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MOLECULAR ZOOGEOGRAPHY OF FRESHWATER FISHES IN THE SOUTHEASTERN UNITED STATES

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ABSTRACT

Restriction fragment length polymorphisms in mitochondrial DNA (mtDNA) were used to reconstruct evolutionary relationships of conspecific populations in four species of freshwater fish—*Amia calva, Lepomis punctatus, L. gulosus*, and *L. microlophus*. A suite of 14–17 endonucleases was employed to assay mtDNAs from 305 specimens collected from 14 river drainages extending from South Carolina to Louisiana. Extensive mtDNA polymorphism was observed within each assayed species. In both phenograms and Wagner parsimony networks, mtDNA clones that were closely related genetically were usually geographically contiguous. Within each species, major mtDNA phylogenetic breaks also distinguished populations from separate geographic regions, demonstrating that dispersal and gene flow have not been sufficient to override geographic influences on population subdivision.—Importantly, there were strong patterns of congruence across species in the geographic placements of the mtDNA phylogenetic breaks. Three major boundary regions were characterized by concentrations of phylogenetic discontinuities, and these zones agree well with previously described zoogeographic boundaries identified by a different kind of data base—distributional limits of species—suggesting that a common set of historical factors may account for both phenomena. Repeated episodes of eustatic sea level change along a relatively static continental morphology are the likely causes of several patterns of drainage isolation and coalescence, and these are discussed in relation to the genetic data.—Overall, results exemplify the positive role that intraspecific genetic analyses may play in historical zoogeographic reconstruction. They also point out the potential inadequacies of any interpretations of population genetic structure that fail to consider the influences of history in shaping that structure.

HISTORICAL biogeography deals with relationships between the earth's physical history and the geographic arrangements of organisms. For example, a central tenet of vicariance biogeography is that modern-day distributions of related taxa within monophyletic groups reflect the fragmentation of formerly widespread ancestral biotas. It is postulated that concordant geographic distributions (tracks) among independently evolving lineages indicate the influences of a shared geologic history (CROIZAT, NELSON and ROSEN 1974; PLATNICK and NELSON 1978; ROSEN 1975, 1978).

Most historical biogeographic studies have been concerned with taxa at or

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above the level of species. Nonetheless, conspecific populations, if differentiated, can provide historical information about a region (CHERNOFF 1982; ROSEN 1978). Empirical data indicate that restriction analysis of mitochondrial DNA (mtDNA) is a sensitive technique for reconstructing evolutionary relationships among conspecific populations (UPHOLT and DAWID 1977; AVISE *et al.* 1979; AVISE, LANSMAN and SHADE 1979; BROWN and SIMPSON 1981; YONEKAWA *et al.* 1981; LANSMAN *et al.* 1983; SAUNDERS, KESSLER and AVISE 1986). In addition, the geographic patterning of mtDNA restriction site polymorphism observed in a number of studies (AVISE *et al.* 1979, 1984; SPOLSKY and UZZELL 1984; LANSMAN *et al.* 1983; WRIGHT, SPOLSKY and BROWN 1983; SAUNDERS, KESSLER and AVISE 1986) suggests that, at least for some species, dispersal and gene flow have not overridden historical influences on population subdivision.

Here we use restriction analysis of mtDNA to characterize geographic population structure within each of several widespread freshwater fish species in the southeastern United States. By considering the geographic distributions of intraspecific mtDNA phylogenies for several species jointly, we have sought to address the role of historical biogeographic factors in shaping population genetic structure.

In biogeographic reconstructions, a common approach is to compare and interpret biological data against known or suspected geologic cladograms. For present purposes, ideally we would want a precise drainage-system cladogram representing known historical patterns of river interconnection. However, current understanding of the geologic history of the Southeast is insufficient to provide this kind of detail. Thus, another goal of the current study will be to utilize genetic data to develop a regional biogeographic scenario against which further geologic information may be compared as it becomes available.

MATERIALS AND METHODS

Collections: Four species of freshwater fish were collected across the southeastern United States—*Lepomis punctatus*, *L. gulosus* and *L. microlophus* (Centrarchidae) and *Amia calva* (Amiidae). Criteria for choice of these species were (1) a widespread native distribution encompassing the region of interest, (2) habits and sufficient abundance for ease of collection at most locales, and (3) sufficient size for ready yield of highly purified mtDNA from liver or heart tissue.

Fourteen major river systems, extending from the Cooper drainage in South Carolina to the Mississippi River and beyond, were sampled (Table 1). In Table 1 and throughout the paper, these major drainages are given sequential lower case letter designations. Numerical subscripts to these lower case letters represent separate collection locales within a drainage. For example, collections from the Ocmulgee and Oconee Rivers, which are major tributaries of the Altamaha drainage, are denoted d_2 and d_3. Major drainages and collection locales are pictured in Figure 1.

Laboratory procedures: Fish were returned live to our laboratory at the University of Georgia. Mitochondrial DNA was isolated from the fresh liver and/or heart of each individual and was purified in closed-circular form using procedures of cesium chloride/ethidium bromide gradient centrifugation (LANSMAN *et al.* 1981). Following dialysis to remove cesium chloride and ethidium bromide, aliquots of purified mtDNA were digested using 14 restriction enzymes per individual of *A. calva* and 17 enzymes per individual of Lepomis sp. (Table 2). Restriction endonuclease digestions were usually

TABLE 1

Collection locations and sample sizes

Letter code and major drainage	Locale	*Lepomis* *gulosus*	*Lepomis* *micro-lophus*	*Lepomis* *punctatus*	*Amia calva*
a Cooper	Cooper River, Berkeley County, South Carolina	6	6	9	6
b Savannah	Savannah River, Aiken County, South Carolina	5	7	6	6
c Ogeechee	Ogeechee River, Effingham County, Georgia			4	6
d_1 Altamaha	Altamaha River, Long County, Georgia	7	3	7	6
d_2	Oconee River, Clarke County, Georgia	5			
d_3	Ocmulgee River, Twiggs County, Georgia				4
e Satilla	Satilla River, Brantley County, Georgia	1		2	6
f St. Marys	St. Marys River, Charlton County, Georgia			3	2
g St. Johns	St. Johns River, Lake County, Florida	6	6	6	6
h_1 Everglades	Lower Everglades, Dade County, Florida	4	1	4	4
h_2	Lake Osborne, Palm Beach County, Florida	1	4	2	1
i Suwannee	Suwannee River, Gilchrist County, Florida	6	4	6	6
j_1 Apalachicola	Apalachicola River, Liberty County, Florida	4	6	9	5
j_2	Chattahoochee River, Troup County, Alabama	2			1
k_1 Escambia	Escambia River, Santa Rosa County, Florida	6	6	9	4
k_2	Conecuh River, Escambia County, Alabama				3
l_1 Alabama/Tombigbee	Tombigbee River, Lowndes County, Mississippi	6	6	2	3
l_2	Black Warrior River, Greene County, Alabama		3		3
l_3	Alabama River, Wilcox County, Alabama				1
l_4	Uphapee Creek, Macon County, Alabama	7	5	2	
l_5	Conasauga River, Whitfield County, Georgia		2	3	
l_6	Coosawattee River, Gordon County, Georgia		4		
m_1 Mississippi	Big Black River, Madison County, Mississippi	2	3		1
m_2	Yazoo River, Yazoo County, Mississippi	2	6		1
n Calcasieu	Calcasieu River, Calcasieu County, Lousiana	4	5	5	
Totals		74	77	79	75

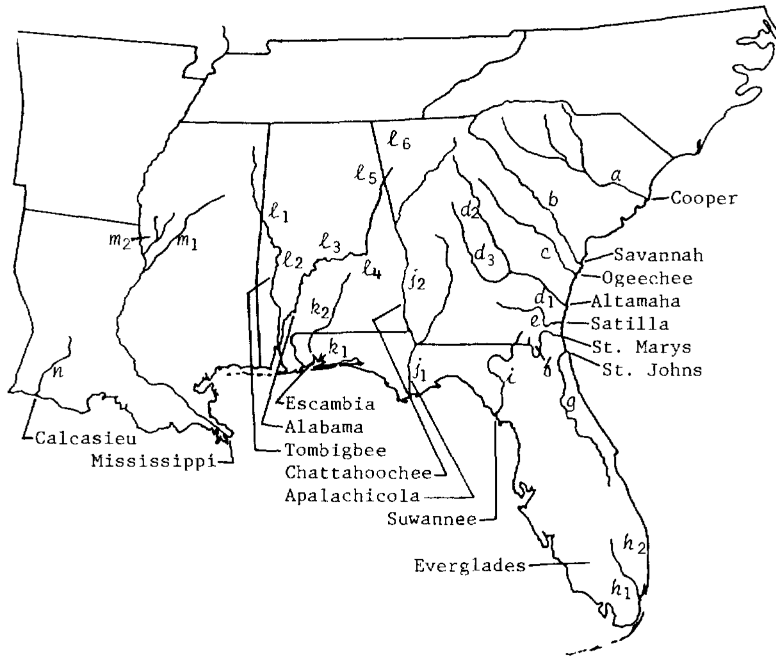


FIGURE 1.—Major drainages and collection locales (labeled as in Table 1) in the southeastern United States.

carried out overnight in assay conditions recommended by the vendor (New England Biolabs). Resulting DNA fragments were labeled with appropriate [α-^{32}P]dXTP(s), using the rapid end-labeling technique of DROUIN (1980), sized by agarose gel electrophoresis (0.7–1.3%) and visualized by autoradiography. The possibility of scoring two nonhomologous fragments as identical due to chance comigration was minimized by rerunning fragments of questionable identity side-by-side at different gel concentrations.

