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HYBRIDIZATION AND INTROGRESSION AMONG SPECIES OF SUNFISH (LEPOMIS): ANALYSIS BY MITOCHONDRIAL DNA AND ALLOZYME MARKERS

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ABSTRACT

We explore the potential of mitochondrial DNA (mtDNA) analysis, alone and in conjunction with allozymes, to study low-frequency hybridization and introgression phenomena in natural populations. MtDNAs from small samples of nine species of sunfish (Lepomis, Centrarchidae) were purified and digested with each of 13 informative restriction enzymes. Digestion profiles for all species were highly distinct: estimates of overall fragment homology between pairs of species ranged from 0–36%. Allozymes encoded by nine nuclear genes also showed large frequency differences among species and together with mtDNA provided many genetic markers for hybrid identification. A genetic analysis of 277 sunfish from two locations in north Georgia revealed the following: (1) a low frequency of interspecific hybrids, all of which appeared to be F_1's; (2) the involvement of five sympatric Lepomis species in the production of these hybrids; (3) no evidence for introgression between species in our study locales (although for rare hybridization, most later-generation backcrosses would not be reliably distinguished from parentals); (4) a tendency for hybridizations to take place preferentially between parental species differing greatly in abundance; (5) a tendency for the rare species in a hybrid cross to provide the female parent. Our data suggest that absence of conspecific pairing partners and mating stimuli for females of rarer species may be important factors in increasing the likelihood of interspecific hybridization. The maternal inheritance of mtDNA offers at least two novel advantages for hybridization analysis: (1) an opportunity to determine direction in hybrid crosses; and (2) due to the linkage among mtDNA markers, an increased potential to distinguish effects of introgression from symplesiomorphy or character convergence.

NORTH American sunfish in the genus Lepomis (Centrarchidae) have long been known for propensity to hybridize, both in laboratory or artificial pond situations (CHILDERS 1967; HESTER 1970; LAGLER and STEINMETZ 1957; WHITT *et al.* 1973) and in nature (BAILEY and LAGLER 1938; BIRDSONG and YERGER 1967; CROSS and MOORE 1952; HUBBS 1955; KEENLEYSIDE 1967). As stated by BREDER (1936), "There is probably no group of fishes, North American at least, in which there would seem to be a concatenation of reproductive and other events so well arranged as to lead to extensive hybridizing; *i.e.*, the species are numerous; there is less geographic separation than usual; spawning occurs at about the same temperature threshold; spawning sites are limited

and similar for most species; nests are exchanged among species." The 11 species of Lepomis can theoretically yield 55 different interspecific hybrid combinations, and more than 20 of these have been reported from nature (CHILDERS 1967). Nonetheless, these species exhibit large genetic distances at protein-coding loci (AVISE and SMITH 1974a,b), and for the most part retain distinct morphological identities throughout their respective ranges (DOUGLAS and AVISE 1982; LEE *et al.* 1980).

The general morphological intermediacy of presumptive Lepomis hybrids, and their frequent low fertility in both sexes, led HUBBS and HUBBS (1933) (see also HUBBS 1955) to conclude that natural hybridization was restricted to the F_1 generation. However, artificially controlled matings and fertilizations among hybrid individuals soon established that "a number of different kinds of hybrid sunfishes . . . are not sterile, are fully capable of producing abundant F_2 and F_3 generations, and can be successfully backcrossed to parent species and even outcrossed to nonparental species" (CHILDERS 1967; see also RICKER 1948). Indeed, artificially reared F_2 and backcross generations are routinely employed by WHEAT, WHITT and CHILDERS (1973) and PHILIPP, PARKER and WHITT (1983) to study linkage relationships and regulatory control of allozyme loci. Thus, as noted by LAGLER and STEINMETZ (1957), "the wild caught individuals presumed to be F_1 by HUBBS and HUBBS (1931), BAILEY and LAGLER (1938), and others may have included also individuals of successive filial generations or offspring of their backcrosses to parental species." It remains an open question whether Lepomis hybrid reproduction occurs commonly in nature and whether introgression between species plays a significant role in evolution of the group.

