

Phylogenetic Relationships Within the Aplocheiloid Fish Genus *Rivulus* (Cyprinodontiformes, Rivulidae): Implications for Caribbean and Central American Biogeography

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We examined the phylogenetic relationships of 16 northern species of the aplocheiloid genus *Rivulus* inhabiting the Caribbean, Central America, and South America. A total of 714 base pairs per taxon were sequenced from two segments of the mitochondrial genome, 12S rRNA and cytochrome *b*. Both parsimony and neighbor-joining analyses suggest an ancient vicariant origin of the Greater Antillean taxa, in addition to a quite recent dispersal of species into the Lesser Antilles from the South American mainland. Combined analyses support the monophyly of the northern South American assemblage as the sister group of a Central American/Columbian biota. However, the monophyly of the Central American biota remains uncertain. Divergence estimates for the Central American taxa are calibrated from the Late Cretaceous separation of the proto-Antilles from the Americas. These data suggest that the extant Central American taxa represent the descendants of at least two separate invasions during the Cenozoic, prior to the closing of the Panamanian isthmus. Times are consistent with the extensive evidence for reptilian and mammalian exchange throughout the Cenozoic.

Introduction

The origin of the West Indian fauna has traditionally been discussed in light of two theories: vicariance biogeography and overwater dispersal. Most current plate tectonic reconstructions suggest the Greater Antilles arose from the drifting of an island arc that transiently connected North and South America during the late Cretaceous (Ross and Scotese 1988; Pindell and Barrett 1990; Pitman et al. 1993). The vicariance theory regarding Antillean biogeography (Rosen 1976, 1985) proposes that the Antillean species, particularly those displaying high degrees of endemism, are the descendants of organisms inhabiting that ancient land bridge. The alternative theory is that of overwater dispersal. Recent molecular data from the Caribbean herpetofauna (Hass and Hedges 1991; Hedges et al. 1992; Hass et al. 1993) suggest that most extant taxa on the Greater Antilles arrived by dispersal. However, members of at least two Antillean genera, *Cricosaura* and *Eleutherodactylus*, probably diverged via vicariance (Hedges 1989; Hass and Hedges 1991; Hedges et al. 1991; Hedges and Bezy 1993).

Other groups of organisms found throughout the region, particularly freshwater fish, can contribute uniquely to our knowledge of Caribbean and Central American biogeography. Their reduced ability to disperse, relative to terrestrial vertebrates, makes them the best indicators of past faunal distributions. While models regarding the biogeography of the Antillean freshwater fish range from purely vicariance (Rosen 1976, 1985) to purely dispersal (Briggs 1984, 1987), none of these has been developed in concert with any rigorous phylogenetic analysis. In fact, there are very few detailed phylogenies of freshwater fish that are informative

for the Caribbean and Central America (e.g., Lydeard et al. 1995a and b).

The diverse cyprinodontiform fishes (killifishes) are of particular interest from an evolutionary standpoint because of their virtual worldwide distribution, extensive karyotypic diversity (Scheel 1968, 1972, 1990), and unprecedented repertoire of reproductive adaptations (Wourms 1972a, b, and c; Parenti 1981; Meyer and Lydeard 1993). Within the Caribbean region, 85% of the freshwater fauna are members of the Cyprinodontiformes (Burgess and Franz 1989). Cyprinodontiforms are termed secondary freshwater fish for the ability of some groups to reside in brackish environments. Although the majority of these are poeciliids, several island endemics are members of the aplocheiloid genus *Rivulus*. This genus (>70 species) ranges through Central America, the Caribbean islands, and South America, as far south as Paraguay (Huber 1992). The genus is represented on the Greater Antilles by *R. cylindraceus* (Cuba and the Isle of Pines) and *R. roloffii* (Hispaniola) and on the Lesser Antilles by *R. cryptocallus* (Martinique). Two other poorly defined species, *R. garciae* and *R. insulaepinorum*, have been described from Cuba and the Isle of Pines (Cruz and Dubitsky 1976), although their meristics suggest they are probably conspecifics of *R. marmoratus* and *R. cylindraceus*, respectively (Huber 1992). There is another undescribed species (probably *R. hartii*; Huber 1992) from Grenada. The unique hermaphroditic species *R. marmoratus* is found in mangrove habitat throughout the region (Davis et al. 1991). On the adjacent mainland, there are 12 recognized species from Central America, at least 1 from Columbia west of the Andes, 3 from coastal and eastern Venezuela, and several species from the Guianas.

