

Fish Biogeography and Molecular Clocks: Perspectives from the Panamanian Isthmus

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I. Introduction

Panama provides a rich landscape against which to study the evolution of fish and molecules. This is because roughly 3 million years ago part of Panama rose to complete the Central American fusion of the North and South American continents. In the process, a marine connection between the eastern Pacific Ocean and the Caribbean Sea was severed. Thus, the Pliocene rise of the Isthmus of Panama initiated, or perhaps continued, an evolutionary experiment of grand scale (Rubinoff and Leigh, 1990). Jordan (1908) developed his "law of geminate species" based on his observations of marine fish "sister" taxa found on either side of the Panamanian isthmus. Seventy years later Vawter and co-workers (1980) used Jordan's geminate taxa to test and support the "molecular clock" hypothesis.

The Central American Isthmus has also played a prominent role in discussions pertaining to the dispersal and diversification of neotropical freshwater fishes. Darlington (1957) suggested that the South American freshwater fish fauna had been derived from Asian immigrants filtering through North and Central America, although several years later he began changing his view (Darlington, 1964). Myers (1966) and Miller (1966) formally recognized the importance of Panama's narrow isthmus as a terrestrial corridor along and

through which the primary freshwater fishes of South America have invaded Central America. Bussing (1976, 1985) presented a more complete analysis of the origin of the region's freshwater fish fauna and supported an early Tertiary arrival in Central America for some characiform fishes. Nevertheless, he also recognized that the vast majority of primary freshwater fish reached Central America sometime in the Pliocene. Because the invasion of Central America by South American freshwater fish is believed recent, molecule-based biogeographic analyses provide one of the best opportunities to chronicle a major colonization episode and the subsequent assembly and diversification of a modern freshwater fish fauna.

This chapter focuses on fish biogeography, particularly the geography of conspecific populations of tropical marine and freshwater fishes. At the core of this discussion lies the idea that molecule-based phylogenetic hypotheses are particularly useful for historical biogeographic analyses of recent earth history events (Bermingham *et al.*, 1992). It is often the case that morphologically monotypic species with ranges that cross one or more centers of endemism exhibit molecular divergence. For example, mitochondrial DNA (mtDNA) phylogeography has demonstrated convincingly that differentiated conspecific populations can provide historical information about a region (Bermingham and

Awise, 1986; Awise *et al.*, 1987; Bermingham *et al.*, 1992, 1996; Awise, 1994; Seutin *et al.*, 1994; Joseph *et al.*, 1995). Furthermore, to the extent that molecular clocks are reliable, one can establish the approximate chronology of branch points in molecular phylogenies (Bermingham *et al.*, 1992; Page, 1991, 1993; Bermingham and Lessios, 1993; Knowlton *et al.*, 1993). In turn, these chronologies can be used to test hypotheses related to geologically dated vicariant events (Sarich and Wilson, 1967; Beverly and Wilson, 1985; Bermingham *et al.*, 1992; Joseph *et al.*, 1995). Molecular data permit one to assess whether both the branching pattern and the general timing of speciation events conform to vicariant models of species diversification.

Fishes collected in the marine and freshwater environments of Panama have provided opportunities to study fish molecular clocks and historical biogeography within a recent time frame of earth history. This time frame is appropriate to empirically document the role that historical contingency plays in the assembly and maintenance of biological communities. Such historical reconstructions help to convey the dynamics of evolutionary changes in species and of ecological changes in communities (Cornell and Lawton, 1992; Ricklefs and Schluter, 1993). It is already the case that studies of tropical species have documented levels of genetic divergence between geographic populations that are generally high in comparison to temperate species (Capparella, 1991; Hackett and Rosenberg, 1990; Escalante-Pliago, 1991; Patton and Smith, 1992; Peterson *et al.*, 1992; daSilva and Patton, 1993; Seutin *et al.*, 1993, 1994; Joseph *et al.*, 1995; Brawn *et al.*, 1996; Bermingham *et al.*, 1996). At the very least, these results have important implications concerning our knowledge of species diversity and the conservation of tropical communities.

II. Temporal Scaling: The Panama Isthmus and Molecular Clocks

The first goal of this chapter is to provide insight into the mechanics and reliability of mitochondrial molecular clocks functioning across shallow spans of time. Generally speaking, we are interested in taxa that have diversified in the Miocene or forward. For the study of such recent speciation events one can only rarely rely on paleontological evidence to yield age estimates of taxic origins (Grande, 1985; Patterson, 1975). Temporal scaling of branch points in phylogenies is critically important for constraining hypotheses on correlation or causation between evolutionary and earth history events (Page, 1991, 1993; Lundberg, 1993) and strongly indicates an exaggerated role for molecular clocks in

studies of species with recent origins. Most biologists recognize the positive relationship between molecular differentiation and time. However, the linearity of that relationship (Zuckerandl and Pauling, 1965) over different time scales and across different taxa and molecules is under constant review. Our own studies of fish mitochondrial clocks (Martin *et al.*, 1992; H. A. Lessios and E. Bermingham, manuscript in preparation; E. Bermingham and S. S. McCafferty, manuscript in preparation) are nascent and mostly unpublished, but provide an affirmative prognosis concerning their utility to fish systematists with an interest in the biogeographical relationships of closely related species groups.

Our studies of fish mtDNA clocks use marine taxa (not only fish) separated by the Central American Isthmus (sharks: Martin *et al.*, 1992; sea urchins: Bermingham and Lessios, 1993; *Alpheus* shrimp: Knowlton *et al.*, 1993; teleost fishes: H. A. Lessios and E. Bermingham, manuscript in preparation; E. Bermingham and S. S. McCafferty, manuscript in preparation). For more than two decades it has been recognized that "geminate" marine species separated by the Central American Isthmus provide an unmatched occasion to study the molecular evolution of taxa that have been separated for roughly 3 million years, a time period of special interest to students of speciation (Lessios, 1979, 1981; Vawter *et al.*, 1980; Bermingham and Lessios, 1993; Knowlton *et al.*, 1993). The marine vicariant event caused by the uplift of the Choco and Chorotega blocks in the region of present-day Panamá (Duque-Caro, 1990) is particularly well dated (Fig. 1). Biostratigraphic correlations of molluscan fossil faunas (Coates *et al.*, 1992), changes in coiling direction of planktonic foraminifera (Keigwin, 1978), extinction of foraminifera (Keigwin, 1982), and changes in oxygen and carbon isotope ratios in the two oceans (Keigwin, 1982) all agree in dating this event between 2.9 and 3.5 million years ago (Ma). Slightly younger dates (2.5–2.7 Ma) from South American mammal fossils found in North America (Lundelius, 1987) and North American fossils found in South America (Marshall, 1988) confirm that the terrestrial corridor connecting the two continents was completed in the Pliocene.

The rise of the Panamanian isthmus permitted a realization of the classic Mayr (1963) "dumbbell" model of population division as it rendered the continuous ranges of marine taxa into two large populations (presumably without secondary contact), resulting in many pairs of closely related marine species, one on either side of Central America. Because they are numerous, phylogenetically diverse, and are thought to have been separated by the same geologic event, the geminate species pairs provide a remarkable array of organisms in which to investigate nucleotide substitution pro-