Data analysis: MtDNA fragment patterns on gels constituted the raw data. Within each species, distinct single-endonuclease patterns were given separate upper-case letter designations. Considering all enzymes, any individual fish could then be assigned a composite letter code describing its observed mtDNA genotype (Table 3). For convenience, we say that any individuals sharing a composite code belong to the same mtDNA clone, although this of course applies only to the particular restriction sites surveyed.

A data matrix was then constructed for each species that consists of presence-absence information for each restriction fragment in each mtDNA clone. These matrices were used to calculate overall percentages of fragments shared between pairs of clones and also to estimate nucleotide sequence divergence (p values) by the approach of NEI and LI (1979). The resulting distance matrices were clustered by the unweighted pair-group method (UPGMA), using the average linkage algorithm of the BMDP statistical software package (DIXON 1981).

The fragment presence-absence matrices were also used to generate Wagner parsimony networks by the Metropolis simulated annealing algorithm (METRO) in the Phylip phylogenetic package distributed by JOE FELSENSTEIN. Confidence limits on branches of the Wagner networks were generated by the bootstrap method (BOOTM in the Phylip package). For *Amia calva*, taxa were input to BOOTM in five distinct orders each run 100 times (total of 500 replicates); for each Lepomis species, three input orders of taxa were each replicated 50 times (total of 150 runs).

FELSENSTEIN'S (1985) discussion of bootstrapping should be consulted by those interested in application of the method to phylogenetics. Simply, the bootstrap method

TABLE 2

Restriction endonucleases, recognition sequences and total numbers of fragments (and fragment patterns) revealed by each enzyme

	Lepomis			*Amia calva*
	gulosus	*microlophus*	*punctatus*	
6-base enzymes				
*Bam*H1 (GGATCC)	5 (3)	3 (2)	6 (4)	7 (5)
*Bcl*1 (TGATCA)	8 (4)	9 (2)	11 (5)	7 (3)
*Bgl*1 ($GCCN_5GGC$)	11 (7)	5 (2)	8 (4)	2 (1)
*Bgl*11 (AGATCT)	* (1)[a]	* (1)	3 (2)	4 (1)
*Bst*E11 (GGTNACC)	3 (2)	4 (2)	8 (5)	* (1)
*Cla*1 (ATCGAT)	* (1)	* (1)	* (1)	3 (2)
*Eco*RI (GAATTC)	5 (3)	5 (3)	* (1)	4 (1)
*Hin*dIII (AAGCTT)	3 (1)	6 (2)	8 (4)	9 (2)
*Kpn*1 (GGTACC)	* (1)	* (1)	* (1)	
*Nde*1 (CATATG)	* (1)	3 (2)	* (1)	
*Pst*1 (CTGCAG)	9 (5)	5 (2)	7 (3)	4 (2)
*Pvu*II (CAGCTG)	6 (4)	* (1)	* (1)	8 (3)
*Sac*1 (GAGCTC)	13 (6)	5 (2)	8 (5)	
*Stu*1 (AGGCCT)	19 (8)	11 (2)	21 (8)	11 (3)
*Xba*1 (TCTAGA)	7 (3)	4 (2)	3 (1)	5 (2)
5-base enzymes				
*Ava*1 (CPyCGPuG)	15 (5)	16 (3)	24 (8)	5 (1)
*Hin*c11 (GTPyPuAC)	15 (6)	11 (3)	15 (6)	9 (3)
Total	119	87	122	78

[a] *, Denotes 0 or 1 cut revealed by restriction endonuclease.

resamples the original data by drawing data points at random, and with replacement, to construct a series of fictional sets of data. The resulting phylogenies are summarized by counting the number of times that the bootstrap estimates contained corresponding monophyletic groups. The percentage of times that a monophyletic assemblage is supported enables one to assess the strength of the phylogenetic hypothesis. Intuitively, the more characters there are supporting a group, the more likely it is that a random sampling of characters will include enough information to define that group.

As pointed out by FELSENSTEIN (1985), the bootstrap method is compromised if characters are correlated. Restriction fragment data (although not restriction site data) are partially correlated in the sense that, for example, loss of one restriction fragment due to gain of a restriction site is accompanied by the gain of two novel fragments (see KESSLER and AVISE 1984). The redundancy of information concerning a single evolutionary event (gain or loss of a restriction site) may inflate bootstrap values. We therefore caution that bootstrap estimates of confidence limits on phylogenies inferred from mtDNA fragment data be used as relative measures only.

In the *Amia calva* data, mtDNA sequence diversity was sufficiently low and the fragment patterns sufficiently simple to permit unambiguous identification of the particular restriction site changes responsible for all clonal differences. For this species, a Wagner network was also constructed from a restriction site presence-absence matrix (it proved to be identical in structure to the network developed from the fragment presence-absence matrix). For the Lepomis species, however, the high diversity of mtDNA fragment patterns and the multiple site differences obviously involved in many fragment pattern interconversions precluded straightforward interpretation of all site changes responsible. Double-digest mapping could, in principle, have identified all re-

TABLE 3

Composite clonal genotypes and number of individual fish in each clone

Clonal designation	Composite clonal genotype[a]	No. of individual fish	Locale
Amia calva			
1	AAAAAAAAAAAAA	30	a–g, i, j_1, j_2
2	AAAAAAAABAAAA	4	a
3	AAAAAAAAABAAA	3	b, c
4	DAAAAAAAAAAAA	1	c
5	AAAAAAAACAAAA	10	d_1, d_3, f, g, i
6	ABAAAAAAAAAAA	2	e
7	AAAAAABAAAAAA	1	e
8	EAAAAAAAAAAAB	3	g, h_2
9	BAAAAAAAAAAAB	5	h_1, h_2, i
10	CAAABAABAAAAC	6	k_1, k_2
11	CAAABAABACAAC	1	k_2
12	CCAABAAAAAAAC	7	l_1, l_2, l_3
13	CCAABAAAAABAC	2	m_1, m_2
Lepomis gulosus			
1	AAAAAAABACBBA	1	a
2	AAAAAAABABBBA	7	a, b, e
3	AAAAAAAAAAAAA	3	a
4	AABAAAAAAAAAA	1	a
5	AACAAAAAADACA	4	d_2
6	AAAAAAAAABACA	2	d_1, d_2
7	AADAAAAABEAAA	3	d_1
8	AADAAAAABCACA	1	g
9	AADAAAAABBACA	6	g, h_1
10	AADAAAAABBAAB	1	g
11	AADAAAAABBAAA	6	d_1, g, h_2, i
12	AADAAABABFAAA	1	g
13	BADAAAAABBAAA	3	i
14	AADAAAAAABAAA	1	i
15	AAAAAAAAABAAA	2	n
16	AAAAAACCDBAAA	1	j_1
17	AAAAAACAEBAAA	1	j_1
18	AAAAAACACBAAA	4	j_1, j_2
19	AAAAAACAACAAA	1	k_1
20	AAAAAACAABAAA	4	i, k_1
21	AAEAAACAABAAA	1	k_1
22	AAAAAABAABAAA	7	k_1, l_4
23	ABAAAABAABAAA	1	l_4
24	ACFBBADCFGCDC	1	l_1
25	ACGBBADCFGCDC	1	l_1
26	ACFBBADDFGCDC	2	l_1
27	ACFBCADBFGCDE	1	l_1
28	CDFBBADBFGCEF	2	m_1
29	ADFBBAEBFGCEF	1	m_2
30	ADFBBADBFHCEF	1	m_2
31	ADFBBADDFGCED	2	n
32	ADGBBADDFHCED	1	l_1

TABLE 3—*Continued*

Clonal designation	Composite clonal genotype[a]	No. of individual fish	Locale
Lepomis microlophus			
1	AAAACAABAAABA	1	b
2	AAAAAAABAAABA	40	a, b, i–n
3	AAAACAABAABBC	5	k_1
4	AAAACAAAAAAAA	14	a–d_1, j_1, m_1
5	ABBBBBBABBACB	4	d_1, i
6	BBBBBBBABBADB	1	i
7	BBBBBBBABBACB	12	b, g, h_1, h_2
Lepomis punctatus			
1	AAAAABAAAAAA	34	a–i
2	AAAAAAAAAAAA	7	a
3	AAAAADBAAAAA	1	g
4	AAAAABBAAAAA	1	g
5	AAAAABAAAAFA	1	d_1
6	AAAAABAABAAA	1	h_1
7	ABBAABABCABB	3	i
8	ABAABBABDABB	1	i
9	BCCACCBCEACE	9	j_1, l_5
10	BCCACCBCEACC	1	j_1
11	BCCACCBEEAHD	1	j_1
12	BCCADCBDEADE	7	k_1
13	BECADCBDEADE	3	k_1, l_1
14	BCCADCBBHAGE	1	k_1
15	DECBECCBGAEF	4	n
16	CDDBACBBFAEF	3	l_4, l_5
17	CECBACBBHAEF	1	n