In this study we examine mitochondrial DNA (mtDNA) divergence in Lepomis by analyzing restriction fragment patterns for nine species. These data will contribute to a developing picture of mtDNA differentiation among vertebrate congeners. We will focus attention on populations of five species cohabiting lake and river settings in north Georgia that by morphological evidence appear to be involved in occasional hybridization. Specifically, the goals are to identify and use maternally transmitted mtDNA markers (as well as biparentally transmitted allozyme markers) to (1) identify the particular pairs of species involved in each hybridization event, (2) determine the direction of each hybrid cross and relate this to mating behaviors for the species, and (3) examine the possibility of recent introgressive hybridization. We will also be concerned with the broader question of the general utility of mtDNA genetic markers, alone or in conjunction with allozymes, to study natural hybridization and introgression phenomena.

MATERIALS AND METHODS

Throughout the spring and summer of 1983, specimens were collected by electrofishing or hook and line from two major study locations near Athens, Georgia: (1) the Oconee River (21 collection sites within a 20-km straight-line radius from central Athens; no two collection sites closer than 1500 m stream distance), (2) Lake Oglethorpe (a small, artifically dammed pond; no distinctions made between collecting areas in the pond). Additional samples were taken from the localities listed in Table 1.

TABLE 1

Preliminary samples of nine species of Lepomis from which mtDNA was analyzed by 15 restriction enzymes

Species	Common name	*N*	Location (county, state)
auritus	Redbreast	5	Oglethorpe, Georgia
cyanellus	Green	7	Oglethorpe, Clarke, Georgia
gibbosus	Pumpkinseed	2	Allendale, South Carolina
gulosus	Warmouth	5	Macon, Alabama; Clarke, Georgia
marginatus	Dollar	2	Aiken, South Carolina
megalotis	Longear	2	Macon, Alabama
microlophus	Redear	4	Allendale, South Carolina
punctatus	Spotted	4	Allendale, South Carolina
macrochirus	Bluegill	10	Oglethorpe, Georgia; Greene, Alabama; Martin, Florida

Fish were returned live to the laboratory. Fresh liver, heart, spleen, kidney and muscle served as tissue sources for mtDNA purification by differential centrifugation and banding in CsCl gradients (LANSMAN *et al.* 1981). Closed circular mtDNA was digested separately with the following restriction enzymes (and recognition sequences): (1) *Ava*I (CPyCGPuG), (2) *Bam*HI (GGATTC), (3) *Bcl*I (TGATCA), (4) *Bgl*I (GCCn$_5$GGC), (5) *Bgl*II (AGATCT), (6) *Bst*EII (GGTnACC), (7) *Cla*I (ATCGAT), (8) *Hin*dIII (AAGCTT), (9) *Pst*I (CTGCAG), (10) *Pvu*II (CAGCTG), (11) *Sac*I (GAGCTC), (12) *Xba*I (TCTAGA), (13) *Hin*cII (GTPyPuAC), (14) *Kpn*I (GGTACC), (15) *Nde*I (CATATG). Digested fragments were "end-labeled" with α-^{32}P-dCTP using the large fragment of DNA polymerase I (BROWN 1980) and separated by molecular weight on agarose gels. Gel concentrations were varied between 0.9 and 1.4% to optimize resolution of fragments of differing size. The labeled fragments were detected by autoradiography (LANSMAN *et al.* 1981). *Cla*I and *Nde*I apparently produced zero (or one) cuts in mtDNA from any Lepomis and are not considered further. For unknown reasons, digestion products of *Pvu*II could not be resolved for three species, *auritus*, *cyanellus* and *microlophus*; and *Bgl*I products were not scored in *marginatus*.