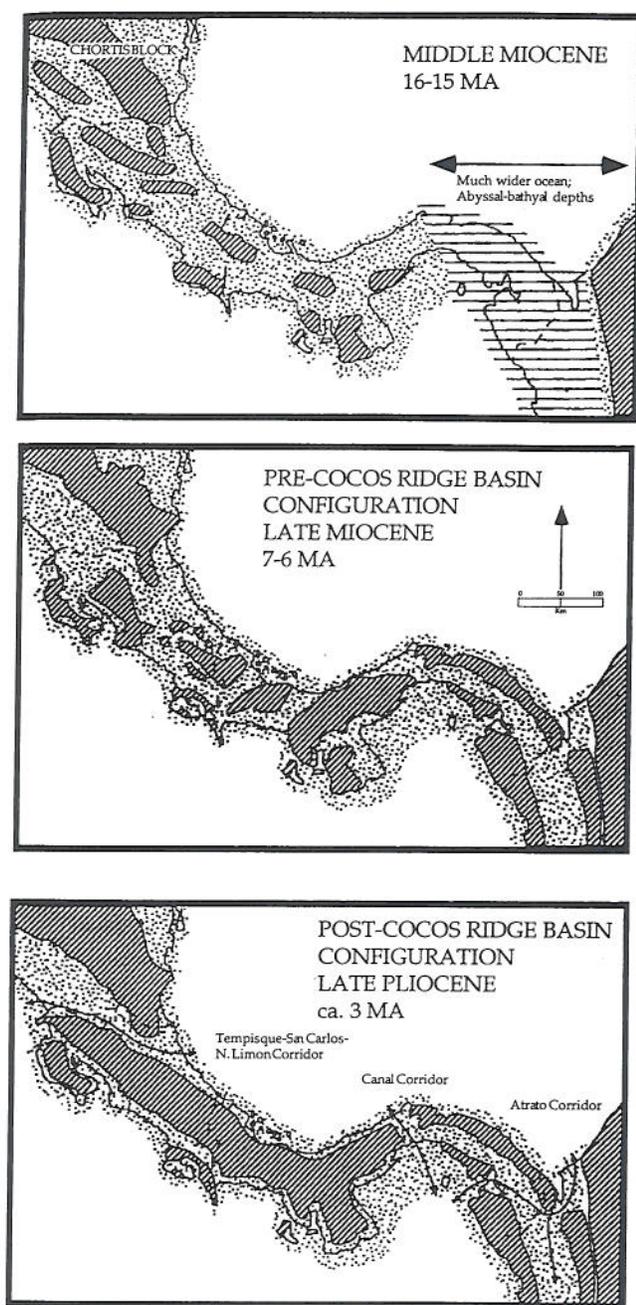


FIGURE 1 Paleogeographic reconstructions of the Central American Isthmus region in the middle Miocene, late Miocene, and late Pliocene. Emergent land is represented by oblique parallel lines, shelf sediments by dots, and abyssal oceanic sediments by horizontal parallel lines. After Coates and Obando (1996).

cesses and rate variation in molecular clocks. Geminate taxa are often found to have similar geographic ranges and ecological distributions in their respective oceans. In addition, most geminate taxa have planktonic larvae, increasing the probability that the rise of the Panama Isthmus interrupted gene flow "simultane-

ously" in species that had been previously panmictic (see later; Shulman and Bermingham, 1995).

The most rigorous framework for testing the molecular clock hypothesis follows an approach similar to that outlined by Muse and Weir (1992). Under the assumption of a molecular clock, transisthmian taxa should accumulate an approximately equal number of nucleotide substitutions since the two species last shared a common ancestor. In other words, $\hat{E}t_P = \hat{E}t_A$, where \hat{E} is the substitution rate at the DNA or protein level, t is the time the two species last shared a common ancestor, and the subscripts P and A denote the ocean affinity of each species. Because t is identical for the species compared, the hypothesis becomes $\hat{E}_P = \hat{E}_A$. The variance in accumulation of nucleotide substitutions across independent geminate pairs is approximately equal to the mean number of substitutions per site since a single time of origin for all taxa, $\hat{E} = \text{Var}(\hat{E})$. Muse and Weir (1992) review test statistics that can be used to refute the hypothesis of a molecular clock.

Molecular clock studies utilizing the isthmus depend on two assumptions concerning "geminate" taxa that usually go untested. The first regards geminate pairs as sister taxa. This assumption can be tested through a rigorous phylogenetic analysis of related species. Improved phylogenetic analyses of geminate species and closely related congeneric taxa have the added benefit that they permit a "relative rate" approach (Sarich and Wilson, 1967) for testing equalities of evolutionary rates as suggested by Muse and Weir (1992). The second assumption requires that geminate taxa were separated at the same time. Critical testing of this assumption would require a detailed and taxonomically rich fossil record. Except in rare cases, however, a paleontological perspective on geminate origins is simply not available. Therefore, failure to accept the null hypothesis in isthmian-based tests of molecular clocks may be due to differences in substitution rates across geminate species or because geminate pairs have split at very different times.

The absence of a good fossil record led Bermingham and Lessios (1993) to suggest an approximate test, here termed the "concordant measures" approach, of the second assumption. The approximation uses multiple, "independent" data sets to identify pairs of transisthmian species unlikely to have been split by the Pliocene rise of the isthmus. For example, concordant and relatively deep measures of divergence in mtDNA, allozymes, and behavior in three of seven "geminate" pairs of *Alpheus* shrimp led us to argue that these species pairs must have separated prior to the rise of the isthmus (Knowlton *et al.*, 1993). On the other hand, congruent and shallow allozyme and mtDNA divergence values in some fish geminates (where interspe-

cific genetic divergences are approximately equal to intraspecific genetic divergences) suggest circumtropical gene flow in these species.

That some "geminate" taxa were almost certainly separated by events other than the Pliocene completion of the Panama Isthmus should not be surprising given that the identification of geminates has resulted more from investigator gestalt than objective morphological criteria. Thus, allozyme and mtDNA studies may provide the most objective (and best comparatively grounded) data available for determining the probable geminate (and sister group) status of transisthmian taxa. Molecular clock studies that include "geminate" pairs that allozyme and mtDNA data concordantly suggest pre- or postdate the Pliocene isthmian barrier are extraordinarily conservative (and potentially flawed). Ultimately, we wish to compare the nucleotide substitution process in transisthmian pairs of similar age without diminishing our inferences through the inclusion of older and younger taxa less easily associated with particular earth history events.

In the remainder of the discussion on fish molecular clocks, a combination of "concordant measure" approximations and statistical methods are used to center our focus on transisthmian pairs of equivalent "age." Using homologous mtDNA cytochrome oxidase I (COI) sequence data for multiple transisthmian pairs of *Alpheus* shrimp (Knowlton *et al.*, 1993) and fishes (E. Bermingham and S. S. McCafferty, manuscript in preparation), nucleotide substitution processes can be compared across arthropods and fishes. Comparison

of branch length distributions between transisthmian pairs suggests that *Alpheus* shrimp have greater levels of between-geminate sequence divergence than fishes. When we focus on the cluster of species that "concordant measures" graphical analysis suggest diverged coincident with the Pliocene rise of the Isthmus, shrimp nucleotide substitution rates are roughly twice those of fishes whether determined for all nucleotide sites or fourfold degenerate sites. These results suggest that there are differences in substitution rates between shrimp and fishes. Other studies have reported differences in substitution rates between arthropods and vertebrates (Britten, 1986; Vawter and Brown, 1986), and various hypotheses including generation time (Li *et al.*, 1987; Gaut *et al.*, 1992) and metabolic rate (Thomas and Beckenbach, 1989; Avise *et al.*, 1992; Martin and Palumbi, 1993) have been proposed to account for rate variation in nucleotide substitution patterns (see Hillis *et al.*, 1996).

Insufficient data exist to test alternative hypotheses of rate variation. Nevertheless, a notable difference between the sequences from shrimp and fishes is the relative frequency of AT and GC nucleotides. To determine if nucleotide composition may be related in some fashion with rates of divergence in geminate shrimp versus fishes, the nucleotide skew (Perna and Kocher, 1995a) and nucleotide bias (Irwin *et al.*, 1991) were studied in the two groups. Figure 2 graphically shows the GC skew, AT skew, and nucleotide bias at supposedly neutral fourfold degenerate sites for eight geminate pairs of fishes (see later) and four geminate pairs of *Alpheus*

