) subtriangular or rectangular, less than 50 % (modified from Costa, 1990b).
- [39] Basihyal, cartilaginous portion, length relative to total basihyal length: (0) about half; (1) about one third; (2) about one tenth (modified from Parenti, 1981 and Costa, 1998: ch.56).
- [40] Urohyal, dorsal process, shape: (0) unbranched; (1) branched; (-) process absent (Costa, 2011a: ch.34).
- [41] Urohyal, anterior process, expansion: (0) absent; (1) present (Costa, 1998: ch.52).
- [42] First gill arch, ventral gill-rakers, number: (0) 7–12; (1) 13–15 (modified from Costa, 1998: ch.74).
- [43] First epibranchial, width of proximal tip relative to distal tip: (0) about 5–6 times; (1) about 1–3 times (Costa, 2011a: ch.36).
- [44] First epibranchial, flexion: (0) unbowed; (1) bowed (modified from Costa, 1995).
- [45] First epibranchial, torsion: (0) untwisted; (1) twisted (Costa, 2011: ch.38).
- [46] Second epibranchial, subdistal process: (0) present; (1) absent (Costa, 2004: ch.18).
- [47] Third epibranchial, uncinat process, angle formed with distal process: (0) about 90°; (1) about 45–60° (Costa, 2011a: ch.41).
- [48] Third epibranchial, uncinat process, development: (0) well-developed; (1) rudimentary (Costa, 1998: ch.62).
- [49] Interarcual cartilage, length relative to first epibranchial length: (0) long, about one third or more; (1) minute, less than one fifth (modified from Costa, 1998: ch.65).
- [50] Second pharyngobranchial, distal condyle, orientation: (0) distally; (1) laterodistally oriented (Costa, 2011a: ch.44).
- [51] Second pharyngobranchial, distal border, notch close to condyle: (0) absent; (1) present (Costa, 2011b: ch.43).
- [52] Second pharyngobranchial, medial border, expansion: (0) not expanded; (1) expanded (modified from Costa, 2005: ch.44).
- [53] Second pharyngobranchial, medial flap: (0) absent; (1) present (Costa, 2011a: ch.46).
- [54] Second pharyngobranchial, teeth: (0) present; (1) absent; (?) present or absent (Costa, 1998: ch.67).
- [55] Third pharyngobranchial, middle teeth, shape: (0) narrow and pointed; (1) broad, approximately molariform (Costa, 1990b).
- [56] First hypobranchial, medial edge: (0) unbranched; (1) branched (modified from Costa, 1990b).
- [57] First hypobranchial, lateral edge, articular facet extension relative to apical cartilage of first ceratobranchial: (0) restricted to articulation area: (1) extended (Costa, 2004: ch.23).
- [58] Second hypobranchial, medial edge: (0) unbranched or with slight division; (1) deeply branched (Costa, 2011b: ch.49).
- [59] Fourth ceratobranchial, ventral process, orientation: (0) ventral; (1) posterior (Costa, 2011a: ch.51).
- [60] Fifth ceratobranchial, anterior region, shape: (0) about straight to slightly curved; (1) bent laterally (modified from Costa, 2005: ch.38).

— *Neurocranium and circum-orbital series*

- [61] Lateral ethmoid, medial extent expressed by cartilaginous space width between medial margin of bone and vomer and parasphenoid relative to anterior parasphenoid width: (0) wider; (1) narrower (modified from Parenti, 1981).
- [62] Lateral ethmoid, anterior retrorse process: (0) rudimentary; (1) well-developed (Costa, 1990b).
- [63] Lateral ethmoid, anterior retrorse process, extent relative to lateral ethmoid main axis: (0) not reaching; (1) contacting, often fused (Costa, 1998: ch.82).
- [64] Parasphenoid, lateral profile in ventral view, middle portion: (0) without projections or with slightly projection; (1) with prominent projection (new character).
- [65] Pterosphenoid, process towards parasphenoid, often contacting it: (0) absent; (1) present (modified from Costa, 1998: ch.83).
- [66] Frontal, lateral border, shape and ossification: (0) approximately straight, well ossified; (1) concave, poorly ossified (modified from Costa, 1998: ch.83).
- [67] Parasphenoid, posterior portion, abrupt narrowing: (0) absent; (1) present (Costa, 2005: ch.7).
- [68] Supraoccipital, posterior processes, medial fusion: (0) not fused; (1) fused (Costa, 2011a: ch.59).
- [69] Dermosphenotic, development: (0) well developed; (1) minute (modified from Parenti, 1981).
- [70] Vomerine teeth: (0) present; (1) absent (Costa, 1998: ch.76).

