A Molecular Phylogeny for Aplocheiloid Fishes (Atherinomorpha, Cyprinodontiformes): The Role of Vicariance and the Origins of Annualism

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Annual aplocheiloid killifish embryos possess a rare ability among vertebrates to enter stages of developmental arrest (diapause) when subjected to adverse environmental conditions. Previous morphological analyses have presented disparate hypotheses regarding the evolution of the intriguing life history associated with this phenomenon. We present a novel hypothesis of aplocheiloid relationships based on 1,099 bp of sequence data from three mitochondrial genes (cytochrome *b*, 12S rRNA, and 16S rRNA). Phylogenetic analysis using maximum parsimony, neighbor-joining, and maximum likelihood produce strongly congruent topologies. Our data confirm the monophyly of the Neotropical family Rivulidae, while demonstrating a paraphyletic Old World assemblage. The basal sister group position of Indo-Malaysian and Madagascaran taxa relative to a monophyletic South American/African dichotomy strongly indicates the role of vicariance in the diversification of these fishes in spite of their definition as secondary freshwater fish. The distribution of annualism onto this topology implies a single early origin for this suite of characters, prior to the divergence of South American and African taxa. If so, then annualism has since been lost several times during the evolution of genera now residing in permanent aquatic habitats. Paleoclimatic knowledge complements this scenario based on molecular characters.

Introduction

The Order Cyprinodontiformes, commonly referred to as killifish, contains over 800 species of freshwater fish distributed circumtropically (west of Wallace's Line) and including some temperate regions of North America and Europe. Cyprinodonts are designated secondary freshwater fish for the ability of some species to reside in brackish environments. The most recent classification of these fish based on morphological characters (Parenti 1981) recognizes two major divisions within the order: the Aplocheiloidei and the Cyprinodontoidei. The aplocheiloids form an assemblage of approximately 350 species found in both the Old World and the New World (fig. 1 and table 1).

Parenti (1981) divided the suborder Aplocheiloidei into two families: the Old World Aplocheilidae and the Neotropical Rivulidae. Each of these families is composed of species that reside in permanent waters and those that inhabit temporary aquatic environments, influenced by the annual dry season. Those species residing in temporary bodies of water exhibit perhaps the most interesting reproductive adaptation within the Cyprinodontiformes, known as annualism (Myers 1942, 1952). Annual fishes spawn in contact with the mud-bottom of ponds or temporary pools in which the eggs, protected from drying by a thickened chorion, are laid. Development of these embryos is delayed by interposition of one to three diapauses, enabling them to survive the unpredictable dry season until the rains rewet the habitat. This phenomenon is exhibited to varying degrees by genera in both South America and Africa (table 2). Despite numerous behavioral, reproductive,

and phenotypic similarities shared by these genera, annualism is hypothesized to have arisen a minimum of two times: once in the Neotropics, and once in the Old World (Parenti 1981; Costa 1990*a*, 1990*b*). Mapping of annualism onto a phylogenetic hypothesis would allow one to determine whether it represents an independently derived trait on both continents, or if it is plesiomorphic and has been subsequently lost by some genera.

The breakup of Gondwanaland during the Cretaceous has been suggested as the mechanism for the divergence of Old and New World fauna (Parenti 1981). However, dispersalists cite the ability of some cyprinodonts to disperse through brackish or saltwater environments as their primary mechanism of radiation, and further hypothesize dispersal from a more recent center of origin in the Neotropics (Briggs 1987; Lundberg 1993). The credibility of this hypothesis is undermined by the fact that very few aplocheiloids are tolerant of saltwater environments. Recent phylogenetic analyses of northern species of the aplocheiloid genus *Rivulus* inhabiting the Caribbean region suggest that most island endemics arrived by vicariance events, although short-distance dispersal was indicated for the Martinique endemic *Rivulus cryptocallus* (Murphy and Collier 1996).

Because of the paucity of cyprinodont fossils with which to gauge past distributions, tests of vicariance-versus-dispersal hypotheses are dependent on phylogenetic analysis. Lundberg (1993) observed that Parenti's phylogeny (fig. 2*a*) depicts terminal clades representing genera occupying India–southeastern Asia and Madagascar–Seychelles (i.e., *Aplocheilus* and *Pachypanchax*, respectively) nested within an older clade, the divergences of which correspond to South American–African vicariance. If a vicariance model is invoked, the divergence pattern would suggest that the split between Africa and South America (106–95 MYA) occurred prior to the rifting of India–Madagascar from eastern Africa (150–135 MYA), an event inconsistent with known geological chronology (Reyment and Dingel 1987; Parrish 1993; Pitman et al. 1993; Smith, Smith, and Funnell

FIG. 1.—Geographical distribution of the suborder Aplocheiloidei.

1994). Thus, Lundberg (1993) supported a dispersal mechanism from Africa to Asia–Madagascar. A reassessment of the phylogenetic relationships of this group can potentially address these conflicting hypotheses. A topology congruent with Parenti's would lend support to the dispersal hypothesis. On the other hand, an earlier divergence of the Asian–Madagascaran taxa from the remaining aplocheiloids would be consistent with vicariance. We report molecular phylogenetic evidence from members of the major aplocheiloid groups to address questions of both biogeography and the origin of annualism.

Materials and Methods

A comprehensive list of the species analyzed, along with their current classifications and sources, can be found in table 1. The aim of this study is to sample the major genera of the Aplocheiloidei from major geographic areas in order to provide a framework for subsequent studies of more specific taxa. Most of the aplocheiloid genera sampled here are large; therefore, assessing the monophyly of any of these genera is beyond the scope of this study. Therefore, two to three morphologically diverse taxa were sampled from speciose genera—always from different defined species groups (see Scheel 1990; Huber 1992; Costa 1995a).