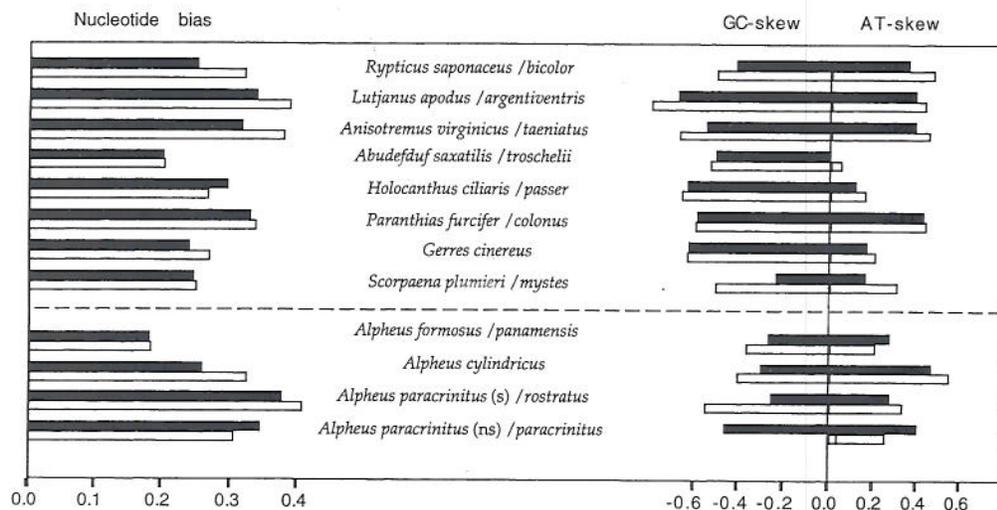


FIGURE 2 Nucleotide composition statistics for comparisons of geminate fishes and *Alpheus* shrimp. The left-hand portion of the figure shows the nucleotide bias at fourfold degenerate sites for each geminate pair. The filled bars represent nucleotide bias from the Atlantic taxa whereas the hollow bars represent the Pacific taxa within each comparison. Nucleotide bias is estimated according to Irwin *et al.* (1991). The right-hand side of the figure shows GC and AT skew at fourfold degenerate sites calculated according to Perna and Kocher (1995a).

(Knowlton *et al.*, 1993). The average nucleotide bias is 0.291 ± 0.058 for geminate fishes and 0.314 ± 0.084 for geminate shrimp. The average AT skew is 0.289 ± 0.158 versus 0.344 ± 0.115 and the average GC skew is -0.573 ± 0.129 versus -0.322 ± 0.180 for geminate fishes and shrimp, respectively.

Although there is little difference in nucleotide bias between the two groups, there appears to be slight differences for both AT and GC skews. These skew values are clearly discernable in the frequency of the four nucleotides at fourfold synonymous sites (fourfold synonymous sites were determined independently for each geminate pair). Fishes and shrimp both show the characteristic reduction of guanine residues at fourfold sites. However, a significant reduction of cytosine residues was observed at fourfold sites in shrimp as compared to fish. Martin (1995) hypothesized that an increase in AT nucleotides accompanies an increase in DNA damage rates resulting from mutagenic oxygen radicals that are by-products of metabolism. The accelerated rate of nucleotide substitution and shift toward A+T nucleotides observed in shrimp relative to fish may be indicative of differences in rates of endogenous DNA damage between these taxa. What-

ever the underlying mechanisms are that set the pace and pattern of nucleotide substitution, the comparison between shrimp and fishes underscores the fact that rates of molecular evolution can differ among taxonomic groups.

Turning our attention to a more complete examination of the transisthmian fishes, mtDNA COI sequence divergence was analyzed in the 19 "geminate" fish pairs listed in Table I. A test of the molecular clock based on the maximum likelihood method (Felsenstein, 1993) was used to determine if any of the geminate pairs were not evolving in a clock-like fashion. Log likelihoods were calculated for a topology with both the molecular clock constraint enforced (ML_{MC}) and relaxed (ML_{NC}). The likelihood values were tested using $2^*(\ln ML_{MC} - \ln ML_{NC})$ which is distributed as a χ^2 with $(n - 2)$ degrees of freedom where n is the number of terminal nodes. No significant differences were found for any of the 19 geminate fish pairs, leading to the assumption that the mtDNA COI region in the separate geminate taxa is evolving in a clock-like fashion.

To test whether all 19 of the geminate fish pairs were evolving at an equal rate, maximum likelihood was used to estimate divergence in pairwise compari-

TABLE I Divergence Values and Maximum Likelihood Test Results for Geminate Marine Fishes^a

Species	n ^b	K2 ^c	Ks ^d	ML ^e	p ^f	Cluster ^g
<i>Melichthys niger</i>	2/4	0.0018 ± 0.0028	0.0040 ± 0.0080	0.0035 ± 0.0023	0.350	A
<i>Diodon hystrix</i>	3/5	0.0058 ± 0.0050	0.0120 ± 0.0120	0.0082 ± 0.0021	0.379	A
<i>Alutera scripta</i>	4/2	0.0087 ± 0.0030	0.0230 ± 0.0050	0.0400 ± 0.0042	0.391	A
<i>Mulloidichthys martinicus</i> vs <i>M. dentatus</i>	5/5	0.0126 ± 0.0030	0.0320 ± 0.0060	0.0363 ± 0.0024	0.909	A
<i>Abudefduf sordidus</i> vs <i>A. concolor</i>	6/8	0.0142 ± 0.0030	0.0410 ± 0.0080	0.0324 ± 0.0016	0.446	A
<i>Anisotremus surinamensis</i> vs <i>A. interruptus</i>	6/6	0.0162 ± 0.0041	0.0310 ± 0.0040	0.0359 ± 0.0011	0.982	A
<i>Rypticus saponaceus</i> vs <i>R. bicolor</i>	4/8	0.0317 ± 0.0033	0.0850 ± 0.0060	0.1338 ± 0.0040	0.518	B
<i>Lutjanus apodus</i> vs <i>L. argentiventris</i>	6/11	0.0348 ± 0.0060	0.1020 ± 0.0108	0.1268 ± 0.0031	0.092	B
<i>Anisotremus virginicus</i> vs <i>A. taeniatus</i>	3/3	0.0436 ± 0.0052	0.1270 ± 0.0178	0.1633 ± 0.0082	0.073	B
<i>Abudefduf saxatilis</i> vs <i>A. troschelii</i>	10/8	0.0449 ± 0.0036	0.1360 ± 0.0110	0.1752 ± 0.0015	0.998	B
<i>Holocanthus ciliaris</i> vs <i>H. passer</i>	4/8	0.0487 ± 0.0016	0.1460 ± 0.0030	0.2204 ± 0.0042	0.319	B
<i>Paranthus furcifer</i> vs <i>P. colonus</i>	2/10	0.0479 ± 0.0033	0.1460 ± 0.0080	0.1564 ± 0.0024	0.788	B
<i>Gerres cinereus</i>	4/3	0.0507 ± 0.0029	0.1530 ± 0.0070	0.1955 ± 0.0062	0.893	B
<i>Scorpaena plumieri</i> vs <i>S. mystes</i>	3/5	0.0550 ± 0.0050	0.1670 ± 0.0120	0.2829 ± 0.0117	0.344	B
<i>Chromis multilineata</i> vs <i>C. atrilobata</i>	4/8	0.0935 ± 0.0089	0.3020 ± 0.0280	0.5591 ± 0.0128	0.566	C
<i>Chaetodon striatus</i> vs <i>C. humeralis</i>	4/8	0.1040 ± 0.0030	0.3570 ± 0.0110	0.6293 ± 0.0107	0.062	C
<i>Priacanthus cruentatus</i>	6/4	0.1071 ± 0.0100	0.3910 ± 0.0090	2.6690 ± 0.0429	0.324	E
<i>Thalassoma bifasciatum</i> vs <i>T. lucasanum</i>	4/6	0.1087 ± 0.0050	0.3630 ± 0.0170	1.9390 ± 0.0671	0.369	D
<i>Ophioblennius atlanticus</i> vs <i>O. steindachneri</i>	8/8	0.1237 ± 0.0048	0.4310 ± 0.0190	1.5240 ± 0.0433	0.131	D

^aBased on ca. 650 bp of the COI gene region of mitochondrial DNA sequenced using standard manual (Lessios *et al.*, 1995) or automated methods (S. S. McCafferty and E. Bermingham, manuscript in preparation).

^bNumber of individuals sequenced (Atlantic/Pacific).

^cAverage geminate divergence at all sites based on the two parameter model of Kimura (1980).

^dAverage corrected percentage divergence at synonymous sites (Li *et al.*, 1985).