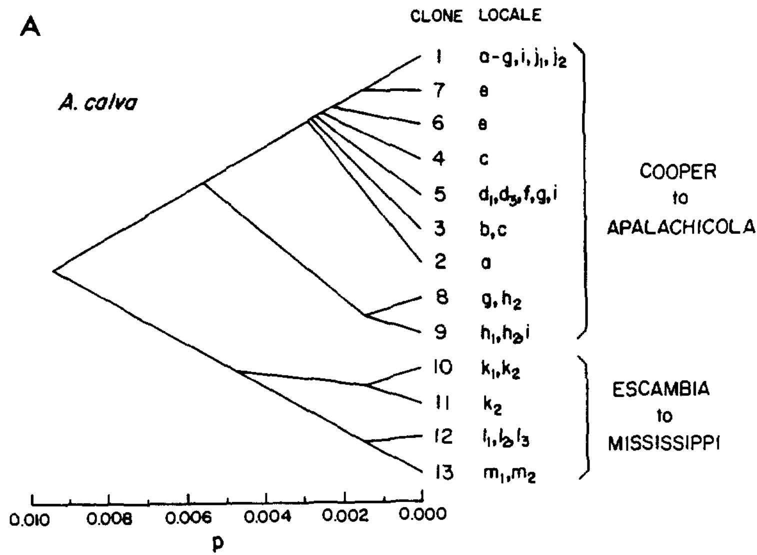
[a] Letters, from left to right, refer to digestion profiles produced by the endonucleases listed in same order in Table 2 (enzymes producing zero or one cut are not included).

striction site changes, but for our purposes this additional effort seemed unwarranted. Nonetheless, for all pairs of clones we could calculate *minimum* numbers of restriction site differences (by assuming two restriction site differences for any mtDNA profiles distinguished by multiple site changes). This information is included in the Wagner network topologies.

RESULTS

This study entailed mtDNA isolations from 305 individuals and a total of 4960 restriction digests, not counting reruns. More than 400 restriction fragments, exhibited in 184 different single-enzyme digestion profiles, were observed (Table 2). Space does not permit a complete description of these profiles, but all are pictured in BERMINGHAM (1986). Altogether, 69 mtDNA clones were observed in this study (Table 3). Molecular aspects of mtDNA polymorphism in bowfin and sunfish are presented elsewhere (Amia, BERMINGHAM, LAMB and AVISE 1986; Lepomis, AVISE *et al.* 1984 and AVISE and SAUNDERS 1984). The following sections describe the major features of the phylogenetic relationships of the mtDNA clones in each species.

Bowfin, *Amia calva*: Thirteen mtDNA clones were observed among the 75 bowfin sampled. In the UPGMA phenogram (Figure 2A), two major genotypic



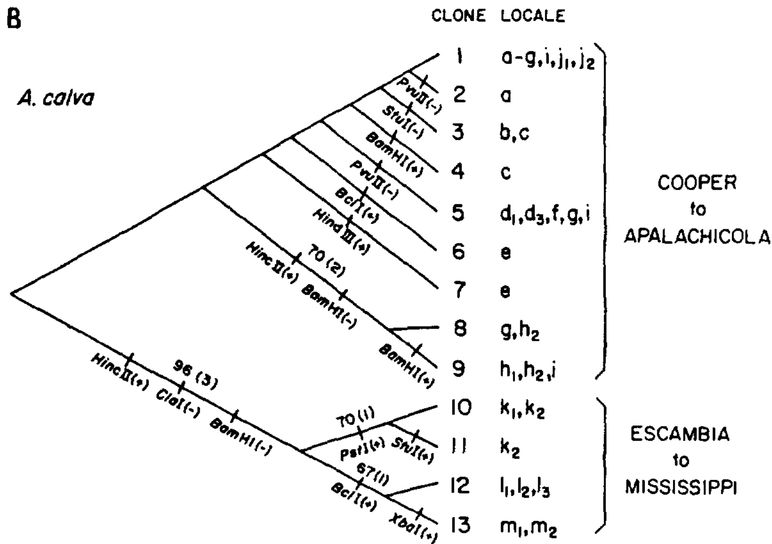


FIGURE 2.—A, Phenogram of mtDNA genotypes in *A. calva* generated by UPGMA cluster analysis of nucleotide sequence divergence ($\bar{p}$) estimates. MtDNA clones are numbered as in Table 3, and locales are lettered as in Table 1. B, Wagner parsimony network of mtDNA genotypes in *A. calva* generated from the presence-absence site matrix. MtDNA clones are labeled as in Table 3, and locales are lettered as in Table 1. First numbers along branches indicate the proportion of times that a group was distinguished in the bootstrap analysis (only proportions greater than 50% are shown). Inferred restriction site changes are shown along all branches. Numbers in parentheses indicate *minimum* numbers of restriction site differences along the path. The network was arbitrarily rooted in a position that facilitates visual comparison with the UPGMA phenogram.

clusters, distinguished by about 1% sequence divergence, were apparent. These genetic groups are strongly patterned geographically, such that all fish collected east of and including the Apalachicola River belong to one cluster, whereas all fish collected west of the Apalachicola River belong to the other cluster. Within the western group, subsidiary clusters distinguish the Escambia River bowfin from those in the Alabama/Tombigbee and Mississippi drainages; and within the eastern group, a subsidiary cluster distinguishes some of the bowfin collected in central Florida. Nonetheless, within the eastern assemblage, mtDNA clone number 1 was found in all sampled drainages, with the exception of the Everglades, from the Cooper to the Apalachicola; and clones 3 and 5 were also present in more than one drainage (Table 3). In contrast, each mtDNA clonal type sampled within the western form of *A. calva* was confined to a single drainage (Table 3; Figure 2A).

The Wagner network based on the presence-absence restriction site matrix is shown in Figure 2B; it is nearly identical to the UPGMA phenogram. The genetic distinction of eastern *vs.* western forms of *A. calva* is supported at greater than the 95% confidence level by the bootstrap approach. There is, as expected, somewhat less bootstrap support for the genetic distinctiveness of the other mtDNA types.

Because of the simple structure of the bowfin mtDNA data, the restriction site changes underlying all clonal differences could be determined. Thus, the western mtDNA clonal assemblage is distinguished from all eastern genotypes by absence of *Cla*I and *Bam*HI sites, presence of a *Hinc*II site, and either presence of a *Pst*I site (Escambia drainage fish) or presence of a *Bcl*I site (Alabama and Mississippi drainage fish). These and other inferred restriction site changes are shown along the cladogram in Figure 2B. There is only one obvious inconsistency (instance of homoplasy) in Figure 2B: clones 4 and 9 are not adjacent in the network, yet share a unique *Bam*HI site. One of several plausible explanations is that the site may have been gained independently, more than once in evolution.

The major feature, then, of the mtDNA data in bowfin is the genetic divergence exhibited between eastern and western forms. To graphically summarize this pattern in a manner that will facilitate later biogeographic discussion, Figure 3 plots the frequencies of fragment patterns produced by an informative and representative endonuclease, *Cla*I (see also Figure 4).

Spotted sunfish, *Lepomis punctatus*: Seventeen mtDNA clones were observed among the 79 *L. punctatus* assayed. In the UPGMA phenogram (Figure 5A), these clustered into two major assemblages between which mean sequence divergence was about 6.1%. One major cluster consisted of all spotted sunfish collected from the Cooper to the Suwannee Rivers, and the other cluster consisted of all fish from the Apalachicola drainage westward. Within the eastern assemblage, two mtDNA clones (7 and 8) in the Suwannee River were also fairly distinct genetically ($p \cong 0.04$; Figure 5A). However, other *L. punctatus* in the Suwannee River possessed a common mtDNA genotype (clone 1) observed throughout the entire eastern region (Table 3). Within the western assemblage, subclusters in the phenogram did not always accord well with present-day drainage limits. Thus, clone 9 occurred in fish from both the

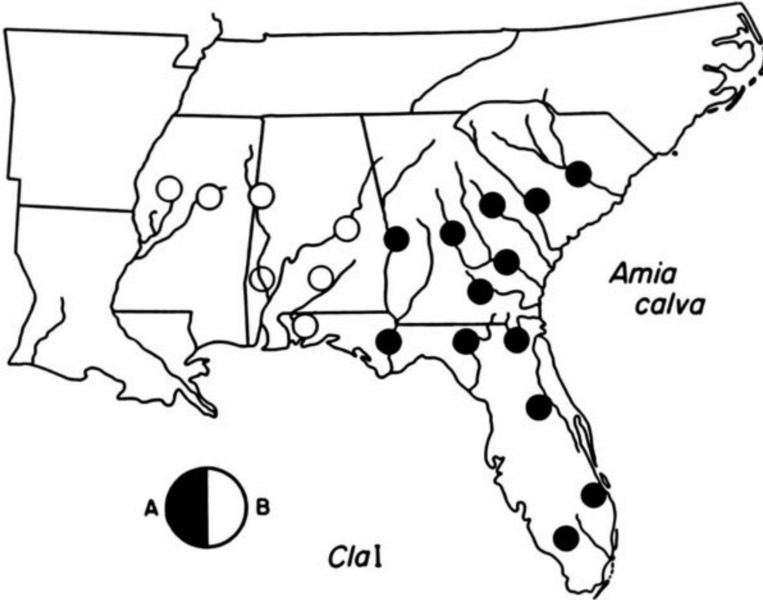


FIGURE 3.—Geographic distribution of *Cla*I mtDNA genotypes (see Figure 4) in *A. calva*. This distribution faithfully reflects the ranges of the two major mtDNA assemblages identified in the entire Amia survey.