— *Vertebrae and unpaired fin skeleton*

- [71] Vertebrae, modal number: (0) 26–32; (1) 34–38 (modified from Costa, 1990b).
- [72] First vertebra, anteriorly directed paired process: (0) absent; (1) present (modified from Costa, 1990b).
- [73] First vertebra, neural process, length relative to neural caudal depth: (0) half or less; (1) twice or more (Costa 1990b).
- [74] Caudal vertebrae, neural prezygapophyses, length relative to vertebral centra: (0) short, one fourth

- or less; (1) three fourths or more (modified from Costa, 1990b).
- [75] Hypurals, middle gap: (0) present; (1) absent; (?) plates often partly or completely fused, gap rudimentary (modified from Costa, 1998: ch.103).
- [76] Preural vertebra 2, hemal spine, width relative to hemal spines of preural vertebrae 4 and 5: (0) distinctively wider; (1) slightly wider (modified from Costa, 2004).
- [77] Caudal fin, middle rays, branching: (0) double; (1) single (new character).
- [78] Dorsal fin, first two proximal radials, relative position: (0) parallel or slightly diverging ventrally; (1) strongly diverging ventrally (new character).
- [79] Dorsal fin, origin position relative to distance between snout and caudal-fin base: (0) nearer posterior quarter than midlength; (1) nearer midlength than posterior quarter (modified from Costa, 1990b).
- [80] Anal fin, anterior proximal radials, shape: (0) narrow, long and straight; (1): short and oval; (2) with broad middle portion and slightly narrow extremities; (3) curved, middle and ventral portions broad, dorsal portion long and narrow (modified from Costa, 1990b).
- [81] Anal fin, first two proximal radials, fusion: (0) not fused; (1) fused (Costa, 2011a: ch.68).
- [82] Anal-fin base in males, length relative to standard length: (0) less than half; (1) about half (modified from Costa, 1990b).
- *Shoulder and pelvic girdle*
- [83] Posttemporal, ventral process: (0) present; (1) absent Costa, 1998: ch.132).
- [84] Posttemporal, prominent lateral keel: (0) absent; (1) present (Costa, 2005: ch.51).
- [85] Supracleithrum, length relative to posttemporal half length: (0) distinctively shorter; (1) distinctively longer (modified from Costa, 1990b).
- [86] First postcleithrum: (0) present; (1) absent (Parenti, 1981).
- [87] Cleithrum, dorsal portion, vertical length relative to vertical length of scapula: (0) about equal; (1) longer; (2) shorter (modified from Costa, 1998).
- [88] Cleithrum, posterior flange: (0) present; (1) absent (Costa, 1998: ch.135).
- [89] Coracoid, middle portion, circular groove: (0) absent; (1) present (new character). Remarks: uniquely in taxa exhibiting the apomorphic condition, the dorsal portion of the scapula is dorsally projected, which is considered as a dependent condition by both characters involving the same structure (shoulder girdle).
- [90] Fourth pectoral radial, ventral expansion: (0) absent; (1) present (modified from Costa, 1998: ch.147).
- [91] Pelvic bone, thickness and processes, development: (0) thick, processes well-developed; (1) thin, ischial and lateral processes rudimentary or absent (Costa, 2011a: ch.78).

- [92] Pelvic bones, relative medial position: (0) separated by interspace; (1) overlapped (Costa, 2011a: ch.79). Remarks: greatly variable in rivulines, difficult to establish limits between overlapped and non-overlapped, although the derived condition is unequivocally found in all plesiolebiasines and *Pterolebias*.
- [93] Pelvic-fin rays, modal number: (0) six; (1) seven; (2) eight; (3) nine (modified from Parenti, 1981; Costa, 1990b).