^eAverage maximum likelihood estimate of divergence at fourfold degenerate sites using a transition/transversion ratio of 3.5 and correcting for unequal base composition and heterogeneity in rates of substitution among sites (γ distribution, $\alpha = 0.25$; Swofford *et al.*, 1996).

^fProbability values < 0.05 indicate that maximum likelihood values with and without the molecular clock enforced are significantly different from each other (i.e. reject the molecular clock; Felsenstein, 1993).

^gGroup association based on a UPGMA of the difference in average ML distance between geminates.

sons between all transisthmian individuals representing each geminate species pair. However, use of all pairwise distances would violate the assumption of independent replication (if we consider the among geminate comparisons simply to be replicates estimating some true parametric value). Instead, minimum and maximum divergence values were used to account for the range of variation within each geminate pair. Variation in substitution rates among sites was accounted for using a γ distribution, unequal frequencies of nucleotides were accounted for using the empirical frequencies, and transition/transversion ratios estimated from data were used with a four-category substitution model (Swofford *et al.*, 1996). Equivalent results were obtained for fourfold degenerate sites using a simple Kimura (1980) two parameter model. Estimates of divergence were used instead of branch lengths from a recent common ancestor because (a) no appropriate outgroup is available for each geminate pair and (b) it has been demonstrated that the sequences are evolving according to a molecular clock. Therefore, on average, the branch lengths from the most recent common ancestor should be approximately equal and the average divergence should be correlated with these branch lengths.

A significant difference was found among group means ($H_{adj} = 35.071$, $p = 0.009$) using a Kruskal-Wallis test (Sokal and Rohlf, 1981). To find homogeneous subgroups within the data, an Unweighted Pair Group Method using Arithmetic Averages (UPGMA) of the Euclidean distance among the average divergences between geminate pairs was used to search for natural clusters in the data (Sneath and Sokal, 1973; Rohlf, 1993). The resulting phenogram should not be confused with a phylogenetic hypothesis of relationship among the taxa. The UPGMA phenogram is merely a tool for grouping geminate pairs that display similar levels of divergence and for this reason has not been presented here. However, the cophenetic correlation of the cluster analysis was 0.94, suggesting that

data lend themselves to hierarchical clustering. Four clusters and one singleton were found. These are marked in Table I as clusters A, B, C, D, or E (the singleton). Each of these clusters is homogeneous within groups when tested using the Kruskal-Wallis test (A: $p = 0.238$; B: $p = 0.178$; C: $p = 0.439$; D: $p = 1.000$; E: NA). When clusters A and B or B and C are combined, they form significantly heterogeneous groups.

The heterogeneity in levels of divergence just described may be due to a number of processes. One explanation would simply recognize that "geminate" pairs exhibit a considerable range of divergence dates. However, it is also well accepted that nucleotide base composition differences among taxa can have a profound effect both on estimates of divergence and on phylogenetic reconstructions (e.g., Saccone *et al.*, 1989; Sidow and Wilson, 1990; Lockhart *et al.*, 1992, 1994; Hasegawa and Hashimoto, 1993; Steel *et al.*, 1993; Perna and Kocher, 1995b). To provide some insight into nucleotide compositional effects on the patterns of divergences found in the geminate fish data set, the levels of divergence at synonymous sites (K_s ; Li *et al.*, 1985) were compared to differences in the nucleotide composition for each geminate pair separately. Differences in nucleotide composition were determined in three ways: the Euclidean distance of the average nucleotide frequency between each geminate pair (termed nucleotide distance), the difference in GC and AT skew (Perna and Kocher, 1995a) between each geminate pair (termed ΔGC and ΔAT), and the difference in nucleotide bias (Irwin *et al.*, 1991) between each geminate pair (the $\Delta bias$). All estimates of nucleotide frequency, skew, and bias were based on the nucleotide composition at fourfold degenerate sites. This fish geminate data set permits 19 independent comparisons for each test.

Significant, although low, correlations are found between levels of divergence at synonymous sites and the nucleotide distance and the Δ skews (Table II). These associations suggest that nucleotide composition differences may indeed have an effect on estimating levels

TABLE II Matrix of Spearman's Rank Correlation between Euclidean Distance in Nucleotide Frequencies (d_n), Difference in GC Skew (ΔGC), Difference in AT Skew (ΔAT), Difference in Nucleotide Bias ($\Delta Bias$), the Average Estimate of Divergence at All Sites (K_2), the Average Divergence at Synonymous Sites Only (K_s), and the Average Divergence Estimated Using the LogDet Paralinear

	d_n	ΔGC	ΔAT	$\Delta Bias$	K_2	K_s	LogDet
d_n	1.000						
ΔGC	0.773	1.000					
ΔAT	0.813	0.511	1.000				
$\Delta Bias$	0.509	0.460	0.278	1.000			
K_2	0.686	0.470	0.515	0.269	1.000		
K_s	0.674	0.457	0.504	0.257	0.994	1.000	
LogDet	0.769	0.608	0.589	0.457	0.926	0.933	1.000

of divergence in the geminate fish data. However, correlations were significant whether the model of divergence used assumed equal frequencies of the four nucleotides (e.g., Kimura's two parameter) or accounted for unequal nucleotide frequencies (LogDet; Lockhart *et al.*, 1994; Swofford *et al.*, 1996). If nucleotide composition effects were the primary determinate of variation in levels of divergence between geminate pairs, then it would be reasonable to expect that the correlation between divergence and nucleotide distance (or Δ skews) should approach zero when differences in nucleotide composition are removed by use of the LogDet metric. Clearly this is not the case, implicating some other process or processes underlying the heterogeneity in levels of divergence found for geminate fish. However, in some other teleost data sets (unpublished results), it does appear that compositional divergence may indeed significantly bias estimates of genetic divergence and phylogenetic relationships at deeper time intervals than what is presented here.

Molecular clocks will remain an enigma until the mechanisms responsible for molecular evolution are fully revealed. The natural experiment initiated by the rise of the isthmus of Panama provides an opportunity to study the process of molecular evolution across a large number of evolutionarily independent taxa, but it is an experiment that has not been fully utilized to date. Nonetheless, even imperfect or imperfectly manipulated experiments yield results that can be used cautiously. One such result from our research relevant to this book is an evolutionary rate estimate for mtDNA COI in fishes. Based on the taxa in group B (Table I), an estimate of roughly 1.2% sequence divergence per million years at all sites between recently separated fish taxa has been derived (3.3% at synonymous sites). Preliminary data on other mitochondrial regions show similar levels of divergence. For example, estimates of roughly 1.3% sequence divergence per million years for the entire ND2 and ATPase6 genes at all sites (3.5 and 3.4%, respectively, at synonymous sites only) have been determined for these Pliocene geminates (E. Bermingham and S. S. McCafferty, manuscript in preparation). Used with caution, these estimates provide a framework for estimating divergence times for a fairly broad taxonomic range of teleost fishes encompassed by our studies of geminate marine fishes.

III. Geographic Scaling: The Panama Isthmus and Caribbean Fish

This section introduces two themes that will be carried through the remainder of this chapter. First,

conspecific populations, if differentiated, can provide historical information about a region (Rosen, 1978; Chernoff, 1982; Bermingham and Avise, 1986). Second, molecules, particularly mtDNA, are well suited for reconstructing the evolutionary relationships among conspecific populations. In other words, for species or species groups demonstrating little or no phylogenetically informative morphological variation, molecules can provide an α taxonomy (albeit that of the molecule being analyzed) that can be easily and immediately placed in a phylogenetic context. Thus, molecules provide an objective measure of the geographic scale over which phylogenetically informative differentiation is occurring.

Biogeographic studies of tropical fishes began with a regional assessment of dispersal, gene flow, and endemism in populations representing eight species of Caribbean reef fishes (Shulman and Bermingham, 1995). The reef fish species varied in two life history traits that may affect dispersal ability and thus population genetic structure: egg type (pelagic and nonpelagic) and length of planktonic (usually larval) life (Table III). Six populations for each species were sampled from widely separated locales in both the northern and the southern current tracks within the Caribbean. mtDNA restriction endonuclease analyses were used to estimate the degree of genetic differentiation among conspecific populations. For two genera (*Abudefduf* and *Ophioblennius*), the putative eastern Pacific geminate taxa (*A. troschellii* and *O. steindachneri*) were analyzed to provide a transisthmian mtDNA divergence measure against which to assess intra-Caribbean mtDNA distances.