Preparations of mitochondrial DNA (mtDNA) were isolated by the sucrose-gradient method of Jones et al. (1988). Amplifications via the polymerase chain reaction (PCR, Saiki et al. 1988) and DNA sequencing were carried out using primers L1091 and H1478 (Kocher et al. 1989) for the 12S rRNA segment, primers 16Sar-L and 16Sbr-H (Palumbi 1991) for the 16S rRNA segment, and primers L14724 and H15149 (Kocher et al. 1989; Meyer et al. 1990) for the cytochrome b segment. Protocols for amplification and sequencing and the sequences for the following taxa have been previously published: Rivulus stagnatus, Rivulus tenuis, and Cynolebias affinis (for the cytochrome b and 12S rRNA segments, Murphy and Collier 1996). Some sequences were determined using an automated sequencing apparatus (Applied Biosystems 373 Stretch). Purified symmetric products were used in cycle-sequencing reactions using dye-labeled terminators and Ampli-TaqFS DNA polymerase (Applied Biosystems Inc.). These reactions were purified using Centri-sep columns (Princeton Separations) and rehydrated according to the manufacturer's instructions. Both strands were determined for all sequences.

Sequence alignments were performed with CLUSTAL V (Higgins, Bleasby, and Fuchs 1992). These initial alignments were manually inspected and modified, facilitated by comparison with proposed teleost secondary-structure models (Alvez-Gomez et al. 1995). Regions of sequence in which homology of sites was questionable were omitted from the analyses. In parsimony analyses, indels were coded as missing data. Sites containing indels or ambiguities were not included in distance analyses. Various statistics were calculated in MEGA (version 1.01; Kumar, Tamura, and Nei 1993).

Cladistic analyses were performed with PAUP version 3.1.1 (Swofford 1993). In all cases heuristic searches

Table 1
Classification of the Aplocheiloidei *Sensu* Parenti (1981) and *Sensu* Costa (1990a)* for Species Analyzed in the Present Study

	Geographic Origins of Taxa
Family Aplocheiloidei (Old World)	
Genus *Aplocheilus*	
lineatus........................	AS, India
panchax	AS, "Southeast Asia"
Genus *Pachypanchax*	
homolonotus	AS, Madagascar
playfairii	AS, Seychelles
Genus *Epiplatys*	
boulengeri	Sindara, Gabon
chaperi........................	AS, Ghana
roloffi	AS, Liberia
Genus *Aphyosemion*	
australe	AS, Gabon
bivittatum.....................	Funge, Cameroon
geryi ("*Roloffia*")...............	Robis 1, Sierra Leone
maeseni ("*Roloffia*")	Lola, Guinea
Genus *Fundulopanchax*	
mirabile	Takwai, Cameroon
occidentale ("*Roloffia*")..........	AS, Sierra Leone
sjoestedti	AS, Nigeria
Genus *Nothobranchius*	
kafuensis	Kayumi, Malawi
kirki	Chilwa, Malawi
Family Rivulidae (Neotropical)*	
Subfamily Cynolebiatinae	
Genus *Cynolebias*	
affinis.........................	Churco Ruta, 5 km 399, Uruguay
antenori	Ceará, Brazil
Genus *Leptolebias*	
citrinipinnis	AS, Brazil
Subfamily Rivulinae	
Genus *Austrofundulus*	
limnaeus.......................	Maracaibo Basin, Edo Zulia, Venezuela
Genus *Rivulus*	
stagnatus	Gluck Island, Guyana
tenuis	Tacotalpa, Mexico
Genus *Trigonectes*	
rubromarginatus	BS 94/2 Aruanã, Brazil

NOTE.—As = aquarium strain of nonspecific origin.

Table 2
List of Aplocheiloid Genera and Their Characteristics with Regard to Annualism (Wourms 1972c)

Continent	Genus	Lifestyle	D1	D2	D3
South America (Rivulidae) ...	*Austrofundulus*	Annual	F	O	O
	Cynolebias and related genera[a]	Annual	F	F	O
	Moema	Annual	?	?	?
	Neofundulus	Annual	?	?	?
	Pituna	Annual	?	?	?
	Pterolebias	Annual	F	O	O
	Rachovia	Annual	F	O	O
	Rivulus	Nonannual	—	—	—
	Terranatos	Annual	F	O	O
	Trigonectes	Annual	?	?	?
Africa (Aplocheilidae)	*Aphyosemion*	Nonannual	—	—	—
	Epiplatys	Nonannual	—	—	—
	Fundulopanchax	Annual	F	F/O	O
	Nothobranchius	Annual	F	F	O
	Pachypanchax	Nonannual	—	—	—
	"*Roloffia*"	Annual/nonannual	F	O	O
Asia (Aplocheilidae).........	*Aplocheilus*	Nonannual	—	—	—

NOTE.—D1–3 = diapauses 1–3, F = facultative, O = obligate.
[a] Includes the genera *Campellolebias, Cynopoecilus, Leptolebias,* and *Plesiolebias.*

