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Description of a new annual rivulid killifish genus from Venezuela

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Abstract

We describe a new genus to accommodate the species originally described as *Rivulus stellifer* Thomerson & Turner, 1973, but currently referred to the genus *Rachovia* Myers, 1927. *Rachovia stellifer* has had a complicated taxonomic history and has, at various times since its description, been placed in and out of three genera: *Rivulus* Poey, 1860, *Pituna* Costa, 1989 and *Rachovia*. However, phylogenetic analyses using 3537 mitochondrial and nuclear characters, and 93 morphological characters indicate it is not a member of any of these genera, but place it as a deeply divergent sister species to the genus *Gnatholebias* Costa, 1998. In addition to molecular characters, it is distinguished from the genera *Rachovia* and *Gnatholebias* by 13 and 33 morphological character states, respectively.

Key words: Rivulidae, total evidence, phylogenetic analysis, taxonomic revision

Introduction

In the last three decades, several phylogenetic hypotheses have been proposed for the fish order Cyprinodontiformes, as well as for its taxonomic subsets. Parenti (1981) presented the first cladistic analysis of the Cyprinodontiformes, including an analysis of phylogenetic relationships of the South American family Rivulidae. Nearly 10 years later Costa (1990a) published a phylogeny focusing solely on the family Rivulidae. Costa (1990a) used data similar to those of Parenti (1981), but came to a startlingly different phylogenetic hypothesis. Among the major points of disagreement between Costa's and Parenti's phylogenies is the taxonomic placement of *Rivulus stellifer* Thomerson & Turner, 1973. While Parenti (1981) retains this annual species in the genus *Rivulus* Poey, 1860 as originally described by Thomerson and Turner (1973), Costa (1990b) places this species into the genus *Pituna* Costa, 1989 based on a set of shared derived characters. The other member of the genus is *Pituna poranga* Costa, 1989 from the Cerrado region of the upper Araguaia River system of Brazil. Later, Costa (1998b) revises the genus *Pituna*, synonymizes *P. poranga* Costa, 1989 with the newly rediscovered *Rivulus compactus* Myers, 1927 which is transferred to the genus *Pituna* (*P. poranga* was subsequently removed from synonymy with *P. compacta* by Costa (2007)), and also removes *Pituna stellifer* from the genus *Pituna*, placing it back into the genus *Rivulus*, but suggesting that it might be related to the genera *Rachovia* Myers, 1927 and *Austrofundulus* Myers, 1932. A second major revision of the Rivulidae came with the publication of Costa's (1998a) work, where he transfers *R. stellifer* to the genus *Rachovia* based on a new set of shared derived characters.

Hrbek and Larson (1999) published a molecular phylogeny of the Rivulidae based on a 1972 nucleotide dataset of mitochondrial genes. In this study, *Rachovia stellifer* is found to be the sister species to *Gnatholebias zonatus* (Myers, 1935). *Rachovia maculipinnis* Radda, 1964, the other *Rachovia* species analyzed in Hrbek and Larson (1999) is strongly supported as the sister species of *Austrofundulus limnaeus* Schultz 1949.

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These findings are reiterated by subsequent molecular studies of the Rivulidae (Hrbek *et al*., 2004; Vermeulen & Hrbek, 2005).

Recently Costa (2005) revised the genus *Pterolebias* Garman, 1895 using *R. stellifer* as one of the taxa in this taxonomic treatment. The new set of characters reported by Costa (2005) do not place *R. stellifer* sister to the species *R. maculipinnis*, a phylogenetic relationship expected if *Rachovia* were monophyletic. In addition to *R. stellifer* and *R. maculipinnis*, the genus *Rachovia* also contains the species *R. brevis* (Regan, 1912) (the type species), *R. pyropunctata* Taphorn & Thomerson, 1978 and *R. hummelincki* de Beaufort, 1940 species which with the exception of *R. hummelincki* were previously analyzed by Costa (1998a). In Costa (2005), *R. stellifer* is sister to a clade containing species of the genera *Terranatos* Taphorn & Thomerson, 1978, *Rachovia*, *Austrofundulus* and *Pituna*.

The taxonomy and evolutionary history of *R. stellifer* is clearly complex, and remains unresolved. There are apparent conflicts between published analyses, however, without a rigorous statistical evaluation of these apparent conflicts, it is difficult to determine the source, or even if real conflicts exist. Earlier publications (Parenti, 1981; Costa, 1990a, 1998a) do not report a statistical assessment of the phylogenetic position of *R. stellifer*, while later publications (Hrbek & Larson, 1999; Hrbek *et al*., 2004; Costa, 2005; Vermeulen & Hrbek, 2005) show weak statistical support for the phylogenetic placement of *R. stellifer*. The goal of this study is the reanalysis of the phylogenetic position of *R. stellifer*. To achieve this goal, we analyzed a dataset composed of 3635 molecular characters approximately equally distributed between the evolutionarily independent nuclear and mitochondrial genomes, and 93 morphological characters obtained from Costa (2005).

Material and methods

Sampling design and DNA sequencing

To have combinable datasets, we analyzed all species used in Costa (2005). The species *Aphyolebias boticarioi* Costa, 2004 and *Moema apurinan* Costa, 2004 were unavailable for this study, and were replaced by *Aphyolebias peruensis* (Myers, 1954) and *Moema cf. staecki* analyzed in Costa (1998a). One of the outgroups, *Rivulus janeiroensis* Costa, 1991, was also unavailable, and was replaced by *Rivulus santensis* Köhler, 1906 a member of the same clade of *Rivulus* (Costa, 1998a). The combined dataset included 19 species from 16 genera. As in Costa (2005) we included *Aplocheilus panchax* (Hamilton, 1822), *Scriptaphyosemion guignardi* (Romand, 1981), *Kryptolebias brasiliensis* (Valenciennes, 1821) and *Rivulus santensis* as outgroups. Table 1 lists all species and locations used in this study, as well as GenBank accession numbers for genes analyzed in this study.

Laboratory protocols

Specimens were collected and preserved in 95% ethanol or were quick-frozen in liquid nitrogen. Total genomic DNA was extracted from muscle tissue of the right caudal peduncle using Qiagen™ DNeasy spin-column tubes. Alternatively, proteinase K/SDS dissolution with phenol-chloroform extraction followed by 70% ethanol precipitation was applied to the tissue.