For each of the eight species of Caribbean reef fishes, the predominant mtDNA haplotype was widespread. Mean sequence divergence observed among conspecific Caribbean mtDNA haplotypes in each of the eight fish species was low, less than 0.7% for all but one species. This level of divergence is roughly one order of magnitude less than mtDNA divergence between Caribbean/East Pacific sister taxa. Even relatively rare mtDNA haplotypes tended to be broadly distributed. Populations located in different major surface currents were no more differentiated from one another than populations occupying the same current track. These results suggest that there is considerable gene flow throughout the Caribbean and that current tracks in the Caribbean have not acted as barriers to gene flow through evolutionary time (Shulman and Bermingham, 1995). This study suggests that the low levels of population subdivision found, although of potential ecological importance, are relatively insignificant in an evolutionary context (Moritz, 1994; McMillan and Bermingham, 1996).

Comparisons to sister taxa separated by the Central

TABLE III Life History Data for Eight Species of Coral Reef Fishes^a

	Egg location ^b	Length of larval life in days ^c		Reference	<i>p</i> value ^d	Geographic Structure ^e
		Mean	Range			
<i>Stegastes leucostictus</i>	Benthic	20.1	19–21	Wellington and Victor (1989)	<0.001 (17.2)	Very strong
		28.5	27–30	Thresher and Brothers (1989)		
<i>Ophioblennius atlanticus</i>	Benthic	28.6	28–29	E. Brothers (personal communication)	0.347 (0.3)	None
<i>Abudefduf saxatilis</i>	Benthic	18.2	17–20	Wellington and Victor (1989)		
		27.2	25–29	Thresher and Brothers (1989)	0.595 (0)	None
from drift algae		33.9	30–55 ^f	E. Brothers (personal communication)		
<i>Gnatholepis thompsoni</i>	Benthic	81.5	59–122	E. Brothers (personal communication)	0.009 (8.2)	Very strong
<i>Haemulon flavolineatum</i>	Pelagic	15	13–20	McFarland <i>et al.</i> , (1985)	0.455 (0.1)	None
<i>Halichoeres bivittatus</i>	Pelagic	24.1	22–26	Victor (1986)	0.002 (7.9)	Strong
<i>Holocentrus ascensionis</i>	Pelagic	48.7	46–50	E. Brothers (personal communication)	0.561 (0)	None
<i>Thalassoma bifasciatum</i>	Pelagic	49.3	38–78	Victor (1986)	0.920 (0)	None

^aAdapted from Shulman and Bermingham (1995).

^bType of egg (pelagic/nonpelagic).

^cMean and range of larval life span taken from published sources and personal communications.

^dProbability of significance of between-population variation in genetic diversity (and percentage of variation between populations) from the AMOVA model (Excoffier *et al.*, 1992).

^eRelative scale of the evidence for geographic structuring.

^fCollected from Sargassum in Florida.

American Isthmus allowed us to exclude the possibility that mtDNA similarity across conspecific populations in the Caribbean was due to reduced rates of mtDNA evolution. Mitochondrial DNA evolutionary rates consistent with those observed for transisthmian fish (E. Bermingham and S. S. McCafferty, manuscript in preparation) and other fish species (Martin *et al.*, 1992; Meyer, 1993) indicated that the mtDNA haplotypes surveyed in each species probably coalesce in the Pleistocene. In comparison to conspecific populations of freshwater fish species (*Lepomis* spp.) broadly distributed across the southeastern United States (Bermingham and Avise, 1986), it is apparent that mtDNA lineage extinction in these coral reef fishes has been especially rapid. In the freshwater fishes, mtDNA lineages were presumably buffered against loss as a result of genetic isolation in discontinuous riverine habitats. It is apparent that an analogous process has not acted across discontinuous coral reef habitats.

The high rates of mtDNA lineage extinction in coral reef fishes probably resulted from two processes. First, the extremely low, and presumably highly stochastic, survival rate of pelagic fish larvae (Leis, 1991) suggested that female reef fish vary considerably with regard to the number of daughters that replace them. High variance in reproductive success among females has been theoretically shown to cause the rapid pruning of mtDNA trees leading to decreased times to coalescence (Avise *et al.*, 1984). Second, Pleistocene reduc-

tions in sea level almost certainly led to smaller reef areas and presumably lower population sizes in most reef-associated organisms (Shulman and Bermingham, 1995). These processes working in concert have probably resulted in long-term effective population sizes that are considerably smaller than the present-day population sizes of many Caribbean reef fishes.

The study of Caribbean reef fishes also focused on the effects of two early life history attributes on the genetic architectures of coral reef fishes. Using AMOVA and its associated permutation testing routine (Excoffier *et al.*, 1992), a statistically significant population subdivision was observed for three Caribbean fish species (Table III): *Stegastes leucostictus* (nonpelagic eggs; short planktonic life), *Gnatholepis thompsoni* (nonpelagic eggs; long planktonic life), and *Halichoeres bivittatus* (pelagic eggs; short planktonic life). However, the between population variance accounted for only 8–17% of the total variance (the remaining 83–92% occurred within geographic populations). The results suggested that neither egg type nor length of larval life was a simple predictor of geographic structure in reef fish populations. In reconciling the apparent paradox of extensive gene flow among Caribbean reef fishes with their striking species richness, one might consider that present-day sea levels, currents, and patterns of gene flow are not representative of the past marine environments in which much of the species richness observed today developed (Vermeij, 1978).

IV. Geographic Scaling: The Panama Isthmus and the Circumtropical *Abudefduf* (Teleostei: Pomacentridae) Species Groups

To continue our investigations of the geographic scale of genetic differentiation in tropical marine fishes, the molecular α taxonomy and phylogenetic relationships for two circumtropically distributed species groups in the damselfish genus *Abudefduf* have also been described (Lessios *et al.*, 1995; E. Bermingham *et al.*, manuscript in preparation). To the extent that marine species richness can be explained by orthodox models of allopatric speciation, the Caribbean results suggest that tropical fish diversification in the marine realm might punctuate periods of relative stasis. Building on the transisthmian and Caribbean studies, we hoped to investigate this possibility through historical biogeographic comparison of two circumtropically distributed species groups in the genus *Abudefduf*, the so-called *A. saxatilis* and the *A. sordidus* groups (Hensley, 1978).

The *A. saxatilis* species group contains *A. saxatilis* (Caribbean Sea and Atlantic Ocean), *A. troschellii* (Eastern Pacific), *A. abdominalis* (Hawaii), and *A. vaigiensis* (Indo-West Pacific including the Red Sea). The *A. sordidus* group includes *A. taurus* (Caribbean Sea and Atlantic Ocean), *A. concolor* (eastern Pacific excluding the Gulf of California), *A. declivifrons* (Gulf of California), and *A. sordidus* (Hawaii and the Indo-West Pacific including the Red Sea).

Both these species groups are characterized by a complete circumtropical distribution and each contains a transisthmian pair of species. In a study of the transisthmian members of the *A. sordidus* group, it was shown that the recognized taxonomy (Thomson *et al.*, 1979; Allen, 1991) of the *A. sordidus* group underestimated the diversity of the group by one species based on combined analysis using allozymes, sequence data from a fragment of the mitochondrial COI region, and morphological data (Lessios *et al.*, 1995). Our investigation resurrected *A. declivifrons* (Gill, 1862), which was also identified by Hensley (1978). Three points need to be emphasized. First, molecules can reinforce careful morphological and meristic analyses (and vice versa), leading to a more robust α taxonomy. Second, molecular data naturally lead to a phylogenetic assessment of the sister group status of transisthmian species. Third, phylogenetic hypotheses relating geographic populations and closely related species are critical to historical biogeographic analyses of the earth's recent marine history.