The most recent phylogenetic hypotheses put forth regarding cyprinodontiforms (Parenti 1981; Costa 1990; Meyer and Lydeard 1993; Parker and Kornfield 1995) are focused primarily on higher-level relationships, although some work has begun on intrageneric relationships (Bernardi and Powers 1995; Lydeard et al. 1995a,

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1995b). Parenti (1981) suggested that *Rivulus* represents a paraphyletic assemblage. However, this was based solely on five northern taxa, one member (*R. stellifer*) of which has been moved to the genus *Pituna* (Costa 1991). Unfortunately, little biogeographic information can be gleaned from these analyses. We present DNA sequence information from two slowly evolving mitochondrial genes for 16 members of the genus *Rivulus* from the Antilles, Central America, Venezuela, and the Guianas. These data generate a robust phylogeny to which past conjectures concerning the biogeography of these fish are compared.

Materials and Methods

Taxon Sampling

All specimens surveyed in this analysis were either wild caught fish or their aquarium-bred descendants. The species examined and their sources are as follows: *Cynolebias affinis*, Uruguay, aquarium stock (AS); *Cynolebias whitei*, Brasil, AS; *Rivulus amphoreus*, Tafelburg, Surinam; *Rivulus cryptocallus*, Martinique, AS; *Rivulus cylindraceus*, Cuba, AS; *Rivulus cylindraceus*, Isle of Pines; *Rivulus fuscolineatus*, Lake Arenal, Costa Rica; *Rivulus hartii*, Paria peninsula (JET88-28), Venezuela; *Rivulus hildebrandi*, Golfito, Costa Rica; *Rivulus immaculatus*, km 110 on road between El Dorado and Sta. Elena de Uairen (JET88-5), Venezuela; *Rivulus isthmensis*, Costa Rica, AS; *Rivulus magdalenae*, Columbia, AS; *Rivulus roloffii*, Banao, Dominican Republic; *Rivulus stagnatus*, Gluck Island, Guyana; *Rivulus tenuis*, Tacotalpa, Mexico; *Rivulus tenuis*, Rio Chajmaic, Guatemala; *Rivulus uroflammeus seigfriedi*, Golfito, Costa Rica; *Rivulus weberi*, El Llano-Carti road, Panama.

Laboratory Methods

Segments from two relatively conserved mitochondrial genes were amplified and sequenced from all taxa listed. A 360-base pair (bp) region of the cytochrome *b* (*cytb*) gene and a 354-bp aligned segment of 12S rRNA were chosen because of their broad application to ichthyofaunal systematics (Kocher et al. 1989; Thomas and Beckenbach 1989; Meyer and Wilson 1990; Normark et al. 1991; Sturmbauer and Meyer 1992; Block et al. 1993; Meyer et al. 1994; Zhu et al. 1994). These same DNA sequences have also been useful in addressing questions regarding Caribbean biogeography for reptilian species sharing distributions similar to those in the present study (Hedges et al. 1991; Hedges and Bezy 1993).

Mitochondrial DNA (mtDNA) was extracted from liver or muscle tissue of either frozen or live specimens following the sucrose-gradient method of Jones et al. (1988). One μL of mtDNA was used as template in symmetric amplifications via the polymerase chain reaction (PCR, Saiki et al. 1988). The primers L14724 and H15148 (Kocher et al. 1989; Meyer et al. 1990) for *cytb*, together with the 12S rRNA primers L1091 and H1478 (Kocher et al. 1989) were used to amplify and sequence 714 total bp (360 bp of *cytb*, and 354bp of 12S rRNA). PCR reactions (25 μL) were performed under the fol-