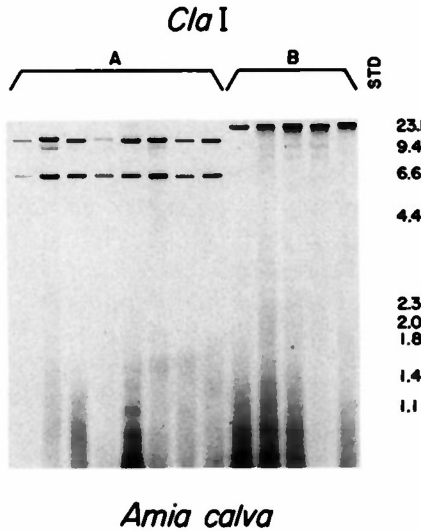


FIGURE 4.—Representative *Cla*I digests of mtDNA from *A. calva*. Although this autoradiograph shows examples of both size polymorphism and heteroplasmy (*e.g.*, lane 2 from left), neither of these characteristics has interfered with the analysis of site or fragment data (see BERMINGHAM, LAMB and AVISE 1986).

Apalachicola and Alabama/Tombigbee drainages; and clone 16, which was phenetically (and cladistically, see beyond) allied to clones 15 and 17 from the far western Calcasieu drainage, was also observed in the Alabama/Tombigbee system.

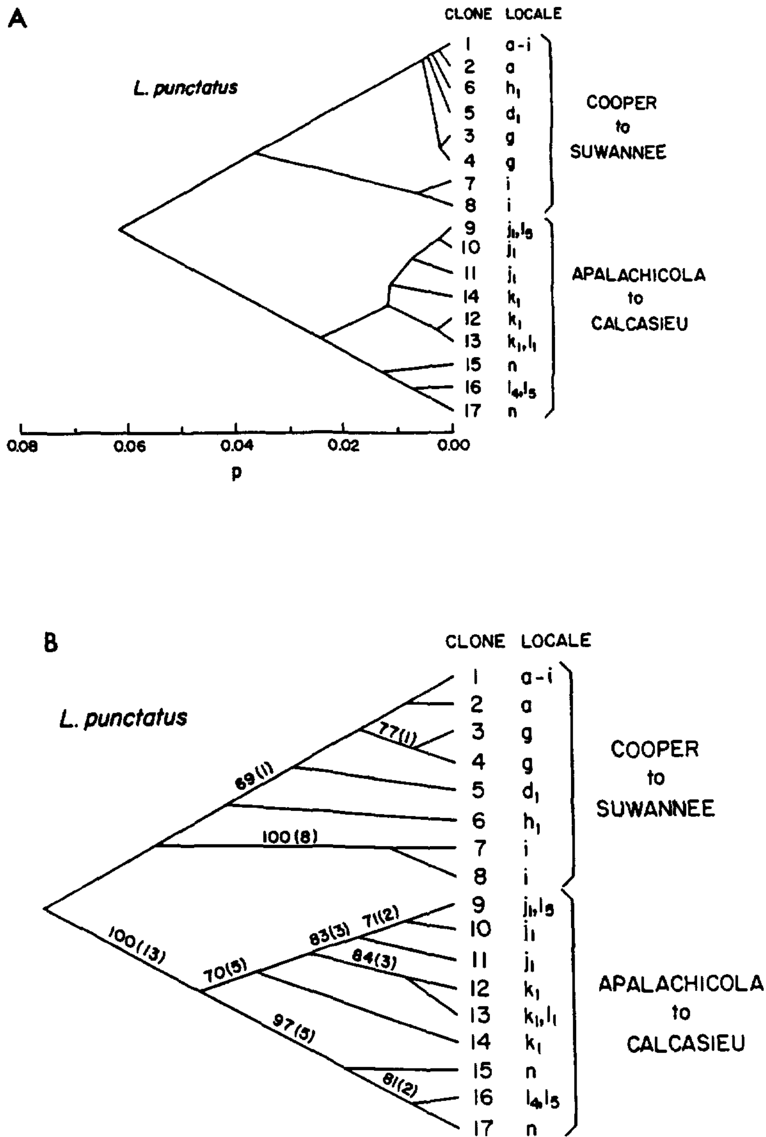


FIGURE 5.—A, Phenogram of mtDNA genotypes in *L. punctatus* generated by UPGMA cluster analysis of nucleotide sequence divergence estimates (see legend to Figure 2A). B, Wagner parsimony network of mtDNA genotypes in *L. punctatus* generated from the presence-absence fragment matrix (see legend to Figure 2B).

The branching pattern in the qualitative Wagner network (Figure 5B) is nearly identical to that in the quantitative UPGMA phenogram. The major east-west break is evidenced by a minimum of 13 restriction site changes, and it is supported at the 100% level by the bootstrap results. The Suwannee River mtDNA clones 7 and 8 appear allied to the eastern *L. punctatus* forms, although they differ from closest relatives by a minimum of eight assayed restriction sites.

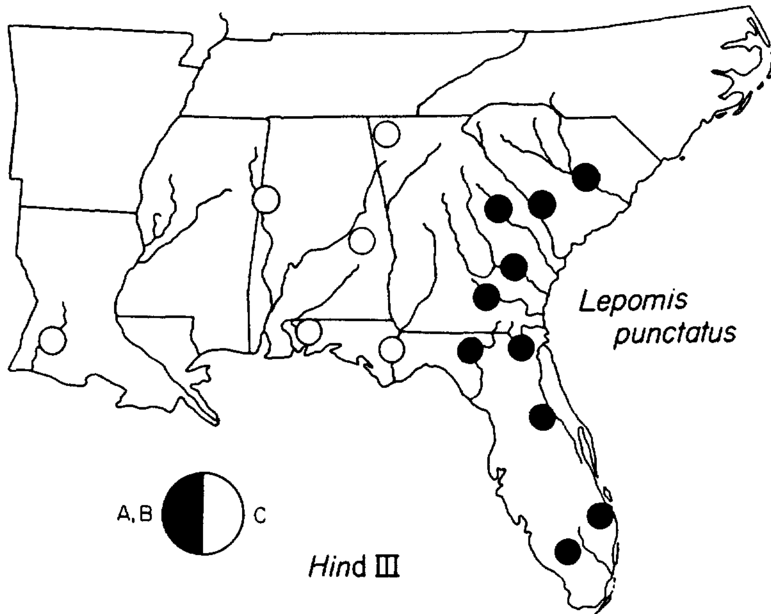


FIGURE 6.—Geographic distribution of *Hin*dIII mtDNA genotypes (see Figure 7) in *L. punctatus*. This distribution faithfully reflects the ranges of the two major mtDNA assemblages identified in the entire *L. punctatus* survey. *Hin*dIII fragment pattern A, which differs from B by a single-site gain and was observed only in the Cooper drainage, is for clarity plotted here as indistinguishable from the common eastern pattern B.

Overall, an east-west genetic break in *L. punctatus* is remarkably concordant in geographic placement with the genetic break in *A. calva*, with the exception that spotted sunfish collected from the Apalachicola drainage belong to a western, rather than eastern, mtDNA assemblage. The major pattern of genetic divergence in *L. punctatus* is summarized in Figure 6, which pictures results for a representative endonuclease, *Hin*dIII (Figure 7). The genetic differentiation between eastern and western *L. punctatus* may have been anticipated by earlier morphological comparisons (CARR and GOIN 1955) resulting in subspecies designations.

Redear sunfish, *Lepomis microlophus*: Seven mtDNA clones were observed among the 77 redear sunfish surveyed. UPGMA and Wagner diagrams summarizing relationships among these genotypes are shown in Figure 8. The mtDNA clones fall into two highly distinct arrays, between which mean sequence divergence is about 0.087, and at least 17 restriction sites are diagnostic. Distinction between the two forms is supported at the 100% level by bootstrapping methods, and by these same criteria no other clonal distinctions are significant (Figure 8).