Findings for the eastern Pacific and Caribbean members of the *A. sordidus* group have been augmented

TABLE IV Geographic Location, Sample Size, and Clade Designation of the *Abudefduf saxatilis* Group and *A. sordidus* Group Samples

	Location	n ^a	Clade ^b
<i>A. saxatilis</i> group			
<i>A. saxatilis</i>	Ascension Island	4	A
<i>A. saxatilis</i>	Belize	2	B
	Bocas del Toro, Panama	2	B
	Los Roques, Venezuela	4	B
	Brazil	2	B
<i>A. abdominalis</i>	Hawaii	4	C
<i>A. troschellii</i>	Clarion Islands	2	D
	Isla San Pedro, Mexico	2	D
	Isla Cocos, Costa Rica	2	D
	Panama	2	D
	Galapagos	2	D
<i>A. vaigiensis</i>	Guam	3	F
	Kosrae	2	F
	Papua New Guinea	3	E
	Eastern Australia	7	E/F
	Solomon Islands	2	E
	Western Australia	5	E
	Taiwan	1	E
	Red Sea	5	E
<i>A. sordidus</i> group			
<i>A. taurus</i>	Puerto Rico	2	G
	Panama	4	G
	Los Roques, Venezuela	4	G
<i>A. concolor</i>	Panama	3	H
	Galapagos	2	H
<i>A. declivifrons</i>	Mexico	4	I
<i>A. sordidus</i>	Hawaii	3	J
	Kosrae	2	J
	Papua New Guinea	2	J
	Eastern Australia	2	J
	Western Australia	5	J
	Taiwan	1	J
	Red Sea	2	J

^aNumber of individuals sequenced for approximately 650 bp of the COI gene region of mtDNA using standard manual (Lessios *et al.*, 1995) or automated (S. S. McCafferty and E. Bermingham, manuscript in preparation) methods.

^bClade designation based on phylogenetic analysis of sequence data.

by results which elaborate the historical relationships within both the *A. sordidus* and the *A. saxatilis* groups (E. Bermingham *et al.*, manuscript in preparation). The *Abudefduf* taxa investigated and their collection locations are presented in Table IV and Fig. 3. Analyses of partial mtDNA cytochrome b and COI nucleotide data (~1300 bases in total) have identified two additional phylogenetic (mtDNA) lineages in the *A. saxatilis* group. Here we specifically refer to mtDNA lineages and not species because the complementary allozyme and morphological analyses have not been undertaken (as was the case for the resurrection of *A. declivifrons*).

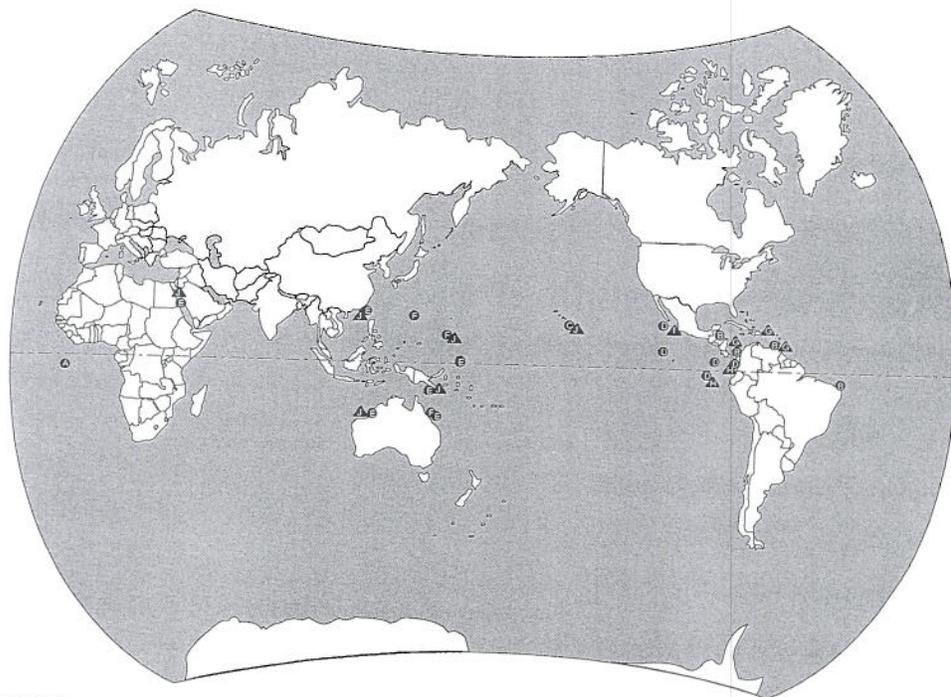


FIGURE 3 Location and clade designation of *Abudedefduf saxatilis* and *A. sordidus* samples. The circles represent locations where members of the *A. saxatilis* group were collected, the triangles are where *A. sordidus* samples were collected. The letters correspond with Table IV.

Genetic analyses provide compelling evidence that *A. vaigiensis* includes two highly divergent mtDNA clades (Fig. 4). A derived clade was found in the western Pacific and Indian Ocean ranging from the Great Barrier Reef to the Red Sea. The more ancestral mtDNA clade was observed only in the western Pacific (Coral Sea, Guam, and Kosrae). In addition, an isolated population of *A. saxatilis* collected on Ascension Island in the mid-Atlantic displays a level of sequence divergence when compared to all other populations of *A. saxatilis* which is similar to that observed between the transisthmian species pair (*A. troschelii* and *A. saxatilis*). Finally, the distinctiveness of the Hawaiian species (*A. abdominalis*, $n = 14$), the geographic extent of the eastern Pacific species (*A. troschelii*, $n = 35$, Galapagos to Gulf of California), and the Caribbean distribution of *A. saxatilis* ($n = 67$, coastal Brazil to Bermuda; Shulman and Bermingham, 1995) are fully supported by both mtDNA sequence and restriction fragment length polymorphism (RFLP) analyses.

In the *A. sordidus* group, mtDNA phylogenetic lineages corresponded closely to currently recognized taxa, including *A. declivifrons* (Fig. 5). Both mtDNA RFLP and sequence analysis confirm the widespread geographic distribution of *A. sordidus sensu stricto* from Hawaii to the Red Sea.

The mtDNA results permit the species and geographic populations in both the *A. sordidus* and the *A. saxatilis* groups to be placed in a phylogenetic context. In turn, these phylogenetic hypotheses can be utilized in historical biogeographic analysis. Visual inspection of Figs. 4 and 5 presents no compelling case for a common historical basis for the pattern of diversification seen in the *A. sordidus* and *A. saxatilis* groups. Both the branching orders and the branching times (under the assumption of a molecular clock) lack congruence except that an Indo-West Pacific mtDNA clade is ancestral in both species groups. If a common mtDNA molecular clock exists for these taxa, the estimated date of divergence of the geminate pairs (*A. troschelii* and *A. saxatilis* versus *A. concolor* and *A. taurus*) is quite different (however, see previous section on geminate comparisons). The relatively reduced mtDNA divergences observed for the *concolor/taurus* transisthmian pair is paralleled by a reduced allozyme distance in this pair as well (Vawter *et al.*, 1980; H. A. Lessios and E. Bermingham, manuscript in preparation).

Two marine species groups can do relatively little to elucidate the historical processes which underlie the diversification of tropical marine fishes across the earth's vast tropical seascape. Yet 15 years after the publication of Victor Springer's (1982) provocative and informative