External morphology

— *Head*

- [94] Membrane between preopercular laterosensory series and isthmus, middle gap: (0) present; (1) absent (modified from Parenti, 1981).
- [95] Branchiostegal membrane in males, extent relative to opercular aperture: (0) poorly extending, slightly visible in lateral view of head; (1) strongly extending, overlapping pectoral-fin base (Costa, 1995). Besides expanded, the branchiostegal membrane in species of *Maratecoara* is bright blue in live individuals (Costa, 1995), here considered as an associated condition.
- [96] Ventral squamation, anterior extent: (0) reaching corner of pre-opercular series; (1) reaching anterior end of pre-opercular series; (2) reaching chin (modified from Costa, 2011a: ch.88; Costa, 2011b: ch.72).
- [97] Frontal scales, geometric arrangement: (0) transverse; (1) circular; (2) irregular (Parenti, 1981).
- [98] Frontal squamation, modal arrangement-pattern: (0) G; (1) E; (2) D; (3) F; (4) S (Costa, 2005: ch.72).
- [99] E-scales, relative position: (0) overlapped; (1) not overlapped (modified from Costa, 1990b, 1998: ch.175).

— *Trunk*

- [100] Flank in males, contact organs: (0) absent; (1) present (Costa, 2005: ch.79).
- [101] Pre-dorsal region in old males, adipose ridge: (0) absent; (1) present (Parenti, 1981).

— *Fins*

- [102] Pectoral fin in males, contact organs: (0) absent; (1) present (Costa, 1998: ch.150).
- [103] Pectoral fin in males, posterior extremity, shape: (0) rounded; (1) pointed; (2) narrowing without forming pointed tip (modified from Costa, 1990b).
- [104] Pelvic fin in males, length relative to anal fin: (0) short, reaching anterior portion; (1) long, reaching at least middle (Costa, 1998: ch.153).
- [105] Pelvic fin in males, tip, filamentous rays: (0) absent; (1) present (extracted from Costa, 1998: ch.153).