lowing conditions: 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 200 μM each dNTP, 1 μM each primer, and 0.5–1 unit *Taq* DNA polymerase (Perkin-Elmer, Cetus). Amplifications (30 cycles: 1 min 94°C, 1 min 55°C, 2 min 72°C) were followed by electrophoresis of 5 μL of product in a 2.5% low-melt agarose (NuSieve-GTG, FMC) TAE gel. The gel was stained with ethidium bromide, after which the bands were excised and melted in 1 mL of sterile, Milli-Q filtered (Millipore) water. Asymmetric amplifications (Gyllenstein and Erlich 1988) of 1 μL of the symmetric melt were performed under the same conditions described above, with the following alterations: reactions were increased to 50 μL , one of the primers was reduced to 0.02 μM , and the cycle number was increased to 35. Five microliters of each single-stranded product was visualized in 2.5% agarose (NuSieve 3:1, FMC) TAE gels. The remainder was washed five times in a 30,000-MW filter (regenerated cellulose; Millipore). Dideoxy sequencing (Sanger et al. 1977) was performed on 15 μL of this product using the *Taq*-Trak sequencing system (Promega). Sequence ladders were generated using 6% polyacrylamide and 8M urea gels, dried, and subjected to autoradiography.

Phylogenetic Analysis

The *cytb* and 12S rRNA sequences have been deposited in Genbank (accession numbers U41768–U41802, U44746). Sequence alignments were performed with CLUSTAL V (Higgins et al. 1992), followed by manual inspection. Indels were coded as missing data. Regions of sequence in which homology of sites was questionable as a result of ambiguities in the alignment were omitted from the analyses. Cladistic analyses were performed with PAUP (version 3.1.1, Swofford 1993), while neighbor-joining (Saitou and Nei 1987) analyses were done in PHYLIP (version 3.5, Felsenstein 1993).

Previous phylogenetic analyses using mtDNA have demonstrated that as divergence time increases, many sites become saturated by multiple transitional substitutions, thus obscuring more ancient divergences (Brown et al. 1982; DeSalle et al. 1987). Transversions, however, appear to accumulate linearly with time (Miyamoto and Boyle 1989; Irwin et al. 1991). Thus, they are commonly given greater weight in phylogenetic analyses in which saturation is evident. We therefore performed analyses with different weighting schemes to examine their effect upon topological stability and confidence levels (see Results). The ratio of transitions to transversions (TS/TV) was plotted against percent sequence divergence (calculated with Kimura's two-parameter method, 1980) for all pairwise comparisons (fig. 1) to assess whether in-group comparisons were demonstrating TS saturation. Weightings were derived from linear regression of intraspecific comparisons and closely related taxa demonstrating a clear TS bias.

All phylogenetic trees were rooted with two species of the South American aplocheiloid genus *Cynolebias* representing outgroup taxa. The large number of taxa necessitated heuristic searches (MULPARS option in ef-

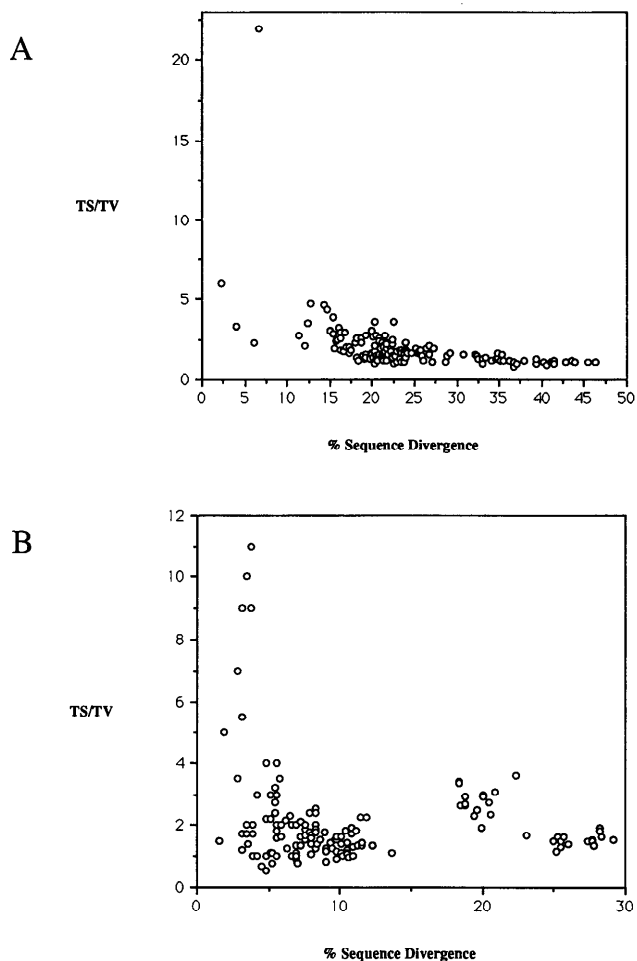


FIG. 1.—Ratio of transitions to transversions plotted against percent sequence divergence for (A) *cytb* and (B) 12S rRNA.