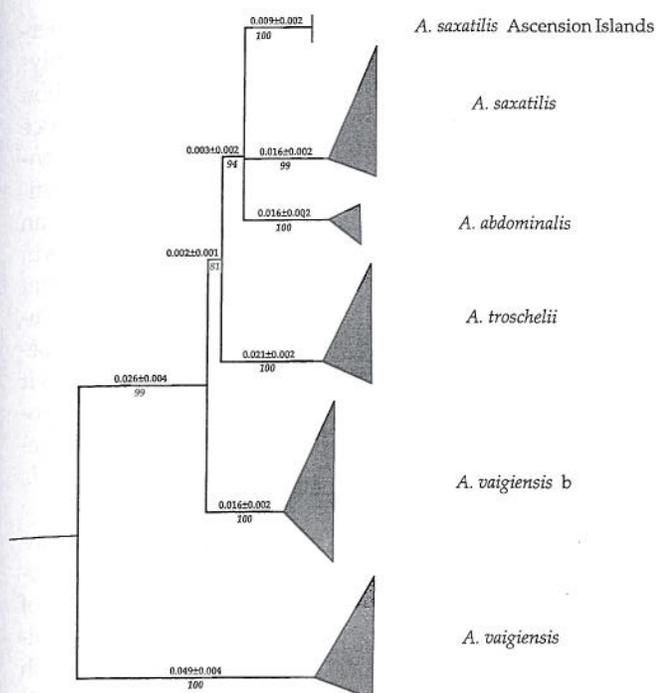


FIGURE 4 Neighbor-joining tree summarizing the relationships among taxa in the *Abudehduf saxatilis* group. Pairwise divergences were estimated using the two parameter model of Kimura (1980) for 570 bp of the COI mtDNA region. The pomacentrid *Amblygliphidodon curacao* was used as an outgroup for rooting the tree. The triangles summarizing the terminal nodes within each clade have a depth proportional to the longest branch length and a height proportional to the number of OTUs found in that clade. Branch lengths plus or minus their standard deviations are shown above the branches. The probability that the branch length was greater than zero is presented below the line. Probabilities were calculated according to Rzhetsky and Nei (1994a) using the program METREE (Rzhetsky and Nei, 1994b). Equivalent topologies were found using weighted and unweighted parsimony analysis, minimum evolution, maximum likelihood, and whether estimates of divergence were corrected for unequal frequencies of nucleotides (Tamura and Nei, 1993; Lockhart *et al.*, 1994), unequal rates of evolution among sites (Tamura and Nei, 1993), or a simple two parameter model (Kimura, 1980).

monograph on Pacific Plate biogeography in which he relied mostly on "inferences and intuitive assessments of relationships" of shorefishes, our studies of *Abudehduf* represent one of the few phylogenetic assessments of species relationships in a genus with a Pacific Plate and/or circumtropical distribution. Meeting Springer's challenge "to correct, elaborate, falsify or corroborate" his study of pattern and process in tropical shorefish speciation requires that phylogenetic hypotheses be accumulated for many more species groups. Our investigations of *Abudehduf* relationships suggest that mtDNA-based phylogenies provide one useful means to that end.

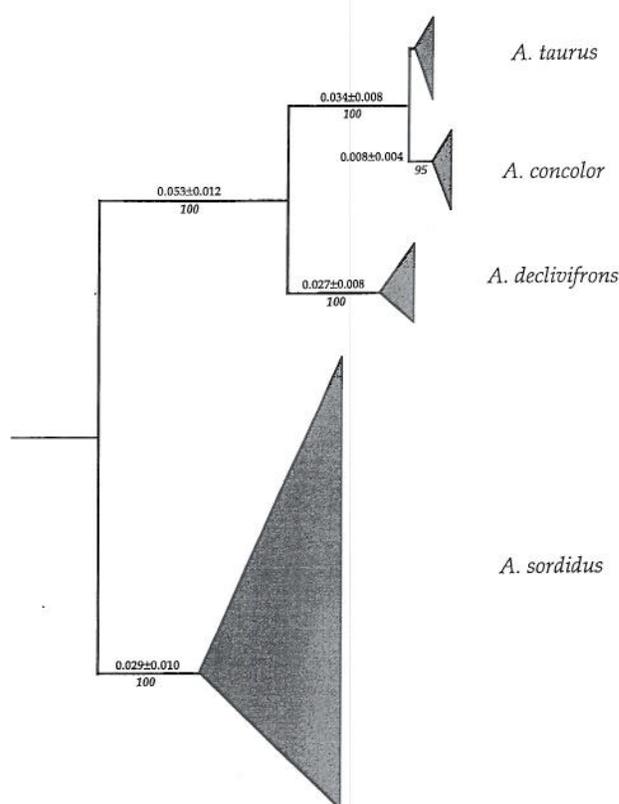


FIGURE 5 Neighbor-joining tree summarizing the relationship among the various taxa in the *Abudehduf sordidus* group. Refer to the legend for Fig. 4 for details.

V. Geographic Scaling: The Panama Isthmus and Neotropical Freshwater Fishes

Generally speaking, it is also the case that phylogenetic analyses of geographic populations and closely related species of tropical freshwater fishes are unavailable. Therefore, we have virtually no knowledge of the geographic scale of genetic differentiation across tropical freshwater organisms. Although our studies of Panamanian freshwater fish are preliminary, they have revealed a considerable degree of phylogeographic structuring in species exhibiting distributions that span large distances across physically isolated drainages. Levels of mtDNA differentiation between populations of some neotropical species are typical of interspecific or even intergeneric differences among temperate fish species. Such marked genetic divergence among populations of neotropical fish suggests that high levels of genetic divergence between populations may be a general feature of tropical species.

Figure 6 shows a phylogenetic reconstruction of 80 *Roeboides* (Characiformes) individuals representing

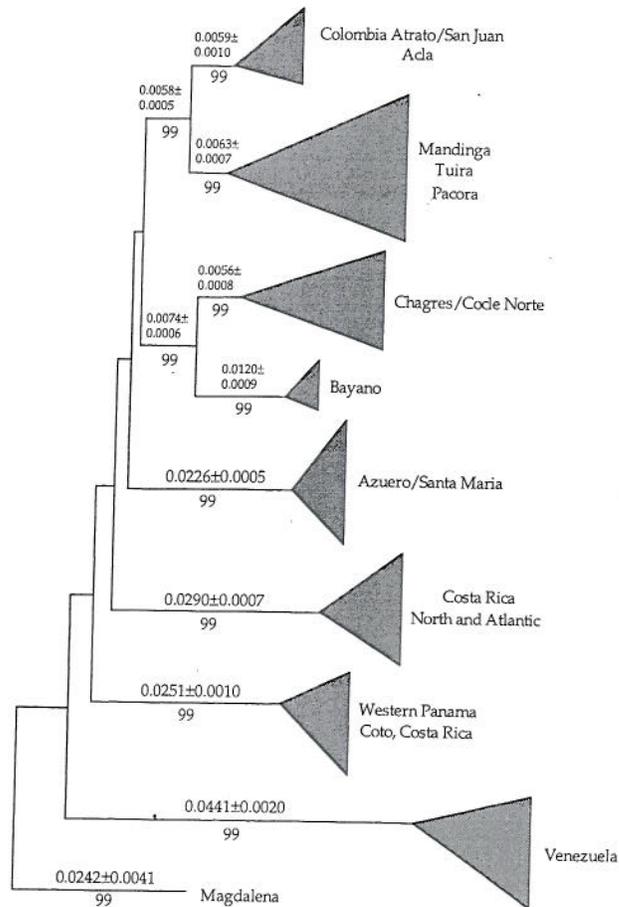


FIGURE 6 Neighbor-joining tree summarizing the relationship among 80 individuals of *Roeboides* collected from Costa Rica, Panama, and South America. Pairwise divergences were estimated using the two parameter model of Kimura (1980) for 852 bp spanning the entire mtDNA ATPase 6 and 8 genes. *R. affinis* individuals from Peru were used as outgroup sequences in rooting the tree (not included in figure). Refer to the legend for Fig. 4 for tree details.

five nominal species collected widely from Venezuela to northern Costa Rica (refer to the map in Fig. 7). Several points can be made using the *Roeboides* phylogeny that are generally valid for most of the neotropical freshwater fish species that have been studied. First, reciprocal monophyly of mtDNA lineages is observed between different regional assemblages of *Roeboides*. Second, although mtDNA clades were easily identified and typically separated from their geographic or genetic neighbors by roughly 2.5% sequence divergence, phylogenetic resolution was weak. This can be taken as evidence of rapid colonization of the emerging Panama landscape, followed by *in situ* diversification of mtDNA lineages. Third, the group identified as Costa Rica, North, and Atlantic includes the species *R. ilsae* and *R. guatemalensis*. The group labeled Mandinga, Tuira, Pacora contains representatives of *R. guatemalensis* and *R.*

occidentalis. Thus, when viewed from an mtDNA perspective, the named *Roeboides* species are both poly- and paraphyletic. Fourth, the geographical distribution of mtDNA variation often shows a poor congruence with the ranges of named species, and it has been concluded that taxonomic distinction provides poor estimates of genetic divergence in lower Central American fish. Finally, to the degree that mtDNA in fish evolves in an approximately clock-like manner, it appeared that not all taxa within a group are historically and evolutionarily equivalent. This finding has important consequences for the study of biogeographic patterns, and it has been pointed out elsewhere that cross-taxa biogeographic analyses would benefit from having phylogenetic branch points sorted by age (Bermingham *et al.*, 1992).