One mtDNA clonal array, which we shall refer to as the Florida form, was present in all fish collected from the Everglades and the St. Johns River, and in three of four fish from the Suwannee River. This Florida form was also found in some redear from the Savannah and Altamaha drainages along the

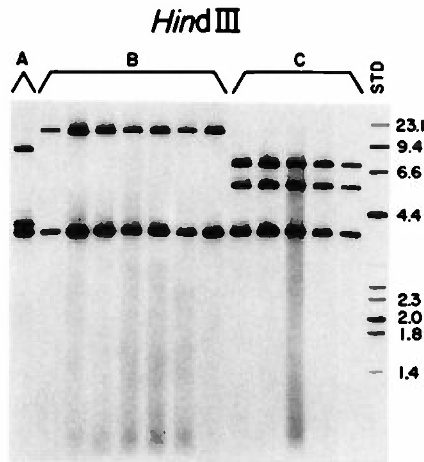


FIGURE 7.—Representative *Hind*III digests of mtDNA from *L. punctatus.*

Atlantic Coast. The other mtDNA clonal array was widespread outside Peninsular Florida, ranging from the Cooper to the Altamaha in Atlantic drainages, and from the Suwannee to the Calcasieu in drainages entering the Gulf of Mexico. These genetic differences may have been anticipated in the subspecies descriptions by BAILEY (1938) based on morphology. The geographic ranges of the two major mtDNA forms are plotted in Figure 9 (see also Figure 10).

By comparison, mtDNA clonal differences within either major lineage of *L. microlophus* were small. Within the northern mtDNA assemblage, two clones (2 and 4), differing by at least three mutation steps, were both geographically widespread. Nonetheless, clone 2 numerically predominated only in collections west of and including the Alabama/Tombigbee drainage, whereas clone 4 was most common east of and including the Apalachicola River (Table 3). In fact, only a single individual with mtDNA genotype 4 was found west of the Apalachicola drainage. Finally, clone 3, also distinguished from clone 2 by a minimum of three restriction site changes, appeared confined to and is perhaps characteristic of the Escambia River.

Warmouth, *Lepomis gulosus*: Thirty-two mtDNA clones were observed in our sample of 74 warmouth sunfish. Again, however, these genotypes were differentiated into two groups distinguishable in all cases by at least 14 restriction site changes (Figure 11). In the Wagner parsimony network, this genetic separation was supported at the 100% confidence level. One phylogenetic assemblage of clones was confined to populations west of and including the Tombigbee drainage, whereas the other assemblage was largely confined to drainages east of the Tombigbee. The only exception to this pattern involved two fish in the Calcasieu sample possessing an "eastern" genotype. Two other warmouth sampled from the Calcasieu River possessed "western" mtDNA gen-

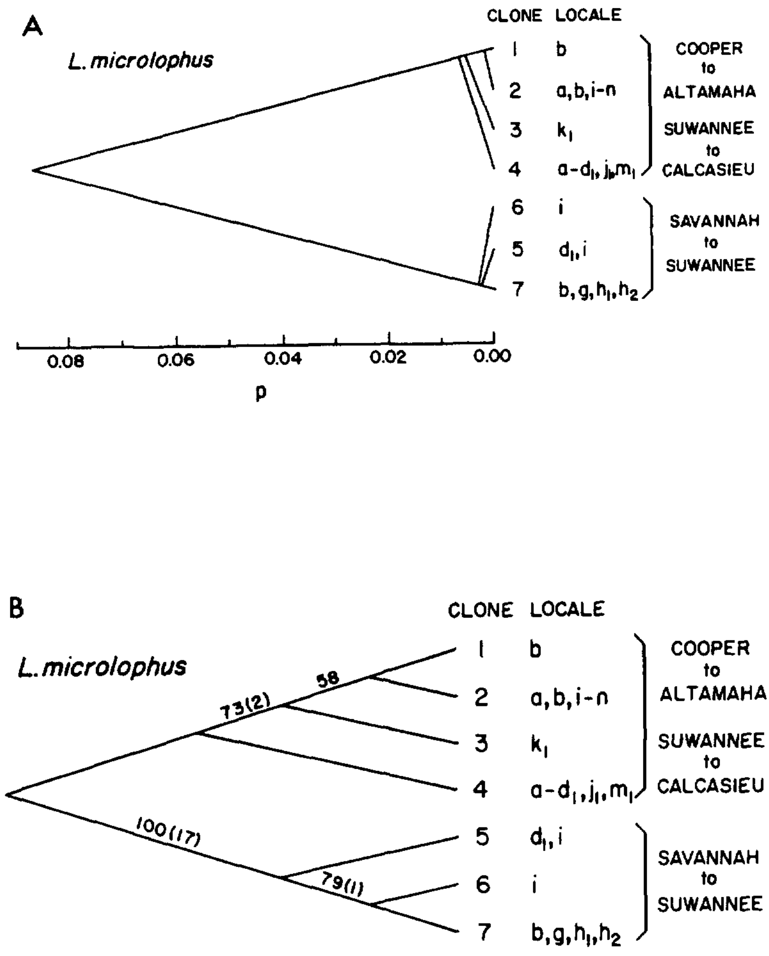


FIGURE 8.—A, Phenogram of mtDNA genotypes in *L. microlophus* generated by UPGMA cluster analysis of nucleotide sequence divergence estimates (see legend to Figure 2A). B, Wagner parsimony network of mtDNA genotypes in *L. microlophus* generated from the presence-absence fragment matrix (see legend to Figure 2B).

otypes, however, leading us to suspect that the aberrant genotypes might reflect a low-frequency natural polymorphism or, perhaps, effects of stocking by man. The position of the major east-west break in *L. gulosus* is summarized in Figure 12 (see also Figure 13).

A wealth of restriction fragment polymorphism was present in *L. gulosus*. For example, five different mtDNA genotypes were observed in the six fish collected from the Tombigbee River, and several other drainages show similar levels of clonal diversity (Table 3). Nonetheless, taxa that appeared closely related genetically were usually geographically contiguous.

The cluster and Wagner analyses revealed many additional but less well supported genetic distinctions within the two major mtDNA lineages (Figure 11). Most warmouth in the Tombigbee (clones 24–27) were two mutation steps

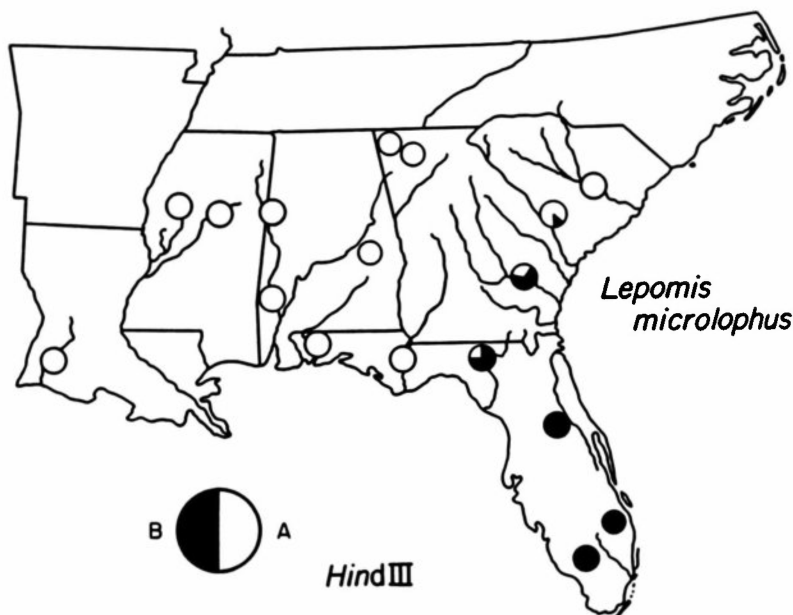


FIGURE 9.—Geographic distribution of *Hin*dIII mtDNA genotypes (see Figure 10) in *L. microlophus*. This distribution faithfully reflects the ranges of the two major mtDNA assemblages identified in the entire *L. microlophus* survey.

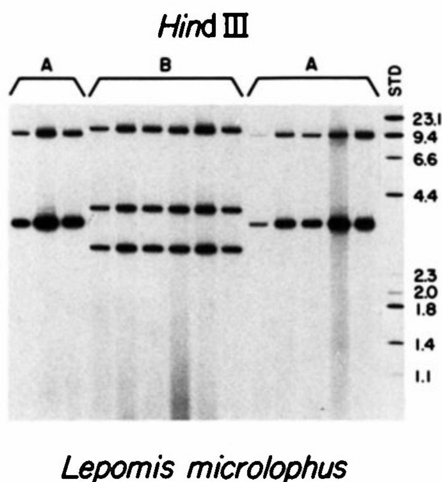
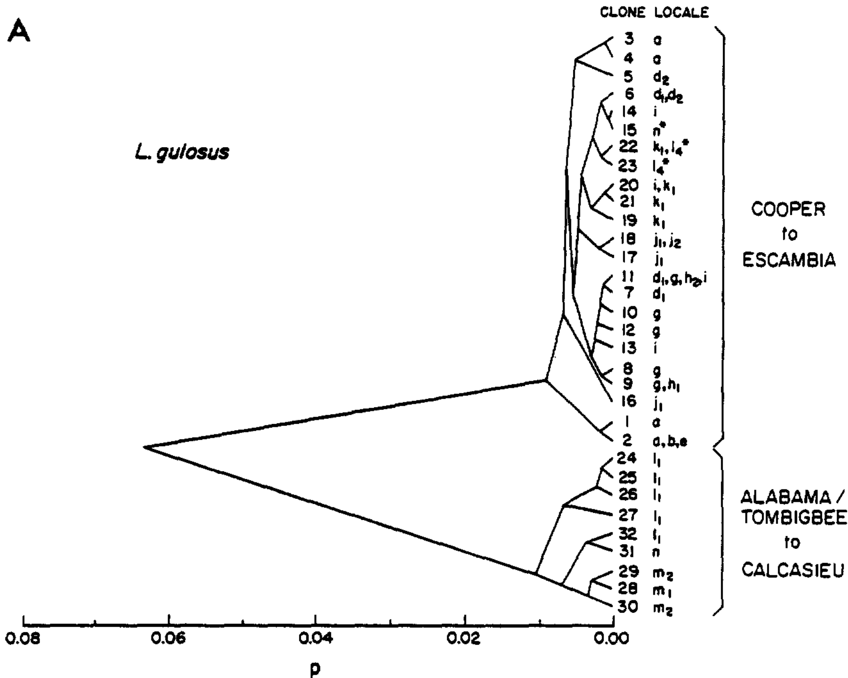