The entire battery of 15 restriction enzymes was employed to assay two to ten specimens for each of nine Lepomis species (Table 1). With the exception of the bluegill (*L. macrochirus*, AVISE *et al.* 1984), restriction sites have not been mapped and we have relied solely on fragment comparisons. Fragments of questionable identity were compared on rerun gels by placement in adjacent slots (we recognize that apparent molecular weight identity does not ensure homology). Molecular weight markers were provided by a mixture of a *Hin*dIII digest of bacteriophage λ and a *Pvu*II/*Hin*cII digest of pBR322. An arbitrary cut-off of about 700 base pairs (bp) was generally established below which we made no attempt to score fragments due to poorer gel resolution and the presence of more intense background counts. Notwithstanding these minor difficulties, we found the general clarity of mtDNA digestion profiles in Lepomis to be outstanding (Figures 1 and 2).

For each pair of species (or individuals in whom they differed in mtDNA genotype), total proportions of shared fragments across all digestions were calculated by $F = 2N_{XY}/(N_X + N_Y)$, where N_X and N_Y are the numbers of fragments in genotypes X and Y, respectively, and N_{XY} is the number of fragments held in common. Shared digestion profiles exhibiting a single band (representing zero or one mtDNA cut) were not included in the calculations. F values were converted to estimates of percent sequence divergence (or number of base substitutions per nucleotide, p), by the approach of NEI and LI (1979).

For the majority of fish collected from the sites of hybridization (Table 2), mtDNAs were typed by digestion with at least two of the following enzymes: *Ava*I, *Hin*dIII or *Xba*I. These enzymes were chosen because the digestion profiles were easily scored and were species diagnostic. All fish were also assayed for allozyme genotype using conventional starch-gel electrophoretic procedures (SELANDER *et al.* 1971) as modified for Lepomis by AVISE and SMITH (1974a). The following enzymes, previously shown to differentiate populations of various pairs of Lepomis species, were scored: (1) aspartate aminotransferase (EC 2.6.1.1; locus *Got-2* of AVISE and SMITH 1977), (2)

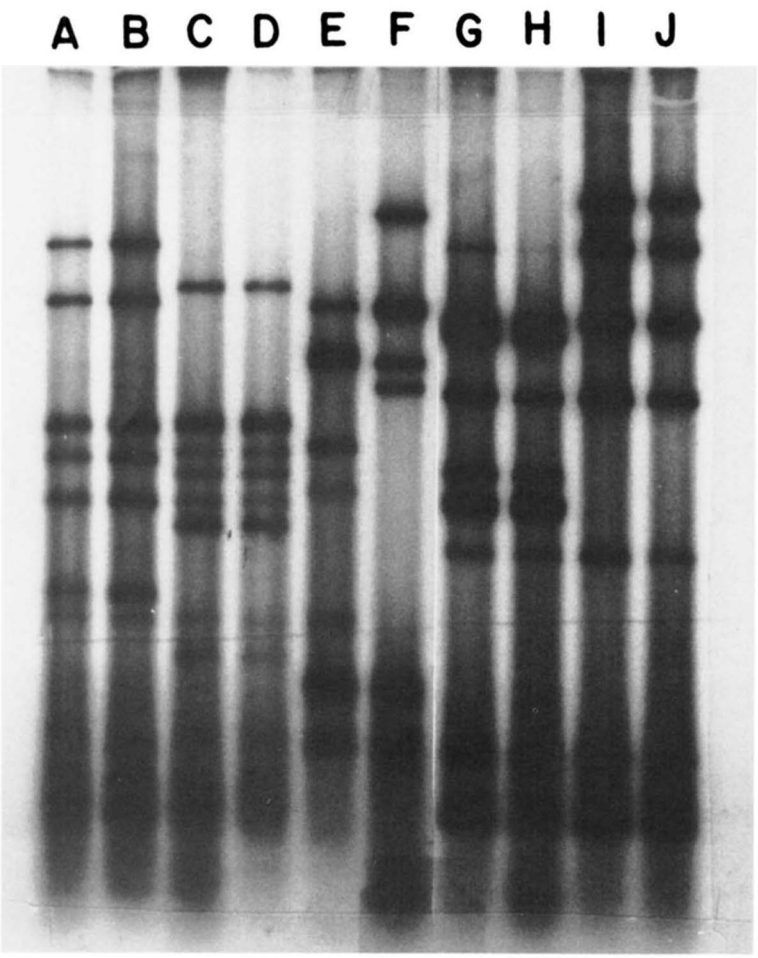

FIGURE 1.—*Hin*cII mtDNA digests of selected Lepomis species. Lanes A and B, *megalotis*; C and D, *marginatus*; E, *macrochirus* genome type "A"; F, *macrochirus* genome type "B"; G and H, *gibbosus*; I and J, *punctatus*.