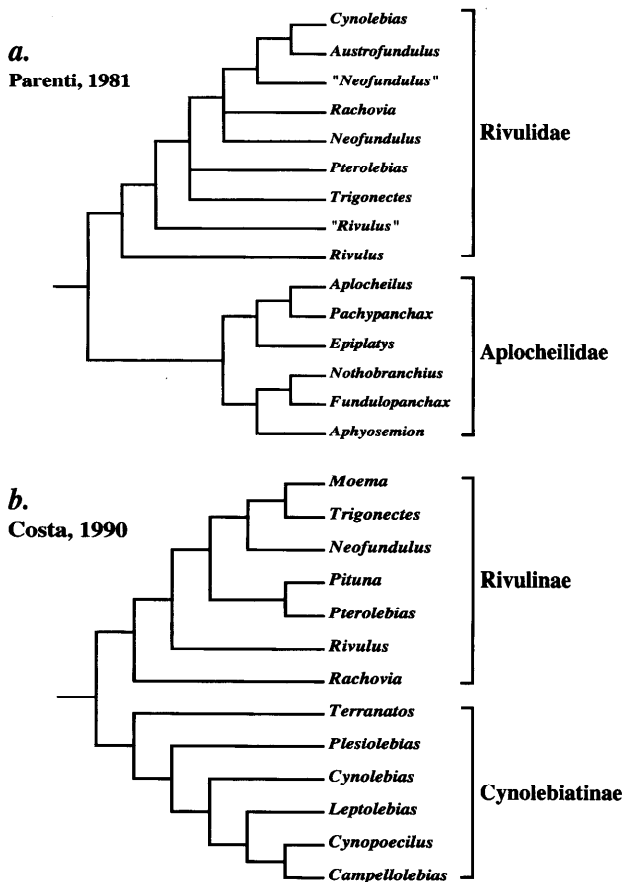*a.*
Parenti, 1981

Cynolebias
Austrofundulus
"Neofundulus"
Rachovia
Neofundulus
Pterolebias
Trigonectes
"Rivulus"
Rivulus

Rivulidae

Aplocheilus
Pachypanchax
Epiplatys
Nothobranchius
Fundulopanchax
Aphyosemion

Aplocheilidae

b.
Costa, 1990

Moema
Trigonectes
Neofundulus
Pituna
Pterolebias
Rivulus
Rachovia

Rivulinae

Terranatos
Plesiolebias
Cynolebias
Leptolebias
Cynopoecilus
Campellolebias

Cynolebiatinae

FIG. 2.—*a*, Phylogeny of the suborder Aplocheiloidei proposed by Parenti (1981) based on cladistic analysis of morphological characters. The following genera were synonymized by Parenti (1981) under *Cynolebias*: *Campellolebias, Cynopoecilus, Cynolebias, Simpsonichthys,* and *Terranatos*. *b*, Phylogeny of the Neotropical family Rivulidae as proposed by Costa (1990*a*, 1990*b*) based on cladistic analysis of morphological characters.

(50 replications, ACCTRAN option in effect, TBR branch swapping, random addition of taxa) were performed. Neighbor-joining (NJ) trees (Saitou and Nei 1987) were generated with MEGA (Kumar, Tamura, and Nei 1993). Maximum-likelihood (ML) trees were calculated in fastDNAML (Olsen et al. 1994). All trees were rooted with representatives of the families Fundulidae (*Fundulus heteroclitus*) and Cyprinodontidae (*Jordanella floridae*), both members of the aplocheiloid's sister group Cyprinodontoidei (Parenti 1981). Support for groupings in parsimony analyses was assessed using the bootstrap (Felsenstein 1985), implemented in PAUP 3.1.1 (Swofford 1993). Five hundred replicates were used for all bootstrap analyses. Interior-branch confidence probabilities (P_C, Rzhetsky and Nei 1992) were calculated in MEGA for NJ trees (Kumar, Tamura, and Nei 1993). It has recently been shown that the interior-branch test gives a more accurate estimate of the statistical confidence for a branch than does the bootstrap P value (Sitnikova, Rzhetsky, and Nei 1995).

We performed initial analyses with equal weighting of transitions (Ts) and transversions (Tv). However, our current knowledge of cytochrome *b* evolution in fishes

indicates that changes in third codon positions become saturated relatively quickly and can contribute significant phylogenetic noise between divergent comparisons (see Meyer 1993, 1994 for reviews). This phenomenon is also prevalent in first-position leucine Ts. Therefore, analyses were also performed using only conservative substitutions for cytochrome *b* (all substitutions excluding leucine Ts in first codon positions and all Ts in third positions; Irwin, Kocher and Wilson 1991). Transitions are also known to saturate with increasing divergence time in mitochondrial rRNAs, although not significantly until divergences of approximately 150 MYA (Mindell and Honeycutt 1990). Because the original aplocheiloid diversification may have been of this age, we also examined the effects of increasing the weight of Tv over Ts for all positions to counteract the potential effects of homoplasy at deeper positions in the phylogeny.

Results
Sequence Characteristics

The combined data contain 1,099 nucleotides (315 bp of 12S rRNA, 442 bp of 16S rRNA, and 360 bp of cytochrome *b*) following removal of 18 unalignable sites from the 12S data set and 78 unalignable sites from the 16S data set. The complete sequences for each gene segment have been deposited in GenBank under accession numbers U73233–U73280. The deleted sites generally correspond to inferred loop regions in the rRNA secondary structure (Alvez-Gomez et al. 1995). In the total analyzed data set, 575 sites were variable, 443 of these considered phylogenetically informative under parsimony. Relative base frequencies for the total data set were 30% A, 27% T, 23% C, and 20% G. Plotting observed transitions and transversions versus sequence divergence for the cytochrome *b* segment indicates a relatively linear relationship at first and second positions (fig. 3*a*), with Ts exceeding Tv in all comparisons. In contrast is the scattered, nonlinear relationship of substitutions versus sequence divergence at third-codon-position changes (fig. 3*b*), in which Ts and Tv are equally abundant, indicating saturation at these sites Similar to figure 3*a*, the rRNA data show a linear relationship, thus suggesting they are not exhibiting any appreciable saturation (fig. 3*c*).