FIGURE 10.—Representative *Hin*dIII digests of mtDNA from *L. microlophus*.

removed from those in the Mississippi and Calcasieu drainages (clones 28–31), the exception being a single individual in the Tombigbee (clone 32) that was genetically allied to the more western assemblage. In the east, several mtDNA clonal arrays bore correspondence to geographic region. Clonal pairs 1-2 and 3-4, distinguishable from each other and from all other genotypes by at least two restriction site changes, were observed only in the Cooper, Savannah and Satilla Rivers. Clonal array 7–13 was observed only in the southerly region



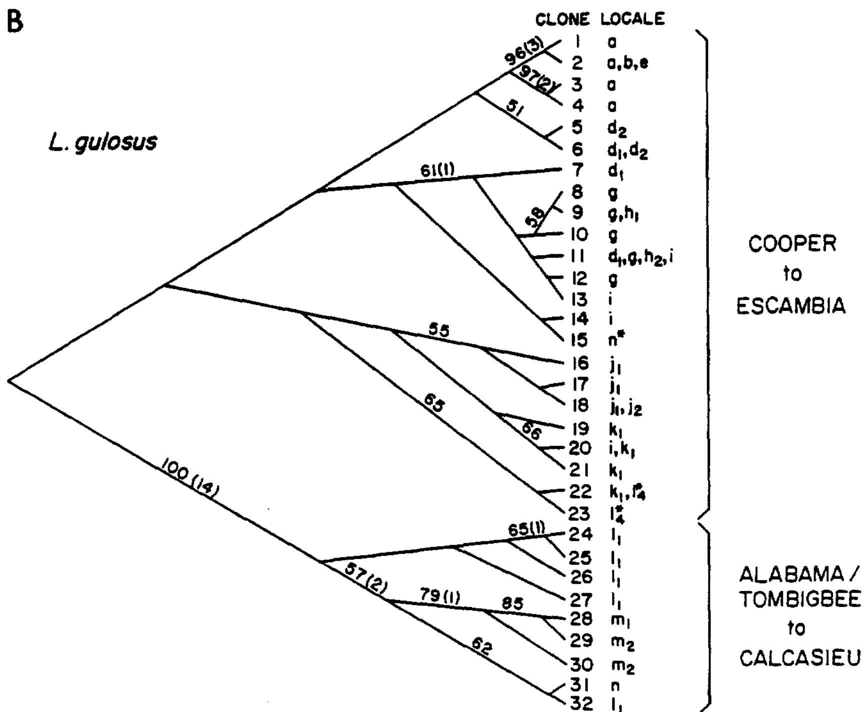


FIGURE 11.—A, Phenogram of mtDNA genotypes in *L. gulosus* generated by UPGMA cluster analysis of nucleotide sequence divergence estimates (see legend to Figure 2A, and also see text for explanation of the geographically disjunct clones n and l_4, marked with an asterisk). B, Wagner parsimony network of mtDNA genotypes in *L. gulosus* generated from the presence-absence fragment matrix (see legend to Figure 2B).

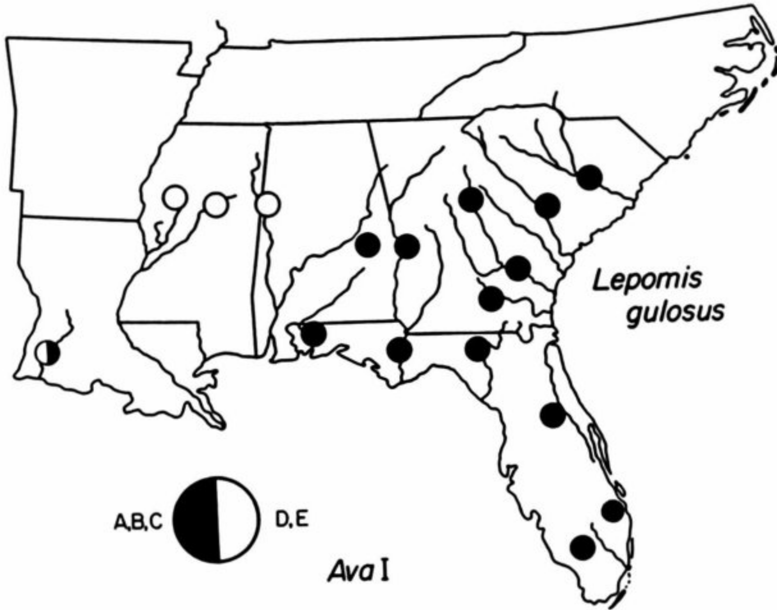


FIGURE 12.—Geographic distribution of *Ava*I mtDNA genotypes in *L. gulosus*. This distribution faithfully reflects the ranges of the two major mtDNA assemblages identified in the entire *L. gulosus* survey. *Ava*I patterns A–C are closely interrelated (by single-site gains or losses), as are patterns D and E. Hence, for clarity, only the distinction of patterns A–C *vs.* D, E is emphasized here (see Figure 13).

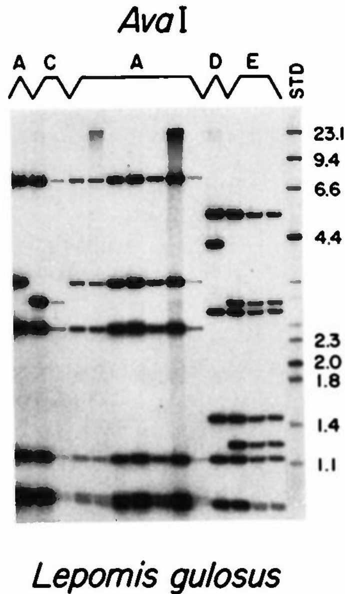


FIGURE 13.—Representative *Ava*I digests of mtDNA from *L. gulosus*.

from the Altamaha to the Suwannee and including peninsular Florida. Another mtDNA assemblage (clones 16–23, recognized in the Wagner network) was made up primarily of fish from the Suwannee to the Escambia River and included individuals collected from a small creek in eastern Alabama that currently flows into the Alabama/Tombigbee system.

DISCUSSION

Geographic patterns of mtDNA restriction site polymorphism have previously been described for a number of individual vertebrate species (reviews in AVISE 1986a,b; AVISE and LANSMAN 1983). The present study extends this approach to a joint consideration of mtDNA clonal phylogenies in several species inhabiting the same geographic region. The purposes of this research have been to (1) provide a data base on intraspecific mtDNA differentiation in freshwater fish species with wide distributions in the southeastern United States, (2) estimate the phylogenetic relationships of mtDNAs within these taxa, (3) compare results to earlier zoogeographic ideas based on species distribution limits and (4) relate these patterns to the geologic history of the region, thus assessing the role that historical biogeographic factors played in shaping the evolution and genetics of the southeastern fish fauna.

Intraspecific phylogenies and geographic distributions

Joint consideration of the genetically inferred intraspecific phylogenies for *A. calva* (Figure 2), *L. punctatus* (Figure 5), *L. microlophus* (Figure 8) and *L. gulosus* (Figure 11) [as well as two previously surveyed species *L. macrochirus* (AVISE and SMITH 1974, figure 3) and *Micropterus salmoides* (BAILEY and HUBBS 1949; PHILLIP, CHILDERS and WHITT 1981, 1982] reveals three major geographic boundary areas characterized by concentrations of phylogenetic discontinuities: (1) Apalachicola drainage region, (2) Alabama/Tombigbee drainage region and (3) a zone separating peninsular Florida from the remainder of the southeastern region. It should be noted that the Apalachicola River may represent a northwestern distribution limit of Floridian genotypes and/or a boundary region dividing eastern (including peninsular Florida) from western forms.

Apalachicola region: Major intraspecific genetic breaks are documented in the Apalachicola region for *A. calva*, *L. punctatus*, *L. microlophus*, *L. macrochirus* and *M. salmoides*. In assayed fish, the Apalachicola River itself marks the distributional limit of the western forms of *L. punctatus* and "pure" *L. macrochirus* and of the eastern form of *A. calva*. The southern clade (clones 5–7) of *L. microlophus* apparently extends to the Suwannee, but not to the Apalachicola or other drainages further west. The region of extensive intergradation between subspecies of *M. salmoides* extends west only as far as the Apalachicola.