esterase (3.1.1.2; *Es-1*), (3) malate dehydrogenases (1.1.1.37; *Mdh-1*, *Mdh-2*), (4) phosphoglucoisomerases (5.3.1.9; *Pgi-1*, *Pgi-2*), (5) phosphoglucomutase (2.7.5.1; *Pgm-1*), (6) 6-phosphogluconate dehydrogenase (1.1.1.43; *Pgd-1*), (7) superoxide dismutase (1.15.1.1; locus *Ipo-1* of AVISE and SMITH 1974b).

RESULTS

mtDNA differentiation: The 13 informative restriction enzymes revealed a grand total of 246 different scorable fragments among the nine assayed species of Lepomis. The proportions of fragments shared between species were in all cases low, ranging from $F = 0.00$ (*auritus/cyanellus*; *marginatus/cyanellus* type "A"; *megalotis/cyanellus* type "B") to $F = 0.36$ (*punctatus/microlophus*) (Table 3); between all possible pairs of species, $\bar{F} = 0.102$. When the multiband profiles of each enzyme were considered, in only a very few cases were mtDNA

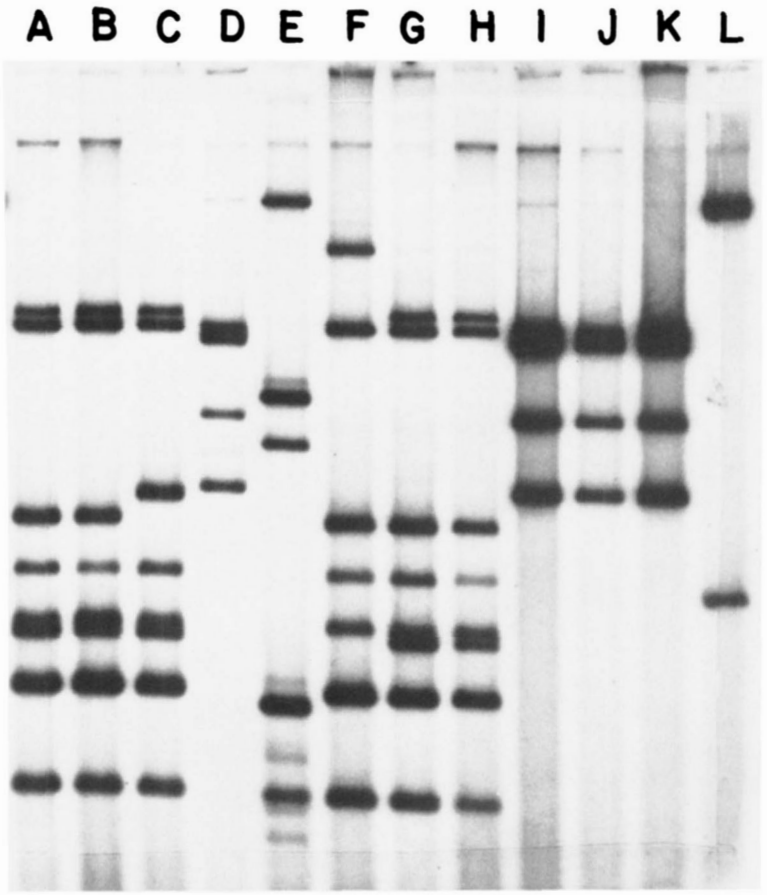

FIGURE 2.—*Ava*I mtDNA digests of Lepomis from the Oconee River. Lanes A through C and F through H, *auritus*; D, I and J, *cyanellus*; E, *gulosus*; L, *macrochirus*. Lane K is an F_1 hybrid between *cyanellus* and *macrochirus*, with a *cyanellus* mother. The variant *auritus* patterns in lanes C and F can both be accounted for by single restriction site losses from the common *auritus* patterns in lanes A, B, G and H.