Phylogenetic Analysis

Maximum-parsimony (MP) analysis yielded one tree (total length [TL] = 2,344, consistency index [CI] = 0.385, retention index [RI] = 0.452) when Ts were given equal weight to Tv (fig 4). Analysis of all rRNA sites and conservative substitutions for cyt*b* yielded four equal-length trees (TL = 1,679, CI = 0.489, RI = 0.553), all largely congruent with the topology in figure 4. The increases in CI and RI values are likely due to the deletion of noisy third-position Ts and first-position leucine Ts in the cytochrome *b* gene segment. Differences among the four trees were due to ambiguity in the placement of *Roloffia maeseni* relative to the other *Roloffia* species, and the relationships between the two *Rivulus* species and *Trigonectes rubromarginatus*. The

**a. Cytochrome b
1st & 2nd Positions**

Observed
Substitutions

%Sequence Divergence

**b. Cytochrome b
3rd Positions**

Observed
Substitutions

%Sequence Divergence

c. 12S & 16S rRNA
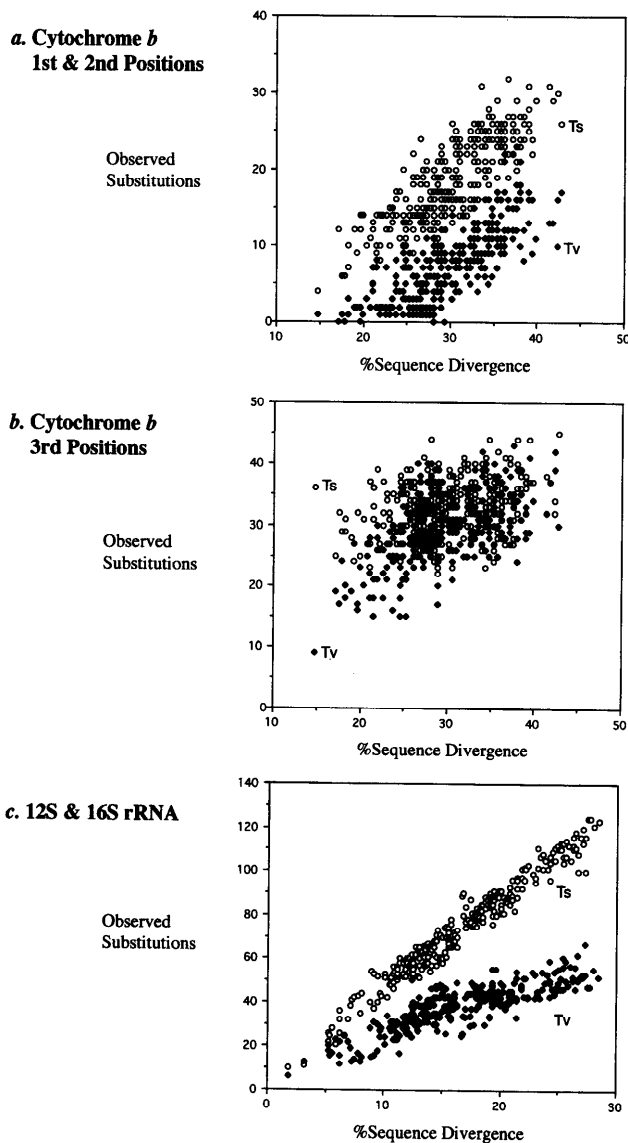
Observed
Substitutions

%Sequence Divergence

FIG. 3.—Plot of the observed number of transitions versus percent sequence divergence (Jukes and Cantor 1969). *a*, First and second positions of the cytochrome *b* segment. *b*, Third positions of the cytochrome *b* segment. *c*, Combined 12S and 16S rRNA data sets.

bootstrap results of this data set grouped *Rivulus stagnatus* and *T. rubromarginatus* as sister species (49% replicates). A single tree (TL = 3,877) was produced when Tv were weighted 3:1 over Ts. This tree was identical to the equal-weighted tree with the exception of *Epiplatys boulengeri* and *E. chaperi* being sister taxa. These two taxa were also grouped together in the bootstrap analysis based on equal weights (40% of all replicates). Further increasing the weight of Tv over Ts had no effect on the overall topology. All analyses show a monophyletic Neotropical clade as the sister group of an African clade. *Pachypanchax* and *Aplocheilus* also consistently form a monophyletic group that occupies the most basal ingroup position.

We used Jukes-Cantor (1969) distances to construct the NJ tree (fig. 5), because in our data set distances ranged almost entirely between 0.1 and 0.3, the Ts/Tv

ratios ranged between 1.15 and 2.78, and the base compositions were not strongly biased (as per Kumar, Tamura, and Nei 1993). These distances differed little from those produced with Kimura (1980) or Tamura (1992) corrections. Under such conditions, the simpler distance correction is preferable due to the lower variance of the estimates (Kumar, Tamura, and Nei 1993). Trees produced using different distance corrections for Ts bias (Kimura 1980) and both Ts and base composition biases (Tamura 1992) were identical to those of figure 4. P_C values for these alternative distance analyses also varied little, if at all, with those shown in figure 4. The NJ topology differs from the MP topology shown in figure 3 only in the interrelationships of the *Epiplatys* species. This tree is identical to the equal-weighted MP bootstrap tree and the 3:1 weighted MP/bootstrap tree. The ML analysis produced the same topology (ln likelihood = −12371.40747) as the NJ tree.