Polymerase Chain Reaction (PCR) amplification was performed on total genomic DNA. Negative controls were run for all reactions. Mitochondrial DNA sequences used in this study consisted of genes encoding part of 12S rRNA, part of CO1 and complete sequences of ND2, transfer RNAs for valine, glutamine, methionine, tryptophan, alanine, asparagine, cysteine and tyrosine, and the origin of light-strand replication. Nuclear DNA sequences consisted of a nearly complete third exon on the Recombination Activating Gene 1 (RAG1).

Mitochondrial DNA sequence data were obtained from Hrbek and Larson (1999) and Vermeulen and Hrbek (2005). New sequence data were obtained using the protocol described in Hrbek and Larson (1999),

however, sequences were resolved on an MJ Research BaseStation automatic DNA sequencer and edited in the program BioEdit (Hall, 1999).

TABLE 1. Species and localities included in this study. Specimens not collected by the authors came primarily from the collections of Jamie Thomerson, Cal Him, Roger Brousseau, Daniel Fromm, Jean Huber and Rosario LaCorte. Some specimens were taken from aquarium stocks (AS). Associated GenBank accession numbers for the 12s rRNA and valine tRNA gene sequence, and for the ND2 gene and adjoining tRNA genes, and for the third exon of the RAG1 gene are listed in that order next to individual species.

Genus	Species	Population	GenBank accession number		
			12S	ND2	RAG1
Rachovia	*maculipinnis*	Papelón, Venezuela	AY850664	AY850639	EF4556714
Austrofundulus	*transilis*	Guanarito, Venezuela	AY850682	AY850659	EF4556715
Trigonectes	*rubromarginatus*	Aruanã, Brazil	AF092335	AF092402	EF4556723
Neofundulus	*paraguayensis*	AS (CI/92), Paraguay	AF092338	AF092405	EF4556722
Moema	*cf. staecki*	Rockstone, Guyana	AF092339	AF092406	EF4556719
Aphyolebias	*peruensis*	Yarina Cocha, Peru	AF092340	AF092407	EF4556718
Renova	*oscari*	Isla Ratón, Venezuela	AF092346	AF092413	EF4556721
Pterolebias	*phasianus*	Corumba, Brazil	AF092347	AF092414	EF4556710
Pterolebias	*longipinnis*	Entre Ríos, Argentina	AF092348	AF092415	EF4556709
Micromoema	*xiphophorus*	Puerto Ayacucho, Venezuela	AF092351	AF092418	EF4556720
Gnatholebias	*zonatus*	La Capilla road, Venezuela	AF092352	AF092419	EF4556711
Gnatholebias	*hoignei*	La Capilla road, Venezuela	EF4556701	EF4556704	EF4556712
Llanolebias	*stellifer*	Arismendi, Venezuela	AF092353	AF092420	EF4556713
Terranatos	*dolichopterus*	La Capilla Road, Venezuela	AF092354	AF092421	EF4556716
Pituna	*poranga*	Aruanã, Brazil	AF092345	AF092412	EF4556717
Kryptolebias	*brasiliensis*	Rio de Janeiro, Brazil	AY946276	AY946281	EF4556707
Rivulus	*santensis*	Santos, Brazil	AF092313	AF092380	EF4556708
Scriptaphyosemion	*guignardi*	AS, Sierra Leone	EF455700	EF4556703	EF4556706
Aplocheilus	*panchax*	AS, India	EF455699	EF4556702	EF4556705

To amplify the third exon of the nuclear coded Recombination Activating Gene 1 (RAG1), we used a semi-nested PCR strategy. PCR was performed in 25 μl reaction volumes containing 11.8 μl of ddH_2O, 1.7 μl of 10 mM $MgCl_2$, 2.5 μl of 10x buffer (200 mM Tris-HCl [pH 8.8], 20 mM $MgSO_4$, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton® X-100, 1 mg/ml nuclease-free BSA), 2.5 μl of each primer (2 μM), 2.0 μl dNTP mix (10 mM), 3 U KlenTaqLA DNA Polymerase, and 1 μl of DNA template (concentration varied between 50 ng and 100 ng). A semi-nested PCR consists of a first round of PCR followed by a second round of PCR of the unpurified PCR product with a new set of primers; one primer is same as the original while the second primer is placed internally to the one used in the first PCR. The temperature profile for both PCRs consisted of 1) pre-heating at 68°C for 60 s, 2) denaturation at 93°C for 10 s, 3) annealing at 55°C for 35 s, 4) extension at 68°C for 150 s, and 5) a final extension at 68°C for 10 min. Steps 2–4 were repeated 25 times. The RAG1 gene transcript was amplified with an external primer pair 5'-CCWGCTGTITGYYTGGCCATIMG-3' (Rag1.HB.F.L1) and 5'-GTGTAGAGCCARTGRTGYTT-3' (Rag1.Mart.R6), and then in the semi-nested reaction the primer Rag1.Mart.R6 was replaced with 5'-TGYTTTCAGCACGTCCTCCARYTC-3' (Rag1r.6). PCR products were evaluated on a 1% agarose gel, and then purified with Qiagen™ QiaQuick spin-columns. The RAG1 product was sequenced using the internal primers Rag1f.2 (5'-GARCGYTAYGAAATATGGAG-3'), Rag1r.1 (5'-GCN-

GAGACTCCTTTGACTCTGTC-3') and Rag1r.2 (5'-GAGAARCGRACAGCCTTYTC-3'). Sequencing reactions followed standard Perkin–Elmer Big Dye v3.1 sequencing protocol for double-stranded cycle sequencing reactions. Sequences were determined on an MJ Research BaseStation automatic DNA sequencer, and edited in the program BioEdit (Hall, 1999).