fect, random addition of taxa, TBR branch-swapping, 50 replications). Confidence limits for groups were assessed by the bootstrap method (Felsenstein 1985); 100 bootstrap replicates were performed for all analyses. Decay indices (DI; Bremer 1988; Mishler et al. 1991) were also computed for parsimony trees. The successive approximations approach was performed to choose among equally parsimonious cladograms, based on the rescaled consistency index (Farris 1969), and implementing a base weight of 1,000.

Results

Sequence Variation and Evolution

The alignments for these gene segments were unambiguous, with the exception of the first 21 base pairs of the 12S rRNA and a second region of five base pairs further downstream. Because several indels combined with multiple base substitutions present in these regions made the alignment questionable, these regions were omitted from the analyses. In the *cytb* segment, 182 sites were variable, 141 of which were phylogenetically informative (for parsimony). For the 328 alignable bases of the 12S data, 123 sites were variable, and 68 of these were phylogenetically informative (for parsimony). Plot-

ting TS/TV vs. sequence divergence (fig. 1) demonstrates a TS bias among closely related ingroup taxa. Saturation is particularly evident among several ingroup comparisons in both the *cytb* (around 20% Seq.Div., fig. 1A) and 12S rRNA (between 5% and 10% Seq.Div., fig. 1B) data sets.

Phylogenetic Analyses

Figure 2 shows the topology of equally weighted maximum parsimony (MP) trees for both the *cytb* and 12S rRNA data sets. Neighbor-joining (NJ) analysis of the *cytb* data resulted in a tree identical to the equal-weighted MP tree. NJ analysis of the 12S rRNA data set agreed in general with the MP tree, except that the *R. hartii*/*R. cylindraceus* grouping was found in a position basal to *R. amphoreus*. This grouping is suspect because of what appears to be a long-branch attraction. Further, this relationship is supported neither when weighting of TVs is increased from 5:1 to 10:1 (see below) over TSs, nor in TV parsimony (data not shown). The relationship is not supported in any other analyses, it is found in <50% of the bootstrap replicates, and this grouping decays one step from parsimony.

Weighted analyses were based on linear regression of TS/TV ratios of closely related taxa not exhibiting saturation and consistently grouped together in terminal portions of the trees. The pairwise comparisons used were *R. isthmensis*/*R. fuscolineatus*, *R. stagnatus*/*R. cryptocallus*, the two *R. tenuis* populations, and the two *cylindraceus* populations. These analyses resulted in a 3:1 ratio for *cytb* and 5:1 for 12S rRNA. When a 3:1 (TV:TS) weighting scheme was applied to the *cytb* data set, two trees were found that differed from the equal-weighted tree only in an ambiguous placement of *R. weberi* and a switch in position of *R. hartii* and *R. amphoreus* (denoted by asterisks in fig. 2). A single tree is found when weighting is increased to 10:1 or TVs only. This tree is identical to one of the two trees from 3:1 weighting. Hence, the *cytb* data appear relatively robust to weighting. Weighting TVs 5:1 over TSs in the 12S data set resulted in two equal-length trees, the consensus tree (not shown) being less resolved (differences denoted by asterisks in fig. 2). Increased weighting of TVs to 10:1 or 1:0 supports a monophyletic South American clade as the sister group of a monophyletic Central American/Columbian clade exclusive of *uroflammeus* and *hildebrandi*. These two clades are completely multifurcating in the TV-only analysis. The latter two taxa occupy a position basal to the previously mentioned taxa. The Antillean taxa remain the most basal lineages, *roloffi* being the deepest. These results indicate that the position of some taxa are not robust to weighting, suggesting possible effects due to homoplasy. However, transitions clearly provide significant phylogenetic signals in terminal relationships.