Discussion
Neotropical Clade (Rivulidae)

The Neotropical family Rivulidae was originally diagnosed as monophyletic by Parenti (1981) on the basis of nine anatomical characters. This finding was corroborated by Costa (1990a) and is convincingly supported by these molecular data (100% of all bootstrap replicates, P_C = 99%). However, character support in Parenti's analysis was lacking for many relationships within this clade, resulting in two trichotomies (fig. 2a). A more recent analysis (Costa 1990a) based on a larger number of characters and taxa resulted in a better resolved phylogeny (fig. 2b). This phylogeny has since been revised (Costa 1995a, 1995b), although the major relationships remain unaltered. While supporting the monophyly of this group, two major lineages were defined within the family: the Rivulinae and the Cynolebiatinae. In Costa's (1990a) results, the nonannual genus *Rivulus* is nested within a clade of annual genera. Therefore, annualism was considered the plesiomorphic condition of the Rivulidae.

Our molecular data support Costa's topology, identifying two distinct subfamilies. *Austrofundulus* (synonymized under *Rachovia*, Costa 1990a, 1990b) represents an earlier divergent genus within the Rivulinae, while *Rivulus* and *Trigonectes* are more derived. Further, the cynolebiatine genera *Cynolebias* and *Leptolebias* are basal and are deeply divergent. Parenti (1981) chose to synonymize all cynolebiatine genera, while Costa maintains six distinct genera (fig. 2b). The substantial molecular divergence between these two genera demonstrates their distinctiveness and supports Costa's division. Although this study contains only seven species from five genera of the Rivulidae, DNA sequence data from a broader sampling of Neotropical genera also support these basic findings (unpublished data).

African Clade

Our data support the monophyly of the African taxa, which is divided into two clades. The first is a monophyletic group of three genera, *Aphyosemion, Fun-*
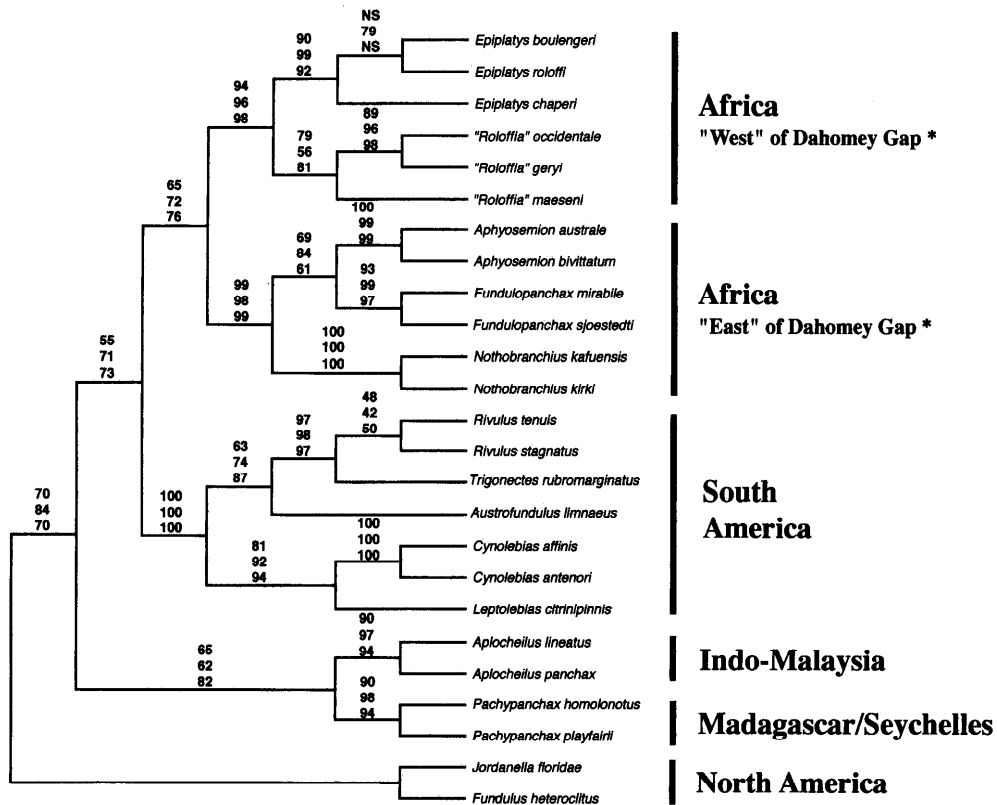
FIG. 4.—Phylogenetic hypothesis for the Aplocheiloidei based on parsimony analysis of the total data set. Bootstrap values (500 replicates) are listed above the branches in descending order for: equal-weighted MP, MP using conservative substitutions for cyt*b* (see *Materials and Methods*) and all sites for the 12S and 16S rRNAs, and MP with Tv weighted three times Ts. The latter analysis did not support the shown relationships within *Epiplatys,* instead grouping *E. boulengeri* and *E. chaperi* (78% of replicates). Asterisks marking the labels "West" and "East" of the Dahomey Gap indicate general distributions of sampled taxa, but note exceptions to these definitions in the *Discussion.*

dulopanchax, and *Nothobranchius,* previously supported based on morphology (Parenti 1981). Our analyses differ from this traditional hypotheses by grouping the geographically proximal *Aphyosemion* and *Fundulopanchax.* The second clade is composed of the genus *Epiplatys* and species of the genus "*Roloffia*" (*sensu* Clausen 1966), a name ruled invalid by ICZN opinion 1010. We use the name "*Roloffia*" here only as a matter of convenience to denote this clearly monophyletic group of taxa.