- [106] Pelvic fins, medial fusion: (0) separated; (1) only base united; (2) base and medial membrane united (Costa, 2005: ch.65).
- [107] Dorsal fin, distal extent relative to caudal fin: (0) short, reaching caudal-fin base or anterior to it; (1) median, reaching middle of caudal fin; (2) long, surpassing posterior edge of caudal fin (modified from Costa, 1990b). In all taxa having a long dorsal fin, the anal fin is nearly so long, thus both conditions here considered as a single evolutionary event.
- [108] Anal fin in males, anterior portion, extent relative to posterior portion: (0) shorter; (1) longer (modified from Costa, 1995).
- [109] Anal fin, basal portion, scales: (0) absent; (1) present (Costa, 2005: ch.75).
- [110] Anal fin in females, distal length relative to fin base: (0) shorter; (1) longer (Costa, 2011b: ch.85).
- [111] Anal fin in females, thickness relative to male anal fin: (0) equal; (1) distinctively thicker (modified from Parenti, 1981).
- [112] Caudal fin in males, shape: (0) rounded; (1) lanceolate; (2) truncate (modified from Costa, 1995).
- [113] Caudal fin in males, posterior margin, isolated filamentous rays projecting beyond caudal fin margin: (0) absent; (1) present (modified from Costa, 1998: ch.159).
- [114] Caudal fin in males, posterior margin, short posterior extensions composed by double filament rays forming fringed border: (0) absent; (1) present (extracted from Costa, 1998: ch.159). Remarks: Costa (1998) considered the posterior filamentous rays of *Gnatholebias* and *Pterolebias* as homologous. In both species of *Gnatholebias*, *Rachovia maculipinnis*, *Austrofundulus leoni*, and in all species of *Maratecoara*, there are true filaments formed by a single ray branch, whereas in species of *Pterolebias*, the extensions are formed by two or more attached ray branches, thus considered as a distinct condition.
- [115] Caudal fin in males, length (excluding filaments and posterior extensions) relative to standard length: (0) clearly less than half; (1) about half or longer (modified from Costa, 1990b).=[116] Caudal fin in older males, squamation, mid-horizontal extent: (0) restricted to basal portion of fin; (1) approximately reaching middle of fin (Costa, 1990b).
- [117] Caudal fin in males, posterodorsal and posteroventral regions, posterior extension: (0) absent; (1) present (Costa, 1998: ch.161).
- *Laterosensory system*
- [118] Supra-orbital series, lateral epidermal trenches: (0) present; (1) rudimentary or absent (Costa, 2011a: ch.104).
- [119] Supra-orbital series, anterior section, neuromast, number: (0) three; (1) four; (2) six or seven (modified from Costa, 1990b).
- [120] Infra-orbital series, neuromasts, arrangement: (0) aligned; (1) on zigzag row (Costa, 2011b: ch.92).
- [121] Preorbital series, arrangement and number of neuromasts: (0) restricted to zone close to orbit, containing 1-3 neuromasts; (1) extending towards supraorbital neuromast series, containing 4-6 neuromasts; (2) contacting supraorbital neuromast series, containing 8 neuromasts (Costa, 2011a: ch.109).
- [122] Preopercular series and infra-orbital series, canal: (0) close; (1) open (modified from Parenti, 1981).
- *Male colour patterns*
- [123] Flank, 3 rows of reddish brown or brown spots along the whole flank alternating with similar shorter rows over light blue or light green ground: (0) absent; (1) present (modified from Parenti, 1981; Costa, 1989).
- [124] Flank, anterodorsal region, three horizontal rows of orange spots: (0) absent; (1) present (Costa, 1995).
- [125] Flank, intense light blue iridescence: (0) absent; (1) present (Costa, 2011a: ch.122).
- [126] Flank, red chevron-like marks: (0) absent; (1) present (modified from Costa, 2006a: ch.90).
- [127] Flank, humeral region, large iridescent blue blotch: (0) absent; (1) present (Costa, 1990b).
- [128] Flank, humeral region, small orange spots: (0) absent; (1) present (modified from Costa, 1998: ch.196).
- [129] Flank, humeral region, dark grey to black spot: (0) absent; (1) present (modified from Costa, 2006a: ch.91).
- [130] Head, iris, dark grey to black bar through centre of eye: (0) absent; (1) present (Parenti, 1981).
- [131] Head, anterior suborbital region, distinctive dark grey to black bar: (0) absent; (1) present (modified from Costa, ch.206).
- [132] Head, supra-orbital region, distinctive dark grey to black bar: (0) absent; (1) present (modified from Costa, 1998: ch.207).
- [133] Head, post-orbital region, dark grey bar adjacent to orbit: (0) absent; (1) present (modified from Costa, 2005: ch.86).
- [134] Head, opercular region, regular reticulate pattern formed by iridescent colour on middle of scales contrasting with dark orangish brown pigment along entire scale margins: (0) absent; (1) present (Costa, 2011a: ch.126).
- [135] Head, lower jaw, black pigmentation: (0) absent; (1) present (modified from Costa, 1998: ch.213).
- [136] Head, lower jaw, bright orange pigmentation: (0) absent; (1) present (Costa, 2011a: ch.129).
- [137] Head, middle opercular region, blue blotch: (0) absent; (1) present (Costa, 1998: ch.211).
- [138] Dorsal fin, distal region, red line on fin rays: (0)