Data analysis

Orthologous protein-coding regions (ND2, CO1 and RAG1) were aligned manually using the PAUP* 4.0b10 text editor (Swofford, 2002). Alignment of protein-coding regions was confirmed by translating DNA data into putative amino-acid sequences in the program BioEdit (Hall, 1999). Alignments of ribosomal and transfer RNAs were constructed manually based on secondary structural models (Kumazawa & Nishida, 1993) and on previous rivulid alignments (Hrbek & Larson, 1999). All transfer RNAs with the exceptions of the $tRNA^{Val}$ of *Pituna poranga*, *Pterolebias phasianus* Costa, 1988 and *Terranatos dolichopterus* Taphorn & Thomerson, 1978 appeared functional. The $tRNA^{Val}$ sequences obtained for the three species mentioned above are thought to be nonfunctional copies due to a lack of tRNA specific anticodon, and thus were coded as missing and excluded from phylogenetic analyses. Ambiguously aligned regions were excluded from phylogenetic analyses. Mitochondrial gene regions were tested for an anti-G bias characteristic of the mitochondrial DNA genes, but not of the nuclear genome, to support our conclusion that we have collected genuine mitochondrial DNA data (Zhang & Hewitt, 1996). Polymorphic sites in the RAG1 nuclear dataset were coded using the standard IUPAC code. Morphological data were obtained from Costa (2005). Data partitions were defined as mitochondrial genome data, nuclear genome data and morphological data. Partitions were tested for phylogenetic incongruence via the Incongruence Length Difference (ILD) test of Farris *et al*. (1994) as implemented in the program PAUP* 4.0b10 (Swofford, 2002).

The most parsimonious phylogenetic topology for molecular and combined data was estimated using PAUP* 4.0b10 (Swofford, 2002) with 100 heuristic searches using random addition of sequences, and implementing the tree bisection and reconnection (TBR) algorithm. Bootstrap resampling (Felsenstein, 1985) was applied to assess support for individual nodes using 2000 bootstrap replicates with 10 random additions and TBR branch swapping. A file containing a constraint at each node and directives for a heuristic search with random addition, TBR branch swapping and 25 replicates was used to find trees not satisfying the particular constraints. Bremer branch-support values (Bremer, 1988, 1994) were calculated by subtracting the length of the shortest tree from the shortest tree constrained not to include the branch being analyzed.

Maximum-likelihood topology for molecular data was also estimated in the program PAUP* 4.0b10 (Swofford, 2002) with 25 heuristic searches using random addition of sequences, and implementing the tree bisection and reconnection (TBR) algorithm. The General Time Reversible (GTR) model of molecular evolution (Rodríguez *et al*., 1990) with rate heterogeneity, rates for variable sites assumed to follow the gamma distribution, with a portion of sites treated as invariable was used. The GTR model was suggested as most appropriate model of molecular evolution for this dataset by the software MODELTEST 3.7 (Posada & Crandall, 1998) under the Akaike information criterion (Akaike, 1974).

Bayesian inference analyses of molecular and combined data were performed in the software MrBayes 3.01 (Ronquist & Huelsenbeck, 2003). Data were partitioned into nine categories representing first, second and third positions of mtDNA (three partitions) and nuDNA (three partitions) protein coding genes, one partition representing mtDNA tRNAs, one partition for mtDNA 12s rRNA and one partition consisting of morphological data. We ran two simultaneous parallel runs of 2,000,000 generations using default long and short chain and heating parameters, sampling trees and branch-length every 100 generations. Bayesian inference was carried out additional two times to access concordance among tree topologies derived from independent runs. Convergence of all estimated parameters was inferred using the Gelman and Rubin (1992) criterion. Log likelihoods stabilized within the first 5% of the run, and we discarded these initial 100,000 trees as burn in steps in the computation of a 50% majority rule consensus tree. Following Huelsenbeck *et al*. (2001), the fre-

quency a clade occurs among the sampled trees was interpreted as the posterior probability of that clade existing. These posterior probabilities are true probabilities under the assumed model of substitution (Rannala & Yang, 1996); thus we considered clades to be significantly supported when Bayesian posterior probabilities were >95%.

All sequence data have been deposited in GenBank (Table 1). Aligned sequence data are available at www.cyprinodontiformes.org, or directly from the first author.

Tests of alternate hypotheses

Alternate hypotheses of phylogenetic relationships were tested within the parsimony framework. Tests were implemented in the programs PAUP* 4.0b10 (Swofford, 2002) by searching for the most parsimonious or most likely phylogenetic hypothesis under the alternate phylogenetic hypothesis constraint. The alternate parsimony hypothesis was compared with most parsimonious topology, and significance of topological differences were assessed using the nonparametric Wilcoxon signed-ranks test (Templeton, 1983).

Institutional codes

Institutional codes follow the usage of Leviton *et al*. (1985): MCNG – Museo de Ciencias Naturales de la UNELLEZ en Guanare; USNM – National Museum of Natural History, Smithsonian Institution, Washington, DC, USA; FMNH – Field Museum of Natural History, Chicago, IL, USA; UMMZ – University of Michigan Museum of Zoology, Ann Arbor, MI, USA; CAS – California Academy of Sciences, San Francisco, CA, USA; MBUCV – Museo de Biologia, Universidade Central de Venezuela, Caracas, Venezuela; ICNMHN – Instituto de Ciencias Naturales, Museo de Historia Natural, Bogota, Colombia.

Results

The ILD test indicated incongruence among the tree data partitions (P = 0.001). When pairwise ILD tests were conducted, incongruence was observed between the morphological and the two molecular datasets (mtDNA *vs*. morphology P = 0.001; nuDNA *vs*. morphology P = 0.028), but not between the mitochondrial and nuclear datasets (P = 0.141).

Combining different datasets, even if they show statistical incongruence, is the only way resolve conflicts among data sets, and to investigate homoplasy. A combined data analysis should, therefore, favor the emergence of congruent phylogenetic signal, allowing different data partitions to contribute to overall phylogenetic resolution (e.g. Chippindale & Wiens, 1994; Wiens & Reeder, 1995; Baker & DeSalle, 1997; Farias *et al*., 2000; Hillis & Wiens, 2000; Hodges & Zamudio, 2004; López Fernández *et al*., 2005). Furthermore, although data partitions may show statistically significant incongruence, there usually is no *a priory* justification of exclusion of any particular data partition. Because of these considerations, we chose to combine all our data, and analyze them jointly.

Bayesian inference resulted in a statistically well supported phylogenetic hypothesis (Fig. 1); many of the same nodes were robustly supported in the maximum parsimony analysis (Fig. 2) as were nearly all nodes in the pruned maximum parsimony analysis (Fig. 3). *Rachovia stellifer* was recovered as a strongly supported but deeply divergent sister taxon of the genus *Gnatholebias*; the monophyly of the genus *Rachovia* including *R. stellifer* was rejected (Templeton test $P < 0.0001$; Likelihood Ratio test $P < 0.001$). The phylogenetic position of the monotypic genus *Terranatos* remained statistically unresolved within the clade containing the genera *Austrofundulus, Rachovia, Micromoema* Costa, 1998 and *Gnatholebias*.