Parenti (1981) considered *Epiplatys* to be the sister group of *Aplocheilus* and *Pachypanchax.* Our results do concur with the sister group status of *Aplocheilus* and *Pachypanchax,* but in contrast place these genera at the base of the aplocheiloid tree, demonstrating the paraphyly of Parenti's (1981) Aplocheilidae. The biogeographical significance of the phylogenetic placement of these two genera is discussed below.

Biogeography

Although some of our hypothesized phylogenetic relationships are different from more traditional views, our area cladogram (fig. 4) is completely congruent with the breakup of Gondwanaland. These data resolve the biogeographical conflict discussed by Lundberg (1993) (see *Introduction*) and further identify four major biogeographical patterns within the Aplocheiloidei. First, the basal position of the *Aplocheilus-Pachypanchax*

clade suggests these taxa began diverging from the ancestor of the African–Neotropical clade with the drifting of the India–Madagascar subcontinent beginning sometime in the Late Jurassic. This initial breakup is thought to have begun ~165 MYA (Rabinowitz, Coffin, and Falvey 1983), though paleogeographic reconstructions suggest this separation may not have been complete until ~150–135 MYA (Smith, Smith, and Funnell 1994). Second, *Aplocheilus* and *Pachypanchax* each form a monophyletic group, and their divergence most likely reflects the breakup of India and Madagascar circa 88 MYA (Storey et al. 1995). Evidence against the dispersal of these fishes from Africa to Madagascar resides in the observation that *Pachypanchax* is endemic to Madagascar (*homolonotus*) and the Seychelles (*playfairii*) and that this genus has no close relatives on continental Africa.

The third biogeographical pattern apparent from the remaining major clades is an African–South American dichotomy (fig. 4), consistent with the final separation of these continents between 106 and 95 MYA (Reyment and Dingel 1987; Parrish 1993; Pitman et al. 1993). Within the African clade we see a fourth geographical distinction in that the members of the "*Roloffia*"-*Epiplatys* clade are most abundant west of the Dahomey Gap, a region of dry savanna habitat that divides the African rain forest into western and eastern components.
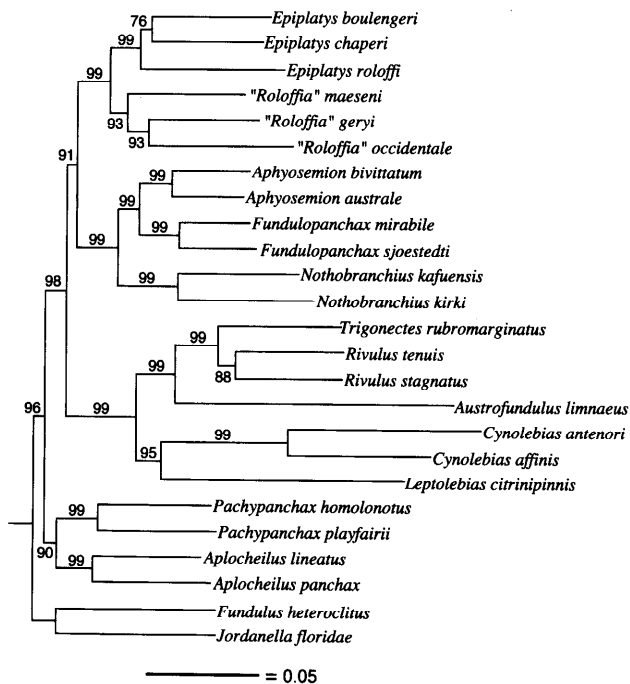
FIG. 5.—Neighbor-joining tree based on Jukes-Cantor (1969) distances of the total data set, excluding sites with indels or ambiguities. A distance scale is shown below the tree. Interior-branch test confidence values (Rzhetsky and Nei 1992) are listed next to their respective branches. The maximum-likelihood tree had the same topology (ln likelihood = −12371.40747).

The species defined here as *"Roloffia"* are restricted to the western component, from Gambia southeast to Ivory Coast and western Ghana. *Epiplatys* species are also abundant west of the Dahomey Gap, but the range of this genus extends eastward to overlap the range of *Aphyosemion* throughout the Zaire Basin. *Aphyosemion* and *Fundulopanchax* are absent west of the Dahomey Gap, with the exception of *F. walkeri* found in Ghana and Ivory Coast.

These disjunct geographic distributions imply some mechanism for the divergence of taxa east and west of the Dahomey Gap. We hypothesize that the presence of an epicontinental sea which extended north from present-day Nigeria and Dahomey and south from the Tethys Sea was the causative factor for the ancient isolation of these clades. Beginning in the Late Cenomanian (~95–92 MYA) and lasting until the Early Eocene (~53 MYA), several trans-Saharan transgressions occurred due to fluctuations in eustatic sea level (Reyment and Dingle 1987; Smith, Smith, and Funnell 1994). The subsidence of these transgressions, and expansion of savanna habitat, may have allowed the eastward expansion of *Epiplatys* through savanna habitat. A more comprehensive analysis of the genus *Epiplatys* will test this hypothesis.