Figure 3 depicts a consensus tree of (1) a successive approximations (SA) tree of four equal-length trees from equal-weighted parsimony, (2) the NJ tree, and (3) a 3:1 (TV:TS) weighted MP tree (same topology as 5:1, 10:1, and TV only). The ambiguity in the South American clade results from the unstable position of *R.*

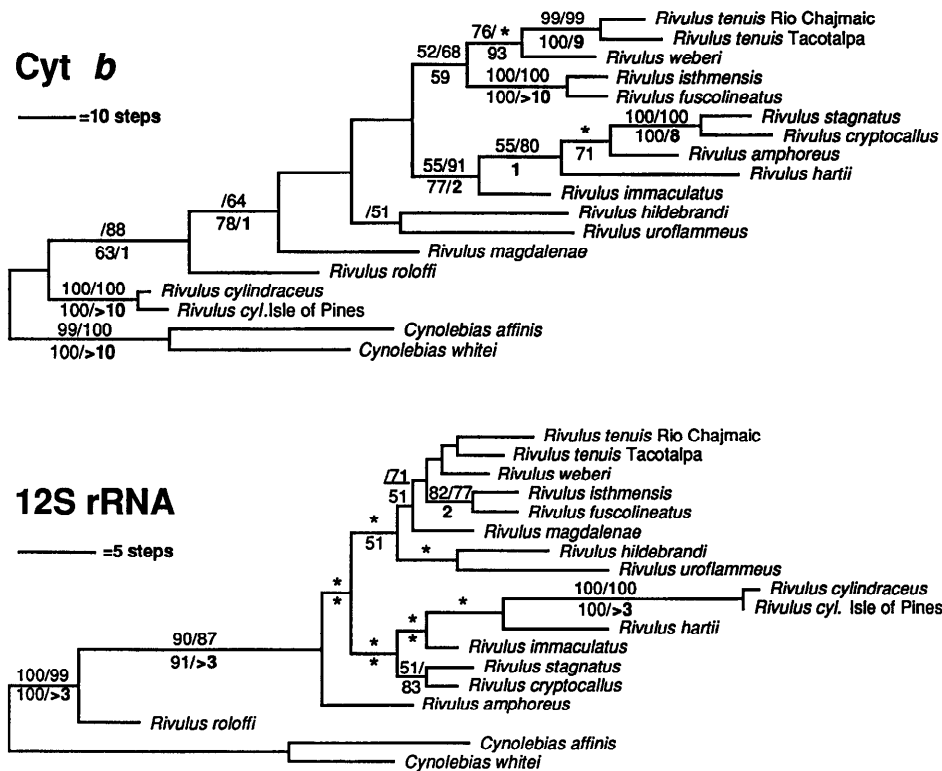


FIG. 2.—Maximum parsimony trees for *cytb* (TL = 543, CI = 0.440, RC = 0.214) and 12S rRNA (TL = 164, CI = 0.561, RC = 0.346) based on equal weighting of TS and TV. Bootstrap values are indicated above the branch for equal-weighted parsimony/weighted parsimony, and below the branch for neighbor-joining. Bremer decay indices are in boldface below the branch. The 12S rRNA tree indicates trees ≥ 3 steps from parsimony (as opposed to ≥ 10 steps) because of computational limits. Asterisks indicate nodes not supported by weighted parsimony or neighbor-joining.

hartii in the three analyses. The NJ tree and weighted MP trees agree in the grouping of *R. hildebrandi* and *R. uroflammeus*, while the SA tree groups *R. hildebrandi* and *R. magdalenae*. The NJ tree also differs from the weighted MP tree in that *R. magdalenae* is the most basal member of the Central American/Columbian

clade, while weighted MP places *hildebrandi* and *uroflammeus* at the base of this clade. *R. roloffii* is identified as the most basal ingroup taxon in all analyses, except weighted MP, in which *R. cylindraceus* occupies this position. However, the weighted MP bootstrap tree supports *roloffii* in the most basal position.