The taxon *Pituna poranga* had an unusual behavior. In the maximum parsimony analysis we recovered a topology where *P. poranga* was placed sister to *Neofundulus paraguayensis* (Eigenmann & Kennedy, 1903) (Fig. 2). In one step longer trees *P. poranga* was placed near the base of the tree. In our previous analyses of

mitochondrial DNA data only, we also observed great phylogenetic instability of *P. poranga* and the annual clade within which it is contained. In the study of Hrbek and Larson (1999) this *P. poranga* containing clade alternated between a sister taxon position to the annual Cynolebiatini and being nested within the annual Rivulini.

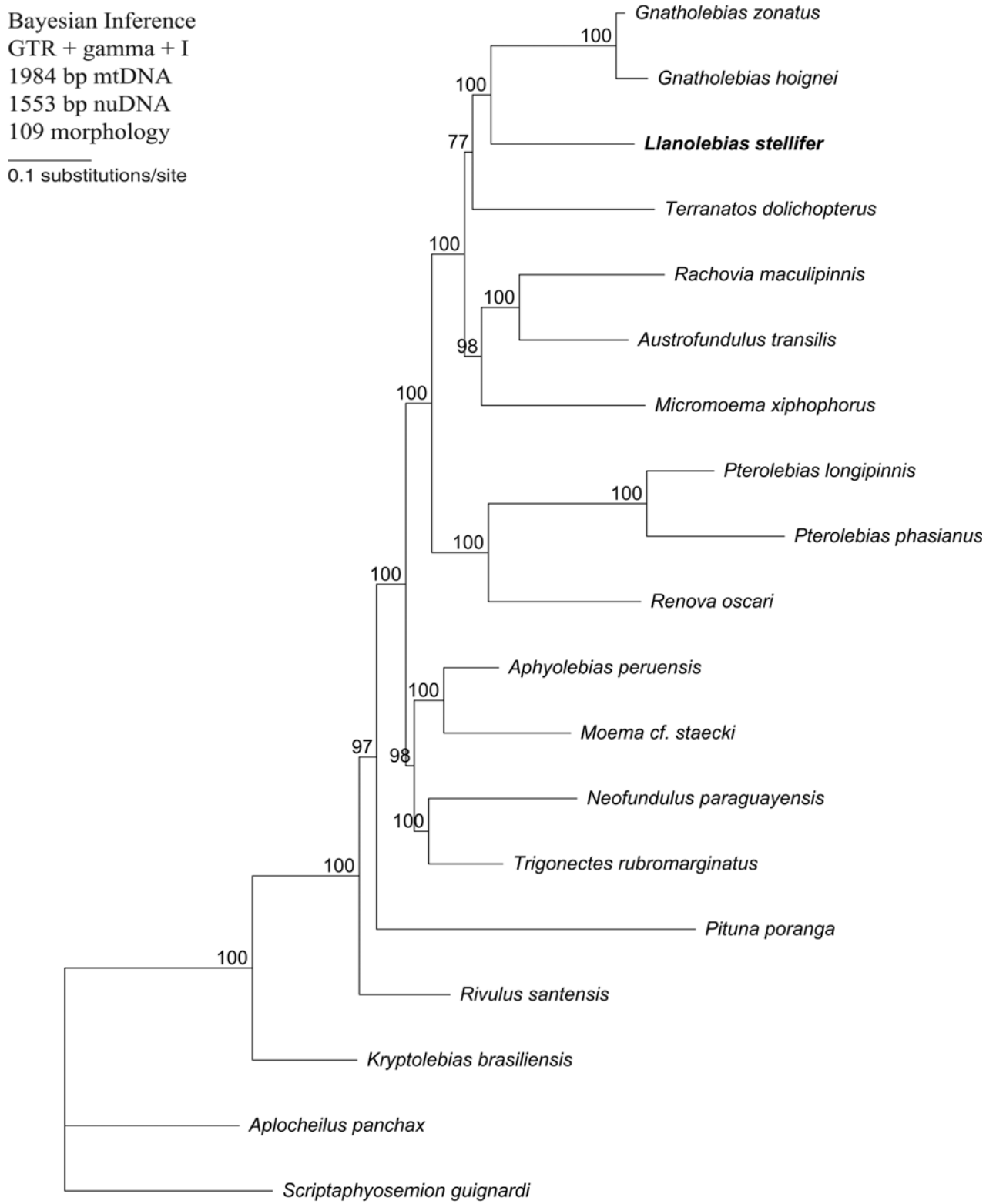

FIGURE 1. Bayesian inference phylogenetic hypothesis based on 2 million MCMC samples. Likelihood of the Bayesian inference majority-rule consensus topology is –ln = 28423.144. Numbers above nodes correspond to posterior probabilities of that node existing.