- absent; (1) present (modified from Costa, 1998: ch.215).
- [139] Anal fin, basal region, dark grey to black ground colour with row of white to yellow spots: (0) absent; (1) present (modified from Costa, 1998: ch.218).
- [140] Anal fin, basal and posterior portions, series of oblique dark gray bars: (0) absent; (1) present (extracted from Costa, 1998: ch.218). Remarks: The derived colour pattern was not distinguished by Costa (1998a) from the above condition.
- [141] Anal fin, sub-basal white to pale yellow stripe: (0) absent; (1) present (Costa, 1988).
- [142] Caudal fin, dorsal portion, submarginal dark reddish brown stripe: (0) absent; (1) present (Costa, 1998: ch.229).
- [143] Caudal fin, dorsal and ventral margins, distinctive yellow stripe: (0) absent; (1) present (Costa, 1998: ch.227).
- [144] Caudal fin, ventral portion, dark orange stripe with narrow light yellow and dark reddish brown upper margin: (0) absent; (1) present (Costa, 2011b: ch.108).
- [145] Caudal fin, ventral portion, yellowish white or pale yellow stripe with broad dark red upper margin: (0) absent; (1) present (modified from Costa, 2011b: ch.109).
- [146] Caudal fin, ventral portion, bright yellow stripe with white and dark red upper margin: (0) absent; (1) present (modified from Costa, 2011b: ch.109).
- [147] Pectoral fin, dark grey to black marks vertically crossing all fin extension: (0) absent; (1) present (modified from Costa, 1998: ch.230–231).
- [148] Pectoral fin, ventral portion, white stripe with ventral narrow black margin: (0) absent; (1) present (Costa, 1998: ch.233).
- *Female colour patterns*
- [149] Flank, dark brown stripes: (0) absent; (1) present (Costa, 2011b: ch.112).
- [150] Caudal peduncle, posterodorsal end, round black spot with white margin: (0) absent; (1) present (modified from Costa, 1998:ch.222).
- [151] Caudal fin, base, dorsal portion, black spot with anterior white margin: (0) absent; (1) present (modified from Costa, 1998:ch.222).
- [152] Caudal fin, narrow black bars: (0) absent; (1) present (Costa, 1988).
- *Brain*
- [153] Telencephalon, length expressed as percentage of brain length: (0) 29–37 %; (1) 39–44 % (new character).
- [154] Telencephalon, posterolateral region, projection: (0) absent; (1) present (new character). Remarks: on all taxa exhibiting the state 1, the telencephalon was medially continuous, considered as a possible dependent condition.
- [155] Telencephalon, relative distance to mesencephalon tectum: (0) in close proximity or in contact; (1) separated by broad interspace (new character).
- [156] Telencephalon, shape: (0) about rectangular; (1) pentagonal (new character).
- [157] Olfactory bulb, extent relative to anterior margin of telencephalon: (0) projecting beyond; (1) not projecting beyond (new character).
- [158] Olfactory bulb, medial connection: (0) basal; (1) lateral (new character).
- [159] Inferior lobe, medial proximity: (0) small interspace; (1) in contact (new character).
- *Myology*
- [160] Levator operculi, attachment to medial face of opercle bone: (0) long, extending over dorsal portion, broadly extending beyond ventral margin of muscle attachment to lateral face of opercle bone; (1) short, slightly extending beyond dorsal margin, not extending beyond ventral margin of muscle attachment to lateral face of opercle bone (new character).
- [161] Levator operculi, dorsal portion, distinctive indentation: (0) absent; (1) present (new character).

Appendix 3.

Data matrix of morphological characters. See Appendix 1 for list of characters.

Gnatholebias zonatus
 100010000011111110201101002001000010001110101111101001100111003010101111110210113100020120010121010001000000010100000100000010000110

Gnatholebias hoignei
 1000100000111111102011010020010000100011101011111010011001110030101011111102101131000201200101210100010000000000100000100000010000110

Rachovia maculipinnis
 100110000011111110201101002011000010001110101111011001100100010000000101110110210113101100000001121001010001000000011100000100000000000100000110

Rachovia pyropunctata
 1000100000111111102001010020110000100011101011110100100100010001000000011101101111010000000112000101200100000001111000000000001000000??? ???

Rachovia brevis
 1000100000111111102001010020110000100011101011110110011001000100010000000111011011110100000000112000171200100000001111000001000000??? ???

Austrofundulus limnaeus
 100010000011111110200101002011000010001110110011001000100010000000111011011012111010000000011210017120010000000111000000100000000000??? ???

Austrofundulus transilis
 10001000001111111020010100201100001000100101011110100110010001000100000001110110110121110100000000112000001200100000001110000000000000100000110

Austrofundulus guajira
 10001000001111111020010100201100001000100101110111010010001000100000001110110110121110100000000110000101200100000001?100000000000000100000110

Terranatos dolichopectus
 100010010111111011020011100200100001000100010011110110011001000110?1001200110111011021010100002100200112100011200100000000000000000000000000110