In addition, minor genetic breaks at the Apalachicola were observed in *L. gulosus* and, again, in *L. microlophus*. Excepting mtDNA clone 20 in *L. gulosus* (which was observed in both the Escambia and Suwannee Rivers), the Apalachicola marks the boundary between a mtDNA clade (clones 7–13) observed in fish from the Suwannee River to the Altamaha and a mtDNA clade (clones

16–21) observed in the Apalachicola and Escambia drainages. Finally, 13 of 14 *L. microlophus* individuals with mtDNA genotype 4 were collected from the Apalachicola River and east.

Alabama/Tombigbee region: Patterns of mtDNA clonal distribution evidence one major phylogenetic break in the Alabama/Tombigbee region. High levels of sequence divergence distinguish most *L. gulosus* from the Tombigbee, Mississippi and Calcasieu Rivers from those east of the Tombigbee. Two fish sampled from the Calcasieu River did have the eastern genotype, but in view of their aberrant geographic placement, we suspect they may have been artificially stocked from an eastern source. Alternatively, the "eastern" genotype may occur as a natural, low-frequency polymorphism in western drainages. *L. gulosus* collected from a small tributary (Uphapee Creek) of the Alabama drainage also possessed the eastern mtDNA genotype. Since Uphapee Creek is near the headwaters and lateral tributaries of the more easterly Escambia and Apalachicola drainages, these fish might represent effects of a recent stream capture event. More intensive geographic sampling would be required to test this possibility.

Minor and less clean genetic breaks in the Alabama/Tombigbee region were also revealed in the *L. microlophus* and *A. calva* data. Of 40 *L. microlophus* with clonal genotype 1, 33 were collected in the Alabama/Tombigbee drainage and rivers to the west (only one fish sampled from those rivers did not have genotype 1), and the remaining seven were collected from five eastern rivers. For *A. calva*, the Alabama/Tombigbee region also serves as a boundary between a mtDNA clade (clones 10 and 11) representing all Escambia River fish and another mtDNA clade (clones 12 and 13) representing all fish from the Alabama, Tombigbee and Mississippi Rivers.

North Florida region: The Florida peninsula is the geographic stronghold of the genetically characterized southern subspecies of both bluegill (*L. m. purpurescens*, AVISE and SMITH 1974) and largemouth bass (*M. s. Floridanus*, PHILLIPP, CHILDERS and WHITT 1981, 1982). It is also the region to which a major mtDNA clade (clones 5–7) in *L. microlophus* was largely confined.

The effect of the Florida boundary region was also documented to a lesser degree in *A. calva* and *L. gulosus*. In *A. calva*, one mtDNA clade (clones 8 and 9) appeared restricted to peninsular Florida and reached highest frequency in the south Florida Everglades. In *L. gulosus*, a fairly distinct mtDNA clade (clones 7–13) had geographic limits (like those of clones 5–7 in *L. microlophus*) that extended from the Altamaha to the Suwannee Rivers.

Other geographic relationships: Further geographic trends in mtDNA distributions are less firmly supported with current data, but are worthy of mention. For example, the Escambia River appeared characterized by an unusually high number of endemic mtDNA clones (although these clones were not separated by major genetic differences from those observed elsewhere). The following clonal genotypes were observed in the Escambia River only: clones 10 and 11 in *A. calva* (present in 7 of 7 fish assayed); clone 3 in *L. microlophus* (5 of 6 fish); clones 12 and 14 in *L. punctatus* (8 of 9 fish); and clones 19 and 21 in *L. gulosus* (2 of 6 fish). In each of the Lepomis species, only one additional, more widespread, clone was represented in Escambia fish.

In contrast, the Suwannee River contained a diverse assemblage of mtDNA genotypes, suggesting a variety of relationships with other southeastern rivers. For example, mtDNA clonal types representing both the northern and Florida forms of *L. microlophus* were collected from the Suwannee, as were fish representing moderately divergent clades in *A. calva* and *L. punctatus*. Each of the four species considered in this report was represented by three or more mtDNA clones in the Suwannee drainage.

Distributional limits of species

SWIFT *et al.* (1985) recently studied the zoogeography of the southeastern fish fauna using a very different data base—geographic distribution limits of species. Ranges of more than 230 freshwater species were analyzed for possible commonalities evidencing zoogeographic effects. Remarkably, the major features of species distributional limits summarized in that report are highly concordant with the intraspecific geographic patternings of mtDNA phylogenies documented in this paper.

The major break in the species-limits analysis occurred in the region of the Apalachicola River. Thus, a phenetic clustering of 31 southeastern drainages based on presence/absence of species revealed two major assemblages: an eastern group composed of all rivers from the Savannah to the Suwannee; and a western group composed of the Apalachicola and all drainages westward to Louisiana (see figure 6 in SWIFT *et al.* 1985). Many species evidence this east-west break in the general Apalachicola region. Among Lepomis, for example, the native distributions of the western species *L. cyanellus* and *L. megalotis* terminate in eastern Alabama, as does the western distributional limit of the eastern species *L. auritus* (LEE *et al.* 1980). These species limits are thus very similar in geographic placement to zones of major intraspecific genetic differentiation in the widespread species *L. macrochirus*, *L. punctatus*, *L. gulosus* and *L. microlophus* (as well as *A. calva* and *M. salmoides*).

Other recognized concentrations of species distributional limits—in the Alabama/Tombigbee and north Florida regions (SWIFT *et al.* 1985; see also REMINGTON 1968)—also generally correspond to zones of differentiation identified in the mtDNA analyses. SWIFT *et al.* (1985) discuss several case histories, as do WILEY and MAYDEN (1985), for fish and other vertebrate fauna in the Alabama/Tombigbee region. An additional noteworthy feature, revealed in both the mtDNA and species distribution data, is a tendency for faunas of rivers in the western region (entering the Gulf of Mexico) to be somewhat more differentiated from one another than are those of rivers in the eastern region (entering the Atlantic). Thus, adjacent drainages in the western region tend to share relatively fewer species than do neighboring rivers in the east (SWIFT *et al.* 1985); and as described, mtDNA clones or clades also tend to show more restricted distributional patterns in the Gulf Coast drainages.

Overall, because the intraspecific mtDNA breaks in several widespread fish species in the Southeast correspond rather closely in geographic position to concentrations of species limits in fish taxa that are not so widely distributed, it seems likely that similar historical factors might account for both phenomena.

Estimation of absolute divergence times

One of the seductive aspects of molecular phylogenetics is its potential for estimating branching times in addition to branching order. The basic premise is that genetic distances between taxa reflect times since lineage separation (WILSON, CARLSON and WHITE 1977). Absolute divergence times are determined by calibrating molecular clocks with dates obtained from fossils or from other independent sources of information. Molecular clocks may be suspect, however, for at least two reasons: they may be uncertainly calibrated, and they may keep irregular time. Nonetheless, recent work utilizing molecular techniques to infer phylogeny in Hawaiian drosophilines (BEVERLEY and WILSON 1985), Australian oscine birds (SIBLEY and AHLQUIST 1983), and neotropical frogs (HEYER and MAXSON 1982) has demonstrated that estimation of branching times can enrich the predictive power of historical biogeographic hypotheses.

Our ability to determine branching times in the present study is compromised by the lack of a good fossil fish record in the southeastern United States and by insufficient knowledge concerning rates of mtDNA sequence evolution in nonmammalian lineages. Rate of mtDNA sequence divergence has been determined only for mammalian (mostly primate) species (BROWN, GEORGE and WILSON 1979; BROWN, 1983). For species that separated less than 8–10 million yr ago, mtDNA sequences were observed to accumulate differences linearly at a rate of 2% per million yr. At greater times of separation, the rate of differentiation slowed, producing an overall curvilinear relationship between mtDNA sequence divergence and time. For this reason, meaningful estimates of branching times should only be attempted when values of sequence divergence are within the linear portion of the curve, *i.e.*, less than about 0.15. All p values in the current study were well within the informative range.

If BROWN, GEORGE and WILSON's (1979) molecular clock calibration for mammals can be applied to the species of fish considered in this study, the major genetic break identified in each of the four Lepomis species occurred in the middle to early Pliocene (3–4.5 million yr BP). In contrast, the two major groups in *A. calva* last shared an ancestor less than one-half million yr ago. Estimates of divergence times for additional mtDNA assemblages within the major lineages are reported in Table 4. We want to emphasize that these ***absolute*** time estimates are quite provisional. However, whether or not the absolute time estimates are valid, the geographical patterns of major genetic breaks within the assayed species remain clear and deserve consideration.

Biogeographic scenarios

Can all of these genetic and distributional data be integrated into a reasonable set of zoogeographic hypotheses for the fish fauna of the southeastern United States? Uncertainty regarding both regional geology and the absolute rates of mtDNA divergence in fishes cautions against overzealous interpretation of the data. Nonetheless, considering the striking commonalities in the intraspecific mtDNA divergence patterns, as well as species-distribution limits, several lines of speculation seem warranted.