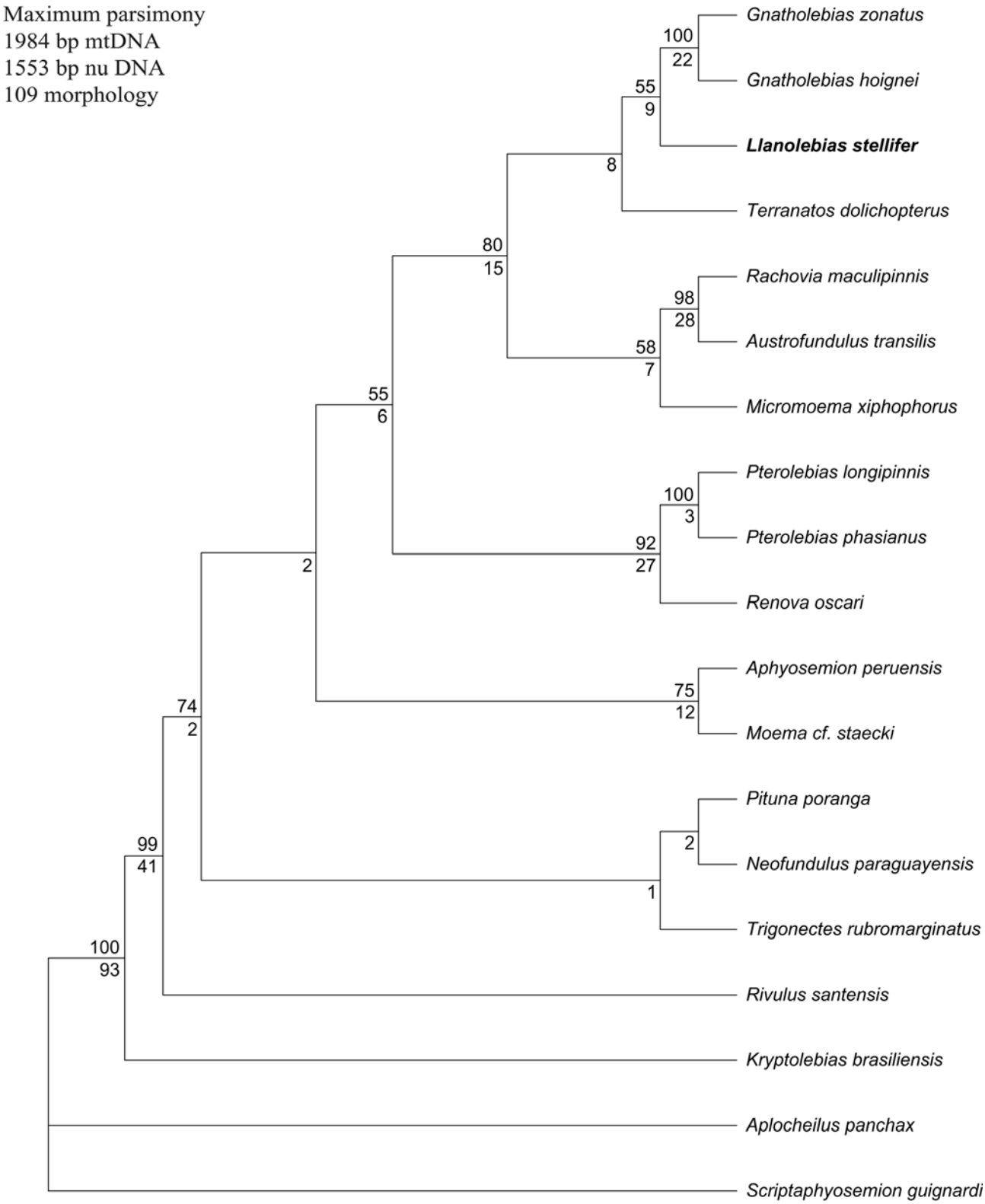

FIGURE 2. Maximum parsimony phylogenetic hypothesis. Heuristic search with 50 random additions and TBR branch swapping resulted in a single most parsimonious topology of 6206 steps. Numbers above nodes are bootstrap values based on 2000 pseudoreplicates (25 random additions each); only values over 50 are reported. Numbers below branches are Bremer support indices which are equivalent to unreversed synapomorphies.

More recent studies (Hrbek *et al*., 2004; Vermeulen & Hrbek, 2005) place this clade in either of these phylogenetic positions. This phylogenetic instability was attributed to long-branch attraction artifacts (Hrbek

& Larson, 1999). Morphological studies place *P. poranga* in various phylogenetic positions as well, including a possible sister species relationship to *R. stellifer* within the annual Cynolebiatini (Costa, 1990a), a sister relationship to *Rachovia* within the Rivulini (Costa, 1998a), or a sister relationship to the genera *Austrofundulus*, *Terranatos.*