Dispersalism Versus Vicariance

Lundberg (1993) recently reviewed the literature regarding potential South American–African relationships in freshwater fishes and concluded that among 13 identified candidates, only three strongly supported vi-

cariance: doradoid catfishes, lepidosirenid lungfishes, and polypterid reedfishes. He rejected a vicariance hypothesis for cyprinodontiforms based on the following arguments: (1) The inconsistency of Parenti's phylogeny with a strict breakup of Gondwanaland; (2) the observation that most cyprinodonts are saltwater tolerant; (3) the observation that the Neotropical distribution of taxa is more general than that of the Old World, implying a west to east dispersal from South America to Africa; and (4) the lack of fossil data supporting a Mesozoic diversification of cyprinodonts. Briggs (1987) concurs with a dispersal scenario and further suggests a Central American origin for the order. We will discuss the weaknesses of each of these arguments below.

First, the topology supported by our molecular data has no timing inconsistency (fig. 4), eliminating the first argument. Second, it is misleading to generalize concerning the ability of cyprinodotiforms to inhabit coastal-brackish environments. While numerous cyprinodontoid genera fit this description (e.g., *Fundulus, Cyprinodon*, various poeciliid genera), as a rule aplocheiloids are restricted to inland river drainages. There are two exceptions to this condition: the widespread hermaphroditic *Rivulus marmoratus* species group, which inhabits coastal mangroves throughout the Gulf of Mexico and along the South American coast to Rio de Janeiro, and the closely related species *Rivulus caudomarginatus* found in similar habitat in Rio de Janeiro (Huber 1992). However, *Rivulus* occupies a relatively terminal position in the aplocheiloid phylogeny, suggesting saltwater tolerance is a secondarily derived condition in these fishes. Other aplocheiloid island endemics from the Greater Antilles (*Rivulus cylindraceus* and *R. roloffi*) and from Madagascar (*Pachypanchax homolonotus*) show no recent phylogenetic affinity to adjacent mainland taxa and represent basal lineages within their respective frameworks (Murphy and Collier 1996; this study). These observations strongly suggest vicariance as the predominant historical force driving divergence of the Aplocheiloidei.

A third argument for dispersalism, made by both Briggs (1987) and Lundberg (1993), is that since the Neotropics contain the highest generic diversity, then this likely was the center of origin for all cyprinodontiforms. However, generic diversity does not reflect species diversity, in which case the Old World Aplocheilidae contains more currently described species (~189; Scheel 1990) than the Neotropical Rivulinae (~160; Costa 1990b). More important though, is the fact that South America and Africa have undergone radically different climatic histories since their separation in the Cretaceous (Goldblatt 1993). The differences in the number of genera and the extent of their distributions are probably the result of 90–100 Myr of different abiotic influences.

Finally, as for the lack of fossil support for a vicariance hypothesis, we emphasize that fossil data represent only minimum ages for specified groups (Parenti 1981; Parker and Kornfield 1995). The oldest known cyprinodontiform fossil is of Oligocene age (Sauvage 1874), while our molecular phylogeny indicates perhaps
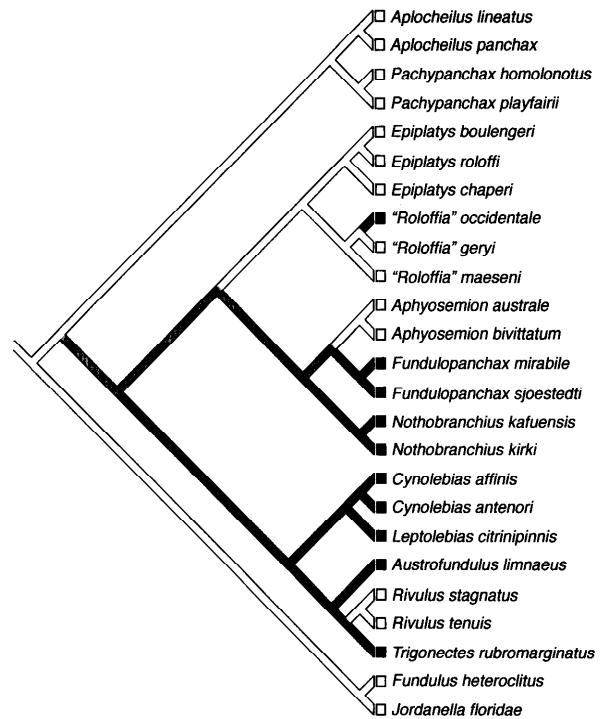
a late Jurassic diversification for the Aplocheiloidei. The sister group status of the Cyprinodontoidei implies that their origin must also be of at least this age. Parker and Kornfield (1995) have presented a Late Jurassic–Early Cretaceous scenario for the cyprinodontoid family Cyprinodontidae, thus further supporting a Mesozoic aplocheiloid diversification.

Annualism

The annual aplocheiloids are distinguished from nonannual teleosts by their unique life history. These fish usually reside in temporary pools found in savanna or even forested regions which regularly dry up. Survival of the population depends on the eggs deposited in the mud bottoms. With the onset of the rainy season, the rains rewet the dried ponds and stimulate hatching of the young. Growth is rapid, with some species attaining sexual maturity in as little as 3–5 weeks (Taphorn and Thomerson 1978).

The most remarkable aspects of annualism are those pertaining to embryogenesis (Peters 1963, 1965; Wourms 1963, 1972a, 1972b, 1972c). During normal teleost development a series of cleavage stages produces two populations of blastomeres: peripheral blastomeres which form the enveloping cell layer (ECL), and deep blastomeres which give rise to the embryo. In nonannual teleosts these deep cells aggregate during epiboly to form the germ ring (e.g., Fundulus heteroclitus; Armstrong and Child 1965). In contrast, annual fishes demonstrate a separation of epiboly and embryo formation (Wourms 1972a). When epiboly commences, the population of deep blastomeres which would normally coalesce and proceed through embryogenesis instead become amoeboid and disperse over the surface of the egg in the space between the ECL and the internal yolk syncitial layer. This dispersed phase normally lasts until 2–3 days after completion of epiboly. The amoeboid blastomeres then reaggregate and continue development through a normal series of stages. This dispersion–reaggregation is correlated with the annual life history (Wourms 1972b). All annual genera exhibit this phenomenon, while nonannuals do not (table 2).