TABLE 4

Provisional estimates of divergence times based on observed mtDNA distances

Species, mtDNA clones compared	*p* values/divergence times			
	Minimum[a]	Maximum[a]	Average[a]	Population average[b]
Amia calva				
1–9/10–13	0.0068/340,000	0.0122/610,000	0.0094/470,000	0.0056/280,000
1–7/8, 9	0.0036/180,000	0.0070/350,000	0.0056/280,000	0.0034/170,000
10, 11/12, 13	0.0032/160,000	0.0065/325,000	0.0048/240,000	0.0033/165,000
1/4 or 12/13	0.0015/75,000			
Lepomis punctatus				
1–8/9–17	0.0513/2,565,000	0.0813/4,065,000	0.0618/3,090,000	0.0440/2,700,000
1–6/7, 8	0.0338/1,690,000	0.042/2,120,000	0.0370/1,850,000	0.0312/1,560,000
9–14/15–17	0.0151/755,000	0.0320/1,600,000	0.0244/1,220,000	0.0138/690,000
9–11/12–14	0.0083/415,000	0.0161/805,000	0.0123/615,000	0.0049/245,000
1/4 or 9/10	0.0020/100,000			
Lepomis microlophus				
1–4/5/7	0.0745/3,725,000	0.1000/5,000,000	0.0867/4,335,000	0.0823/4,115,000
1,2/3 or 1,2/4	0.0046/230,000	0.0068/340,000	0.0057/285,000	0.0048/240,000
6/5, 7	0.0020/100,000	0.0045/225,000	0.0033/165,000	0.0021/105,000
1/2 or 5/7	0.0019/95,000			
Lepomis gulosus				
1–23/24–32	0.0571/2,855,000	0.0773/3,865,000	0.0629/3,145,000	0.0558/1,890,000
1,2/3–6	0.0066/330,000	0.0111/555,000	0.0093/465,000	0.0067/335,000
7–13/16–21	0.0034/170,000	0.0099/495,000	0.0066/330,000	0.0033/165,000
24–27/28–32	0.0069/345,000	0.0147/735,000	0.0111/555,000	0.0064/320,000
3/4 or 24/25	0.0012/60,000			
Lepomis macrochirus[c]	0.0850/4,250,000			

[a] All time estimates based on BROWN, GEORGE and WILSON's (1979) calculation of 2.0% mtDNA sequence divergence per million years. The minimum p and divergence time values reflect the lowest value for any pairwise comparison between mtDNA clones drawn from different clonal assemblages; maximum is the largest value, and average is the mean across all possible pairwise comparisons.

[b] $p = p_{xy} - 0.5(p_x + p_y)$, where p_{xy} is the mean pairwise mtDNA distance between taxa x and y, and p_x and p_y are the mean pairwise distances among mtDNA clones within x and y, respectively.

[c] Data from AVISE *et al* (1984).

The major genetic break evidenced in each of the four species of Lepomis considered in the present study dates to the Pliocene interglacial, during which time the sea stood 50–80 m above present-day sea level (VAIL and MITCHUM 1979). A Pliocene coastline for the southeastern United States has not been reconstructed, and it is not appropriate to merely superimpose the Pliocene marine transgression on today's topography (WINKER and HOWARD 1977a,b). It is likely, however, that many of the Coastal Plain rivers were absent or significantly reduced in size at that time and that the major southeastern drainages (*e.g.*, Alabama/Tombigbee, Apalachicola and Savannah) with headwaters above the Fall Line (an abrupt topographic boundary separating the coastal plain from the uplands) were well isolated. Another potential refuge area was the Ocala highlands region in north-central Peninsular Florida. This area has been fully or partially isolated from the mainland by a marine seaway (the Suwannee Straits) a number of times during the Cenozoic (the last 65 million yr). Thus, the major genetic effects of a Pliocene 1 million year-long, high sea-level stand should have been twofold: (1) extinction of locally differentiated fish in the smaller Coastal Plain rivers, with attendant reduction of overall levels of mtDNA polymorphism within each species, and (2) an opportunity for initiation of significant sequence divergence between those lineages that survived in headwater and/or Floridian refuge areas.

As the seas receded from the Pliocene high sea-level stand, dispersal along the lowlands was presumably facilitated, thus enabling taxa within the major lineages of each species to colonize adjacent coastal rivers (and, possibly, displace mtDNA taxa that had earlier events of population subdivision recorded in their genomes). The fact that not all boundary regions are perfectly concordant suggests slightly different histories of dispersal postdating the separation. This might reflect different dispersal abilities between the species and/or different locations of mainland refugia. As an example, the mainland refuge of the western clade in *L. gulosus* might have been farther west than the mainland refuge of *L. punctatus*. The fact that populations in the Atlantic Coast drainages are usually genetically aligned with those in Florida drainages raises the possibility that mainland refugia for the species considered in this report may have all occurred in Gulf drainages, with the eastern refugium being the Florida peninsula.

It strikes us as somewhat surprising that the two major lineages within each of the four Lepomis species appear to trace to common ancestors within the same relative time-span. If we are to suggest that Pliocene vicariant events are responsible for the initiation of the major mtDNA differences currently observed in Lepomis species, it would appear that we must also posit widespread distributions of ancestral forms (and little mtDNA sequence differentiation) before that time. Perhaps the pre-Pliocene landscape in the Southeast was less refractory to fish dispersal. Freshwater fish dispersal is generally considered to be facilitated during sea level lowering (SWIFT *et al.* 1985), and the late Miocene was marked by a major marine regression, substantially longer in duration than any subsequent sea level lowerings. Another way of considering the problem would be to suggest that the relatively short Pleistocene glacial periods

have not enabled widespread dispersal, and thus, effects of these earlier historical influences on population subdivision have not been erased by subsequent gene flow.

An extension of this same line of reasoning might also reconcile apparent differences in the magnitude of sequence divergence between the major lineages within *A. calva* and *L. punctatus*. Recall that these two species had geographically oriented mtDNA phylogenies that were remarkably congruent with respect to branching patterns, but not branching times. It is possible that this difference is due to different rates of mtDNA sequence evolution in the two species. On the other hand, perhaps *A. calva* disperses more readily than *L. punctatus* and, thus, was relatively widespread before the Pleistocene interglacial 440,000 yr ago [sea level was 10–25 m above present-day levels (CRONIN *et al.* 1981)]. Evidence of earlier historical relationships in *A. calva* would be erased by dispersal and by the fairly rapid effects of mtDNA lineage sorting in populations that are not spatially subdivided (see AVISE, NEIGEL and ARNOLD 1984). Subsequent isolation during Pleistocene interglacials could then initiate differentiation of *A. calva* lineages, while merely accentuating mtDNA differences already present in *L. punctatus*. In this scenario, the effectiveness of a barrier to gene flow varies both as a function of (1) a taxon's dispersal abilities and (2) changes in the landscape over time.

Another possible example of the interplay between geologic history and the opportunity for dispersal involves a well-known Pleistocene headwater capture of the Chattahoochee River (draining into the Gulf of Mexico) by the Savannah River (draining into the Atlantic) (SWIFT *et al.* 1985). In both *L. macrochirus* and *M. salmoides*, secondary hybrid zones in Georgia suggest effects of this fairly recent interregional transfer of genetically distinct forms. Similarly, the distribution of mtDNA clones in *L. microlophus* may reflect a secondary invasion of Atlantic Coast drainages by western genotypes. It is of further interest to note that the three assayed species (*A. calva, L. punctatus, L. gulosus*) that are rare or unusual above the Fall Line in the Savannah and Chattahoochee drainages (DAHLBERG and SCOTT 1971) apparently show no genetic evidence of this kind of secondary eastern invasion. Thus, interregional transfers mediated by headwater captures probably have little or no effect on species largely restricted to the Coastal Plain.

Overall, the geographic correlations documented by intraspecific mtDNA phylogenies, and by distributional limits of freshwater fishes in the southeastern United States, suggest a strong role for historical zoogeographic factors. The fact that these patterns are documented at several taxonomic levels makes it likely that there have been repeated episodes of fragmentation of southeastern fish faunas. Eustatic sea level changes and the creation of refuge areas by alterations in shoreline morphology are a likely cause of the initiation of vicariant differences, with subsequent and characteristic dispersals leading to the final patterns observed. The concordance of geographic patterns across different taxonomic levels is postulated to result from the cyclical nature of sea level change relative to a reasonably static continental morphology.

Particularly during the last 20 yr, a major effort in population genetics has

been directed to understanding the evolutionary factors (usually dichotomized as selection-mediated responses *vs.* genetic drift) to account for observed geographic distributions of genetic traits, such as allozyme frequencies. More recently, with the development and applications of mtDNA approaches, it has been possible to demonstrate a phylogenetic component to population structure; geographically separated populations often appear to occupy different branches of an intraspecific evolutionary tree. As noted by AVISE (1986a), "This pushes the issue of the adaptative significance of observed geographic differences [in mtDNA] to a problem relating to the reasons for the survival and extinction of various female lineages." Results of the present study exemplify the important role that historical biogeography can play in shaping the geographic distributions of such lineages. Thus, any interpretations of population genetic structure that fail to consider the possible influence of history in shaping that structure, may be seriously inadequate.

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