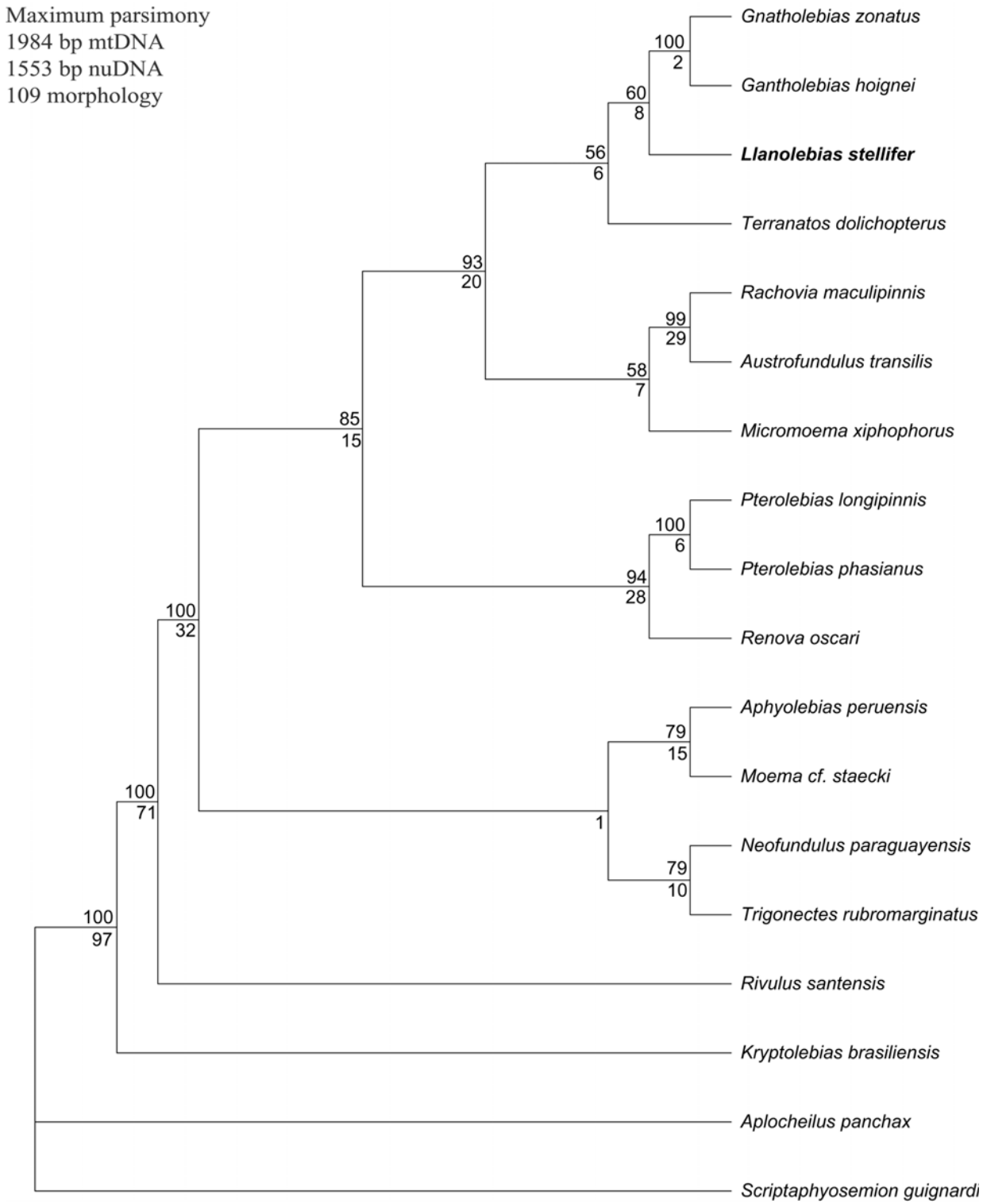

FIGURE 3. Maximum parsimony phylogenetic relationships with the taxon *Pituna poranga* removed. Heuristic search with 50 random additions and TBR branch swapping resulted in a single most parsimonious topology of 5706 steps. Numbers above nodes are bootstrap values based on 2000 pseudoreplicates (25 random additions each); only values over 50 are reported. Numbers below branches are Bremer support indices which are equivalent to unreversed synapomorphies.

and *Rachovia* (not including *R. stellifer*) within the Rivulini (Costa, 2005). The sources of the phylogenetic instability of *P. poranga* and the *P. poranga* clade, and its actual phylogenetic position will require additional studies before a more definitive inference can be made.

Because of the phylogenetic instability of *P. poranga* in the maximum parsimony analyses, we performed a second set of maximum parsimony analyses where this taxon was removed. We recovered a single most parsimonious topology (Fig. 3) that showed higher bootstrap support for nearly all nodes when compared to the topologies with *P. poranga* included.

Discussion

In parsimony and likelihood analyses, the monophyly of the annual Rivulidae of northern South America was strongly supported. Parsimony bootstrap support was 94 while Bayesian support value for this clade was 100%. Strong phylogenetic support was found for this clade in spite of the fact that this clade includes some of the morphologically most divergent killifish species. All of these species are distributed in the Llanos of the Orinoco and Magdalena basins, in the Caribbean coastal savannahs of Colombia and the Maracaibo basin of Venezuela, and also in the inland savannahs of Essequibo River in Guyana. This clade was sister to a paraphyletic group found in the savannahs and flooded forest areas of the Amazon basin and in the Chaco and Pantanal of the Paraguay River basin.

A point of disagreement between maximum parsimony bootstrap (Fig. 2), Bayesian inference (Fig. 1) and pruned maximum parsimony (Fig. 3) phylogenetic hypotheses included the placement of the root within the annual non-Cynolebiatini rivulid clade. Maximum parsimony analysis placed the root on the branch separating *Trigonectes* + *Neofundulus vs*. all other species, while Bayesian inference and pruned maximum parsimony analyses placed the root on a branch separating (*Trigonectes* + *Neofundulus*) + (*Moema* + *Aphyolebias*) *vs*. all other species. The ingroup topology was otherwise identical. Since the rooting in the parsimony analysis was not statistically supported, and the Bayesian inference rooting is highly supported by Bayesian support value of 98%, the Bayesian inference / pruned maximum parsimony topology appears more credible. Indeed, rerooting the maximum parsimony phylogeny to conform to the root placement of the Bayesian inference hypothesis resulted in a non-significantly less parsimonious topology (Templeton test $P = 0.73$). Other relationships showed high statistical support in all analyses, however.

Comparison of molecular and morphological phylogenies

Different sets of data, including morphological and molecular data, can produce differing estimates of phylogenetic relationships among species of the same group. However, only rarely are these difference significant (Hillis & Wiens, 2000). Points of conflict usually result from undersampling of characters or taxa, differences in phylogenetic methods and differences in rooting techniques. However, in cases where topological conflicts are due to differences in rooting, the ingroup topology remains unmodified.

A potential source of conflict among the molecular and morphological estimates of phylogenetic relationships could be the result of undersampling of taxa especially in the morphological dataset. Phylogenetic estimates using morphological data vary widely (Parenti, 1981; Costa, 1990a, 1998a, 2005), while estimates based on molecular data (Hrbek & Larson, 1999; Murphy *et al*., 1999; Hrbek *et al*., 2004) show little variation among studies, and with the phylogenetic estimates obtained in this study. The morphology-inferred relationships are also not supported by bootstrap values, and do not reject the topology inferred from mitochondrial and nuclear data only.

Despite these conflicts, all datasets and analyses speak against the inclusion of *R. stellifer* within a clade together with other *Rachovia* species. We are therefore confident that *R. stellifer* is not a member of the *Rachovia* clade, but rather a deeply divergent sister clade of the genus *Gnatholebias*.

Characters of *Rachovia stellifer*

In the molecular dataset 426 out of 3636 (11.72%) characters exists that distinguish *R. stellifer* from *R. maculipinnis*; 409 are in the mtDNA partition and 15 are in the nuDNA partition. *Rachovia stellifer* is also distinguished by 415 characters in the mtDNA partition and 15 are in the nuDNA partition from both species of *Gnatholebias*. The *Rachovia stellifer* + *Gnatholebias* clade is supported by nine or eight unreversed molecular character states (Bremer support values in Figs. 2 and 3, respectively). For a complete list of molecular characters, see supplementary data available online at www.cyprinodontiformes.org.

Costa (2005) provides a data matrix for a section of the Rivulidae. Costa lists 13 characters distinguishing *R. stellifer* from the other species of the genus *Rachovia* into which *R. stellifer* is currently placed (Table 2). Costa also lists 33 characters which distinguish *R. stellifer* from its molecular sister clade *Gnatholebias* (Table 3). In spite of being clearly distinguishable from species of the genera *Rachovia* and *Gnatholebias*, there appear to be no unreversed morphological autapomorphies for *R. stellifer* (Costa, 2005) that would provide unambiguous diagnostic characters.