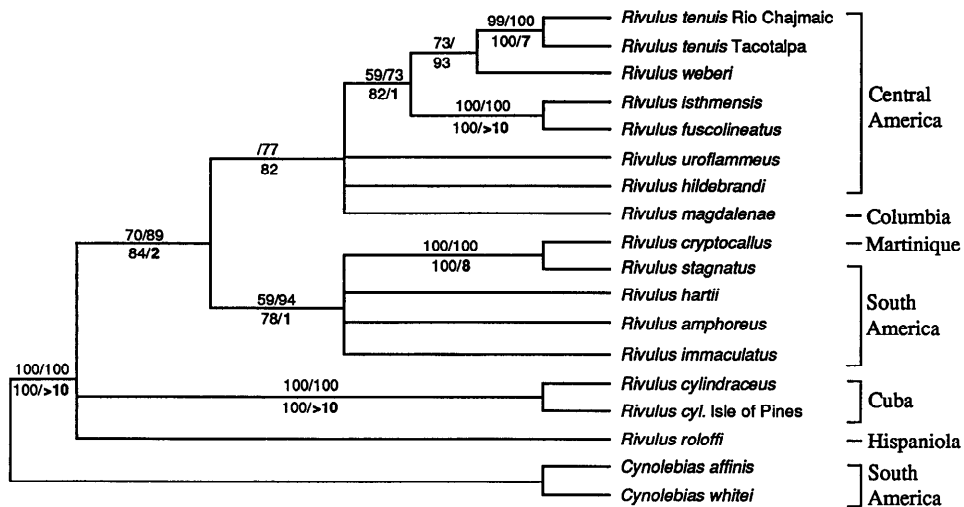


FIG. 3.—Consensus tree derived from (1) successive approximations tree of four minimum-length cladograms (TL = 717, CI = 0.462, RC = 0.233) from an equal-weighted MP analysis, (2) weighted parsimony tree, and (3) neighbor-joining tree of the combined data set. Bootstrap values are indicated above the branch for equal-weighted parsimony/weighted parsimony, and below the branch for neighbor-joining. Bremer decay indices are in boldface below the branch.

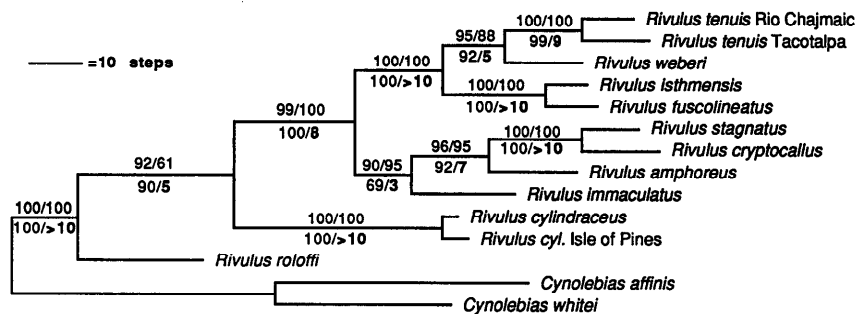


FIG. 4.—Single most parsimonious tree (TL = 498, CI = 0.574, RC = 0.354) for the combined data set based on equal weighting of TSs and TVs, with specific taxa omitted (see Results). Neighbor-joining and weighted MP analyses resulted in the same tree. Bootstrap values above the branch are for equal-weighted MP/weighted parsimony (3:1), and below the branch for neighbor-joining. Bremer decay indices are in boldface below the branch.

Bootstrap values based on 100 replications provided good support for most terminal relationships of the *cytb* tree, while the 12S rRNA tree shows few nodes with high bootstrap values. Decay indices correspond well to most nodes highly supported by the bootstrap. In the combined data set, most relationships identified in the consensus tree were identified in >70% of the bootstrap replicates. There appears to be consistent support for a Central American clade exclusive of *hildebrandi* and *uroflammeus*, which appears to be the sister group of a South American clade, while the Greater Antillean taxa represent the most basal lineages. These three major tenets are highly supported by the bootstrap when an analysis is performed (fig. 4) that omits taxa fluctuating in previous analyses (*hildebrandi*, *uroflammeus*, *magdalenae*, and *hartii*). Thus, the major uncertainty in our data set appears to center around the monophyly of the Central American taxa.