Our results also provide examples of recent expansions of specific lineages within the ranges of old established or extinct populations and suggest a pattern of alternation between geographical expansion and quiescence. Although genetical analyses of additional fish species are required to assess the generality of this pattern, data to date suggest that different populations of the same species may be in different phases of colonizing activity at a specific time. For example, in the Siluriform genus *Pimelodella*, limited geographic distribution of derived mtDNA haplotypes in eastern Pacific Panama contrasting the more widespread distribution of ancestral haplotypes in the rest of Panama has been found. If founder effects resulting in reduced within-population genotypic variability accompany colonization, the pattern of haplotypic diversity suggests that the spread of *Pimelodella* within Panama occurred in two waves: an older one that extended to central Panama and a more recent progression, probably from the Atrato through the Bayano. Because the Tuira is the largest of Panama's rivers, its reduced mtDNA variability may be explained most readily by a founder effect resulting from recent colonization instead of a postfounding bottleneck.

Prominent barriers to dispersal in lower Central America are evidenced by congruence between phylogeographic patterns across species. For example, the Sona Peninsula in central Pacific Panama marks either the end of a distribution (*Hypopomus*) or a genetic disjunction (*Pimelodella*: A. P. Martin and E. Bermingham, manuscript in preparation; *Roeboides*: E. Bermingham and A. P. Martin, manuscript in preparation; *Aequidens*: S. S. McCafferty *et al.*, manuscript in preparation) in the species examined to date. The cause of this phylogenetic break may relate to a transverse range of hills that bisect Panama in this region.

Early results somewhat belie the depauperate condition of the middle American primary freshwater fish

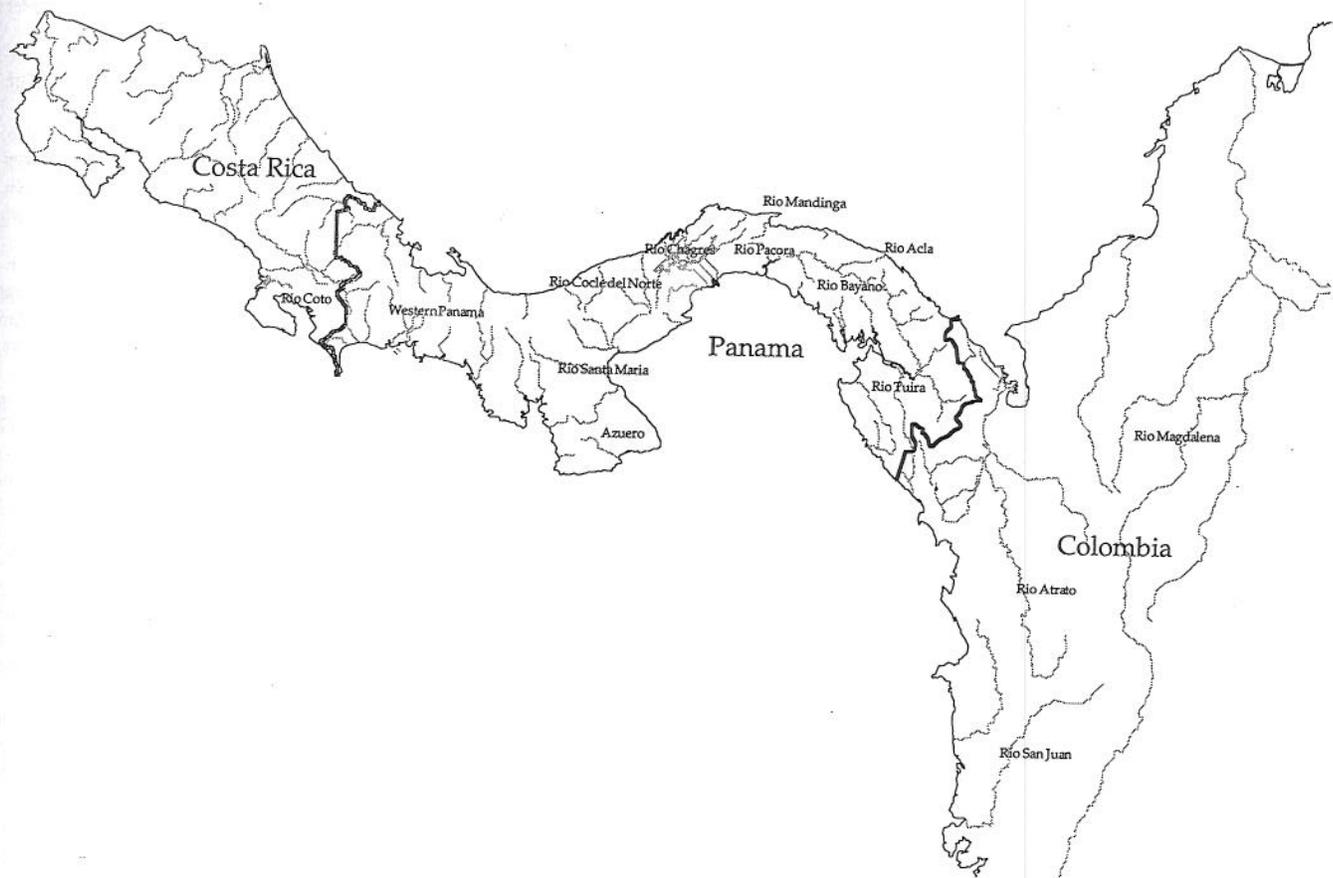


FIGURE 7 Location of major drainages in Costa Rica, Panama, and Colombia where samples of *Roeboides* were collected.

fauna which led Myers (1966) to state that he could "see no escape from the conclusion that Central America possessed no obligatory freshwater ostariophysans until the Pliocene or even the Pleistocene, since which time the most aggressive and ubiquitous of all characoid genera (*Astyanax*) has, in a geological sense, raced northward to the Rio Grande, trailed a little more slowly by *Hyphessobrycon*, *Brycon*, *Roeboides*, *Gymnotus*, and a few others." Furthermore, the significant mtDNA divergence and reciprocal monophyly of fish mtDNA lineages among lower Central American drainage basins foreshadow an analysis of the pattern and rate of freshwater fish exchange that took place before and following the Pliocene completion of the Panamanian isthmus.

VI. Concluding Remarks

Surveying large numbers of individuals across moderate numbers of species with overlapping distribu-

tions should be a goal in evolutionary biology for both theoretical and applied reasons. On the theoretical side, species richness may be influenced more strongly by extrinsic biogeographical relationships and historical circumstances than by such intrinsic, local processes as competition and predation (Ricklefs, 1987; Cornell and Lawton, 1992; Ricklefs and Schluter, 1993). The sheer magnitude of systematic description required in the tropics indicates a pervasive role for molecular systematics if we are to determine the dependence of local richness on regional species richness in tropical ecosystems.

On the practical side, molecular genetic analyses can provide a reasonably rapid means for surveying regional biotic diversity. Indices of species richness, sometimes taking into account abundance, have been the traditional measures of diversity. When used to make decisions regarding the preservation of biodiversity, however, it has been argued that these indices fail because they consider all species to be equal or nearly equal. Erwin (1991), Vane-Wright *et al.* (1991), and others (Crozier, 1992; Faith, 1992; Weitzman, 1992; Solow

et al., 1993; reviewed by Krajewski, 1994) have suggested that phylogenetic history and/or genetic diversity should be used in biodiversity indices to emphasize the phylogenetic and genetic distinctiveness of some groups compared to others. To the degree this view is adopted by conservation biologists, molecular systematics will undoubtedly be called upon to provide measures of taxonomic distinctiveness. The resulting taxic diversity measures, when coupled to detailed knowledge of organismal distribution patterns, can be used to identify priority areas for conservation (Vane-Wright *et al.*, 1991).

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