TABLE 2. Character states of characters listed in Appendix 1 as observed in *Llanolebias stellifer* and *Rachovia maculipinnis.*

	40	54	56	59	63	67	68	72	80	85	86	92	93
Llanolebias stellifer	0	0	1	0	0	0	0	1	0	0	0	0	1
Rachovia maculipinnis	1	1	2	1	1	1	1	3	1	1	2	1	0

TABLE 3. Character states of the above characters listed in Appendix 2 as observed in *Llanolebias stellifer, Gnatholebias zonatus* and *Gantholebias hoignei*

	02	06	07	09	11	14	17	22	31	32	41	44	47
Llanolebias stellifer	1	1	0	1	1	1	0	1	0	1	2	1	0
Gnatholebias zonatus	0	0	1	0	0	0	1	0	1	2	3	2	1
Gnatholebias hoignei	0	0	1	0	0	0	1	0	1	2	3	2	1

	48	54	55	56	62	63	64	65	66	67	68	69	72
Llanolebias stellifer	0	0	0	1	0	0	0	0	1	0	0	0	1
Gnatholebias zonatus	1	1	1	2	1	1	1	2	2	1	1	2	3
Gnatholebias hoignei	1	1	1	2	1	1	1	2	2	1	1	2	3

	74	75	81	84	89	91	93
Llanolebias stellifer	2	0	1	1	0	0	1
Gnatholebias zonatus	0	1	0	0	1	1	0
Gnatholebias hoignei	0	1	0	0	1	1	0

***Llanolebias* new genus**

Fig. 4 (male and female), Fig. 5 (male and female MCNG 25828)

Type species. *Rivulus stellifer* Thomerson & Turner 1973. Holotype: USNM 209254; Paratypes: USNM 209254, FMNH 71667, FMNH 71668, UMMZ 193375, CAS 27556, MBUCV V-7199, ICNMHN 700.

FIGURE 4. A representative female (4a) and male (4b) *Llanolebias stellifer* (photos by Tonnie Woeltjes).

Diagnosis. *Llanolebias* is diagnosed by the numerous molecular characters, however, it possesses no unique morphological characters that will unambiguously distinguish it from all other rivulid species; however, 13 characters distinguishing it from the genus *Rachovia*, and 33 characters distinguish it from the genus *Gnatholebias*. Compared to its sister genus *Gnatholebias*, the single species of *Llanolebias* is a robust, cylindrical-shaped fish, with short fins and in males very small fin-ray extensions, while *Gnatholebias* species have relatively deep, laterally compressed bodies with long fins and long fin-ray extensions. The anal fin base of *Llanolebias* is also short compared to species of *Gnatholebias*, and *Llanolebias* has 14-15 anal fin rays versus 22-26 fin rays in *Gnatholebias*. *Llanolebias* also lacks the fatty predorsal ridge characteristic of older males of *Rachovia*. With the sole exception of females of *Renova oscari* Thomerson & Taphorn, 1995, *Llanolebias* is the only member of the 'lowland annual' clade of Rivulidae (Hrbek & Larson, 1999) with a 'Rivulus' spot in females. *Llanolebias stellifer* also differs ecologically from the two species of *Gnatholebias*. *Llanolebias stellifer* occurs in shallow habitats on the edges of aquatic systems in deep forest, whilst *Gnatholebias zonatus* is from sunny pools in the savannah and *Gnatholebias hoignei* (Thomerson, 1974) is from shaded but deeper pools than *L. stellifer*. *Llanolebias stellifer* is confined to seasonally wet areas in the seasonally dry tropical forest region of the Llanos, and is found in a habitat similar to that occupied by species of

the genera *Aphyolebias* and *Moema* which are found in seasonally wet areas of dense tropical forests of the Amazon and Essequibo basin.

Included taxa. Only the type species.

Etymology. From the Spanish *Llanos* (grassy plains, and in particular the Orinoco River savannahs of Venezuela and Colombia) and the Greek *Lebia* (a small fish and a nominal cyprinodontiform genus). Gender masculine.

FIGURE 5. An adult male (5a) and female (5b) *Llanolebias stellifer* deposited in the MCNG collection (MCNG 25828).

Conclusions

The joint use of molecular and morphological data allowed us to reassess former conclusions regarding the taxonomic status of *Llanolebias stellifer.* Phylogenetic analyses of combined datasets using different phylogenetic reconstruction methods showed strong statistical support for the sister taxon relationship of *R. stellifer* and the genus *Gnatholebias*, and rejected the hypothesis that *R. stellifer* is a member of the genus *Rachovia*. Following the philosophy that species as well as higher level taxonomic categories should be monophyletic (de Queiroz & Donoghue, 1988), the non-monophyly of *R. stellifer* + *Rachovia* spp. lead us to remove the species *R. stellifer* from the genus *Rachovia*. The sister taxon relationship of *R. stellifer* and species of the genus *Gnatholebias* left two possibilities for a taxonomic revision: 1) *Rachovia stellifer* is transferred into the genus *Gnatholebias* Costa 1988 – type species of the genus is *Gnatholebias zonatus* (Myers, 1935), or 2) a or a new genus is created for the species originally described as *Rivulus stellifer* Thomerson & Turner, 1973 and currently called *Rachovia stellifer.* We chose option number two. Although there are no universally accepted definitions as to what constitutes a genus, it has been argued that genus designations should encompass not only monophyletic units, but also morphologically and ecologically distinct groups, thus conveying additional information beyond the species level (e.g. Thomerson & Taphorn, 1992). Parenti (1981), for example, argued that creating monotypic genera adds no taxonomic information, thus monotypic genera should be avoided. While this is true, retaining very divergent taxa within the same genus, even if the genus remains monophyl-

etic, is also not desirable since it renders the genus non-diagnosable (Thomerson & Taphorn, 1992). Rivulus *stellifer* and species of the genus *Gnatholebias* are morphologically distinct units (Thomerson & Turner, 1973; Costa, 1998a, 2005; Table 3), showing sister taxon relationship but also a deep phylogenetic divergence (Figs. 1-3 and Hrbek & Larson, 1999; Hrbek *et al*., 2004; Vermeulen & Hrbek, 2005); *Gnatholebias* is monophyletic (Figs. 1-3 and Costa, 1998a; Costa, 2005). Using the above criteria, we placed the species *Rivulus stellifer* Thomerson & Turner, 1973 into a separate genus here named *Llanolebias*, thus providing a classification congruent with the phylogenetic relationships of the group as well as solving the problem of paraphyly of *Rachovia*.

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Appendix 1

Morphological characters listed in Costa (2005) which distinguish *Llanolebias stellifer* from species of *Rachovia*.