Discussion

The origin of the genus is clearly South American, as opposed to a suggested Central American cyprinodont origin (Briggs 1987). This is supported by two observations: (1) all other Neotropical genera are restricted to South America, which together form a monophyletic group (Parenti 1981; Costa 1990; unpublished data), and (2) the relative northward expansion of the majority of other cyprinodonts inhabiting the Neotropics (Bussing 1985). The phylogenetic hypotheses formulated from the separate and combined analyses suggest a vicariant origin of the Greater Antillean endemics, *R. cylindraceus* and *R. roloffi*. All analyses support quite earlier divergences for both species from other *Rivulus* taxa in this study, with the exception of a grouping of *R. hartii* and *R. cylindraceus* in the 12S rRNA data set, clearly a result of long-branch attraction. Further, *R. cylindraceus* and *R. roloffi* are not themselves closely related, consistent with long isolation on their respective islands. If these species had recently dispersed to the Greater Antilles, a closer alliance with taxa from northern South America or Central America would be expected.

A vicariance hypothesis is also supported by observed high levels of sequence divergence between the Antillean endemics and mainland taxa—averaging

22.1% for *cylindraceus* and 25.6% for *roloffi* for the *cytb* data. We note this in light of recent data from the poeciliid genus *Gambusia* (Lydeard et al. 1995a, 1995b) that suggest a dispersal origin for Antillean species based on low levels of *cytb* sequence divergence (1% to 2.8%) between island and mainland taxa from a segment encompassing the region we sequenced. These authors note, however, that pairwise comparisons between Antillean and mainland representatives of the *punctata* and *nicaraguensis* species groups reach 8.4% and may indicate vicariance. This is unlikely, given the extreme levels of divergence seen in *Rivulus*. If these species did diverge as the result of a vicariant event, then *cytb* must be evolving at at least twice the rate in *Rivulus* to account for the large differences seen between mainland and Antillean members of the two genera. Though we cannot determine this, it seems unlikely, given they are members of the same order. Poeciliids, *Gambusia* in particular, are potentially better colonizers than aplocheiloids because they are viviparous and have a higher tolerance for saltwater environments. Further, poeciliids are far more widespread throughout the Greater Antilles, while the two *Rivulus* species are restricted to the two largest islands, demonstrating their reduced dispersal abilities. These observations further support our hypothesis of vicariance for the Antillean endemics.

The remainder of the *Rivulus* species analyzed form two sister groups, a Central American/Columbian clade and a northern Venezuelan/Guiana clade. Within the former, *R. isthmensis*, *R. fuscolineatus*, *R. weberi*, and the two populations of *R. tenuis* are monophyletic. The monophyly of all Central American taxa is ambiguous, but the consistent deeper placement of *R. uroflammeus* and *R. hildebrandi* suggests these taxa represent a distinct invasion. The Venezuelan/Guiana clade includes the remaining species of *Rivulus*. Interestingly, the Martinique species *R. cryptocallus* is very closely related to *R. stagnatus* from Guyana, rather than to the more proximal species *R. hartii* from coastal Venezuela, Trinidad, and Margarita Island. This observation is consistent with phylogeographic hypotheses of boid snakes found in South America and the Lesser Antilles, in which species from St. Vincent and Grenada were found to have genetic affinities to species from Guyana (Henderson and

Hedges 1995). Thus, it appears that species inhabiting the Lesser Antilles arrived by recent dispersal from coastal South America.

These hypothesized relationships are supported by cytogenetic data (unpublished data). Members of the Central American/Columbian clade are all $2N = 46$, with the exception of *R. uroflammeus* ($2N = 44$). These karyotypes are characterized by chromosomes of relatively uniform size and a high proportion of metacentrics. In contrast, the members of the Venezuelan/Guiana clade are all $2N = 44$. These karyotypes are characterized by greater variation in chromosome size and a lower proportion of metacentrics. A distinctive large submetacentric is shared by *R. cryptocallus* and *R. stagnatus*. Of the two Greater Antillean endemics, *R. roloffii* is $2N = 44$ (unpublished data) and *R. cylindraceus* is $2N = 48$ (Scheel 1972).

Morphological differences are also consistent with the proposed relationships. Within the Central American/Columbian clade, *R. isthmensis*, *R. fuscolineatus*, *R. weberi*, and *R. tenuis* all have very similar meristics, including number of lateral line scales (Huber 1992). The molecularly divergent *R. hildebrandi* has a greater number of lateral line scales and *R. uroflammeus* has fewer relative to the other taxa in the clade. Further, the overall color patterns of *R. hildebrandi* and *R. uroflammeus* are unlike that shared by the other four Central American taxa examined. The Greater Antillean endemics *R. cylindraceus* and *R. roloffii* differ from all other *Rivulus* in this study by having a shorter standard length, reduced number of lateral line scales, greater body depth, and a reduced D/A (position of dorsal fin insertion relative to anal fin insertion) ratio (Huber 1992).