The ability of annual populations to survive dry seasons is attributable to (1) modifications of the chorion and (2) the potential ability to enter diapause at any of three embryonic stages (the dispersed cell phase [D1], the long somite embryo [D2], and the prehatching stage [D3] to prolong their development (Wourms 1972c). Each of these diapauses is defined as either facultative or obligate, with annual genera differing only in the condition of D2 (table 2). Wourms (1972b, 1972c) defines the dispersed cell phase as a "developmental switch" which allows the embryo to enter diapause if environmental conditions are unfavorable. These pluripotent dispersed cells act as a developmental buffer such that destruction of some can be replaced by others through mitosis. D2 and D3 also represent stages that are apparently developmentally insensitive (Wourms 1972c). The ability of some populations of "escape eggs" to avoid diapause presumably allows up to eight categories of eggs to develop in different time frames. This "mul-

| Aplocheilus lineatus |
| Aplocheilus panchax |
| Pachypanchax homolonotus |
| Pachypanchax playfairii |
| Epiplatys boulengeri |
| Epiplatys roloffi |
| Epiplatys chaperi |
| "Roloffia" occidentale |
| "Roloffia" geryi |
| "Roloffia" maeseni |
| Aphyosemion australe |
| Aphyosemion bivittatum |
| Fundulopanchax mirabile |
| Fundulopanchax sjoestedti |
| Nothobranchius kafuensis |
| Nothobranchius kirki |
| Cynolebias affinis |
| Cynolebias antenori |
| Leptolebias citrinipinnis |
| Austrofundulus limnaeus |
| Rivulus stagnatus |
| Rivulus tenuis |
| Trigonectes rubromarginatus |
| Fundulus heteroclitus |
| Jordanella floridae |

	# of Steps			
Weight	1 Origin	2 Origins	3 Origins	4 Origins
Equal	6	5	5	5
2:1	7	7	8	9
3:1	8	9	11	13

FIG. 6.—Distribution of annualism onto the proposed molecular phylogeny. Solid branches represent annual lineages, while hatched branches indicate lineages on which mapping of annualism was equivocal with respect to different hypotheses. Character evolution was traced with MacClade (version 3.05; Maddison and Maddison 1995). The legend below the tree indicates the number of steps required if gains and losses require equal numbers of steps or if gains are given successively greater weight than losses (e.g., 2:1 and 3:1). See text for discussion of alternative hypotheses.

tiplier effect" counteracts any false hatching due to irregular rainfall and ensures some embryos will survive (Wourms 1972c).

Mapping annualism onto our topology does not decisively distinguish between alternative numbers of origins. Three scenarios are suggested, each requiring five steps: annualism being gained independently three times, being gained independently four times, or being gained once early, lost three times, and regained in the ancestors of the large "Roloffia" species (see fig. 6). Alternatively, annualism may have arisen once early and since been lost independently five times—this scenario requiring one extra step. However, these scenarios rest on the unrealistic assumption that it is equally as easy to gain annualism as it is to lose it. Gaining annualism requires the acquisition of the following characters: (1) modifications (thickening) of the chorion that withstand desiccation, (2) the ability to delay development (diapause) at three different stages, (3) behavioral modifications necessary for bottom spawning (i.e., egg burying), and (4) the presumed physiological changes to allow a rapid rate of growth to sexual maturity. Thus, annualism is a complex character, and it seems likely

that the gain of this character should be weighted more heavily than its loss. If we assign increasing weight to gaining annualism relative to losing it, an earlier origin does require fewer steps than does gaining it several independent times (see fig. 6). Furthermore, the presence of three apparently morphologically/physiologically homologous diapause stages in all annual species (Wourms 1972c) is suggestive of common ancestry.

An early, possibly single, origin for annualism is also consistent with our current understanding of Gondwanan paleoclimatology since the Mesozoic. Climatic reconstructions indicate that arid or seasonally dry conditions were prevalent in low–midlatitude regions of Gondwanaland throughout the Mesozoic (Wing and Sues 1992; Coetzee 1993). During the Cretaceous, the ability to postpone development during developmentally insensitive times (Wourms 1972b, 1972c) was favored in populations occupying these seasonally dry climates. Morphology (Costa 1990a, 1990b) and our molecular based phylogeny are congruent with annualism being the plesiomorphic condition for Neotropical aplocheiloids. Thus, annualism arose at least as early as Late Cretaceous. Wetter climates did not return to midlatitude regions until the Cretaceous–Tertiary boundary, and probably not in equatorial regions until the mid-Tertiary (Wing and Sues 1992; Coetzee 1993). Populations exposed to increasingly regular rainfall and persistent habitat would simply not enter diapause. This presumably was the case in the ancestors of the current nonannual genera. Subsequent exposure to seasonally dry conditions would allow a potential reversion to an annual lifestyle (i.e., "*Roloffia*" *occidentale* and sister species), assuming the ancestors already harbored the genetic repertoire to undergo diapause. Continued phylogenetic sampling within the Aplocheiloidei, particularly from the Neotropical Rivulidae, will further our knowledge of the evolutionary history of annualism.

Acknowledgments

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