[40] Dorsally directed process on the base of anterior epipleural ribs (Costa, 1998a): (0) absent; (1) present.
[54] Fourth pectoral radial (Costa, 1998a): (0) not expanded; (1) ventrally expanded.
[56] Number of pelvic-fin rays (Costa, 1990a): (0) six; (1) seven; (2) eight.
[59] Fatty predorsal ridge in older males (Costa, 2005 modified from Taphorn & Thomerson, 1978; Parenti, 1981): (0) absent; (1) present.
[63] Pectoral-fin length (Costa, 2005 modified from Parenti, 1981): (0) 18.6–23.8 % SL; (1) 24.1–31.1 % SL.
[67] Filaments on tip of dorsal and anal fins in males (Costa, 1998a): (0) absent; (1) present.
[68] Filaments on posterior border of caudal fin in males (Costa, 1998a): (0) absent; (1) present.
[80] Pectoral-fin contact organs in males (Costa, 2005): (0) absent; (1) present.
[85] Dark pigmentation pattern on supraorbital region (Costa, 1998a): (0) no distinctive mark; (1) spot adjacent to eye.
[86] Pigmentation pattern on post-orbital and preopercular region (Costa, 2005): (0) no distinctive pattern; (1) two oblique bars; (2) post-orbital vertical bar; (3) two oblique stripes [not ordered].
[92] Dark pigmentation pattern on flank and fins (Costa, 1998a): (0) forming bars, stripes or spots, according to the pattern occurring in males; (1) flank almost plain, pigmentation reduced to minute dots, not presenting the general pattern as in males.
[93] Dark pigmentation pattern on upper portion of caudal-fin base (Costa, 1990a): (0) no distinctive mark; (1) one black spot.

Appendix 2

Morphological characters listed in Costa (2005) which distinguish *Llanolebias stellifer* from species of *Gnatholebias*; *Gnatholebias* is the molecular sister genus of *Llanolebias* (Figs. 1-3 and Hrbek & Larson, 1999).

[2] Ventral portion of lachrymal (Costa, 1998a): (0) short; (1) expanded.
[6] Anterolateral process of parasphenoid (Costa, 1998a): (0) short, free; (1) long, attached to pterosphenoid.
[7] Posterior portion of parasphenoid (Costa, 2005): (0) wide; (1) narrow.
[9] General shape of the premaxilla and dentary (Costa, 1998a): (0) elongate, snout profile sharply pointed; (1) short, snout profile blunt.
[11] Ascending process of premaxilla (Costa, 2005): (0) wide; (1) narrow.
[14] Rostral cartilage (Costa, 1998a): (0) approximately rounded; (1) longitudinal length longer than transversal length.
[17] External medial teeth of premaxilla and dentary (Costa, 1998a): (0) approximately directed as other teeth; (1) laterally displaced, strongly contrasting to other teeth.
[22] Dorsal arm of preopercle (Costa, 1990a): (0) broad; (1) narrow and pointed.
[31] Interarcual cartilage (Costa, 2005 modified from Parenti, 1981): (0) not reduced; (1) reduced.
[32] Number and arrangement of second pharyngobranchial teeth (Costa, 2004): (0) numerous teeth arranged in two rows; (1) few teeth arranged in single row; (2) teeth absent.
[41] Hypurals (Costa, 2005 modified from Costa, 1998a): (0) two dorsal plates and one ventral plate separated by gap; (1) two plates separated by wide gap; (2) two plates in close proximity, sometimes ankylosed; (3) single plate.
[44] Number of vertebrae (Costa, 2005 modified from Costa, 1990a): (0) 29–32; (1): 33–35; (2) 36–38.
[47] Anterior proximal radials of dorsal and anal fins (Costa, 2005): (0) slender; (1) wide.
[48] Orientation of anterior proximal radials of anal fin (Costa, 2005 modified from Costa, 1998a): (0) anteriorly or dorsally directed; (1) posteriorly directed.
[54] Fourth pectoral radial (Costa, 1998a): (0) not expanded; (1) ventrally expanded.
[55] Ischial process of pelvic girdle (Costa, 2005): (0) prominent; (1) vestigial.
[56] Number of pelvic-fin rays (Costa, 1990a): (0) six; (1) seven; (2) eight.
[62] Pectoral fin (Costa, 1990a): (0) rounded; (1) pointed.
[63] Pectoral-fin length (Costa, 2005 modified from Parenti, 1981): (0) 18.6–23.8 % SL; (1) 24.1–31.1 % SL.
[64] Extent of pelvic fin in males (Costa, 1998a): (0) short, its tip not surpassing anterior portion of anal fin; (1) long, its tip reaching the central or the posterior portion of the anal fin.
[65] Pelvic-fin (Costa, 2005): (0) bases separated or in contact; (1) bases united; (2) pelvic fins united along proximal portion of medial margin.
[66] Dorsal and anal fins (Costa, 2005 modified from Costa, 1998a): (0) short, tip rounded; (1) somewhat elongated, tip

pointed; (2) long, tip sharply pointed.

[67] Filaments on tip of dorsal and anal fins in males (Costa, 1998a): (0) absent; (1) present.

[68] Filaments on posterior border of caudal fin in males (Costa, 1998a): (0) absent; (1) present.

[69] Caudal-fin length in males (Costa, 1990a): (0) 32.5-41.5; (1) 42.0-49.0; (2) 52.5-81.0.

[72] Predominant frontal squamation-pattern (Costa, 2005 derived from Hoedeman, 1958): (0) G; (1) E; (2) D; (3) F [not ordered].

[74] Caudal-fin squamation in older males (Costa, 1990a, 1998a): (0) approximately on anterior 10–30 % of fin; (1) approximately on 40 % of fin; (2) approximately on 50–80 % of fin.

[75] Anal-fin base squamation in males (Costa, 2005): (0) no scales on anal-fin base; (1) 1–7 rows of scales on anal-fin base.

[81] General color pattern on flank (Costa, 2005): (0) vertical; (1) oblique; (2) longitudinal [not ordered]

[84] Pigmentation pattern on suborbital region (Costa, 1998a): (0) no distinctive mark; (1) gray to black suborbital bar; (2) red suborbital spot [not ordered].

[89] Dark pigmentation on dorsal portion of caudal fin (Costa, 1998a): (0) not distinctive concentrated; (1) concentrated to form dark brown stripe; (?) variable.

[91] Melanophore pattern on ventral margin of pectoral fin (Costa, 1998a): (0) not distinctive concentrated; (1) concentrated to form stripe.

[93] Dark pigmentation pattern on upper portion of caudal-fin base (Costa, 1990a): (0) no distinctive mark; (1) one black spot.