Present plate tectonic reconstructions show the Greater Antilles originating in the Pacific Ocean as a series of subduction-derived islands that drifted between the Americas, providing a temporary bridge between the two continents circa 80 million years ago (MYA) (Pindell et al. 1988; Ross and Scotese 1988; Pindell and Barrett 1990; Pitman et al. 1993). This arc was broken during the early Cenozoic and drifted to its present position. Present-day Central America also arose in the Pacific as an arc and migrated within close proximity to northwestern South America between 45 and 59 MYA (Ross and Scotese 1988; Pitman et al. 1993). More stable connections probably evolved during the Miocene until the final closure of Panama around 3 MYA.

The most recent biogeographical hypotheses concerning Central American and Caribbean freshwater fish (Briggs 1984, 1987; Bussing 1985) are based primarily on outdated geological models of the region. Bussing ascribed present Central American freshwater fish distributions to three distinct migratory events: (1) an early radiation into Central America during the Late Cretaceous (Old Southern Element), (2) a recent invasion from South America following the closing of the Panamanian Land Bridge 3 MYA (New Southern Element), and (3) a northern element that contains no cyprinodont members.

We can test this hypothesis with our current phylogenetic framework. The lack of fossil evidence pre-

cludes our ability to make precise estimates of minimum ages for the extant Central American fauna. Thus we estimated minimum divergence dates calibrated by the breakup of the proto-Antilles arc 70–80 MYA and using the average substitutions between *R. cylindraceus* and *R. roloffii* from the Central American taxa. We investigated rate homogeneity by the use of Tajima's (1993) test among the Antillean taxa and the Central American taxa. In the *cytb* data, 9 of the 165 three-way comparisons failed to support a constant rate of evolution, 8 of these including *R. hildebrandi*. In the 12S data set, two of the three-way comparisons were rejected, both involving *R. uroflammeus*. Further, these taxa varied in their placement in the phylogenetic analyses; thus, we did not include them in the divergence estimates. Since the five remaining Central American taxa demonstrate rate constancy, we estimated their divergence time to be 40–46 MYA based on this combined data set. Given the deeper phylogenetic position of *hildebrandi* and *uroflammeus* in all analyses, we conclude they arrived in Central America prior to this. Alternatively, they may represent descendants of a simultaneous paraphyletic invasion of Central America.

These findings are contrary to Bussing's definition of Old and New Southern Elements, of which these taxa appear to belong to neither. This is not surprising, given the extensive evidence for mammalian and herpetofaunal exchange throughout the Cenozoic (Estes and Báez 1985; Gingerich 1985; Webb 1985; Marshall and Semper 1993). However, there is a species we did not examine from central and eastern Panama, *Rivulus chucunaque*, that is morphologically very similar to *R. magdalena* from Columbia. Thus, it may represent a recent invasion into Central America following the closing of the Panamanian isthmus (circa 3 MYA), fitting the description of Bussing's New Southern Element. The addition of this taxon and other *Rivulus* species to the present data will further test the proposed biogeographical hypotheses. If *R. insulaepinorum* or *R. garciae* are indeed valid species, they would represent two additional Greater Antillean endemics that could contribute to the vicariance hypothesis. *Rivulus robustus* is sympatric with the northernmost populations of *R. tenuis* near Veracruz, Mexico. Its meristics, however, are quite similar to those of the Antillean endemics. Thus, it may represent a species isolated at the southern tip of North America following the migration of the proto-Antilles arc during the late Cretaceous. Finally, the relationships of the widespread *R. marmoratus* populations, found throughout the Caribbean and as far south as Rio de Janeiro, might provide some insights into the origins of hermaphroditism.

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meus. Jan Jap DeGreef provided *R. tenuis* Rio Chajmaic. Liz Hutchings, William McNiff, Darrell Ullisch, Edward Warner, and Al Castro, members of the American Killifish Association, provided aquarium stock of the remaining species. We would also like to thank Mark Buchheim, The University of Tulsa, for thoughtful discussion and computational assistance throughout the course of this project.

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