digestion patterns shared by species: (1) the *auritus* and the *macrochirus* "B" genomes share a three-band *Hin*dIII pattern (Figure 3); (2) *auritus* and *microlophus* share a two-fragment *Bst*EII profile; (3) *auritus* and *microlophus* share a four-band *Pst*I pattern.

Some of the few bands apparently shared by species may reflect fortuitous comigration of fragments cleaved at nonhomologous restriction sites. However, a few fragments did exhibit surprising conservatism across species. The best example is a 3.7-kb *Pst*I fragment shared by seven of the nine species. Other examples include a 4.0-kb *Xba*I fragment shared by five species (Figure 3) and a 4.2-kb *Hin*dIII fragment shared by four species. It was recently argued (C. F. AQUADRO, N. KAPLAN and K. J. RISKO 1984) from sequence comparisons in mammals that a fraction of sites in the mtDNA molecule is highly conserved by selection, whereas the remainder of the genome evolves extremely rapidly.

TABLE 2

Species compositions of the hybridizing populations, as determined by gross morphological inspection and by mtDNA genotype

Study site	Species (morphological identification)	No. of fish		mtDNA markers characteristic of				
		Collected[a] (% of catch)	Assayed for mtDNA and allozymes	*auritus*	*macrochirus*	*cyanellus*	*gulosus*	*microlophus*
Oconee River (Clarke County, Georgia)								
	auritus	299 (55.3)	30	30				
	macrochirus	178 (32.9)	20		20			
	cyanellus	45 (8.3)	15			15		
	gulosus	10 (1.8)	6				6	
	Hybrids	9 (1.7)	9			7	2	
	Subtotals	541	80					
Lake Oglethorpe (Oglethorpe County, Georgia)								
	auritus	230 (45.1)	32	32				
	macrochirus	247 (48.4)	151		151			
	cyanellus	26 (5.1)	9			9		
	microlophus	2 (0.4)	0					
	Hybrids	5 (1.0)	5	1	1			3
	Subtotals	510	197					

[a] In Lake Oglethorpe, numbers are approximate because some collections were supplemented by catches of independent fishermen.

TABLE 3

Estimates of mtDNA differentiation among Lepomis species

	1	2a	2b	3	4	5	6	7	8	9a	9b
auritus	——	0.000 (77)	0.000 (75)	0.148 (81)	0.027 (75)	0.053 (76)	0.102 (78)	0.225 (80)	0.169 (71)	0.158 (76)	0.206 (68)
cyanellus (genotype "A")	1.00	——	0.788 (66)	0.051 (78)	0.235 (68)	0.000 (73)	0.027 (75)	0.053 (75)	0.125 (64)	0.082 (73)	0.102 (59)
cyanellus (genotype "B")	1.00	0.014	——	0.053 (76)	0.303 (66)	0.056 (71)	0.000 (73)	0.027 (73)	0.129 (62)	0.113 (71)	0.140 (57)
gibbosus	0.141	0.211	0.298	——	0.105 (76)	0.104 (77)	0.078 (77)	0.173 (81)	0.171 (70)	0.052 (77)	0.119 (67)
gulosus	0.329	0.103	0.088	0.253	——	0.029 (69)	0.027 (73)	0.056 (71)	0.067 (60)	0.116 (69)	0.105 (57)
marginatus	0.208	1.00	0.307	0.156	0.333	——	0.189 (74)	0.054 (74)	0.031 (64)	0.057 (70)	0.032 (62)
megalotis	0.157	0.342	1.00	0.177	0.331	0.115	——	0.078 (77)	0.060 (67)	0.054 (74)	0.031 (64)
microlophus	0.102	0.208	0.335	0.123	0.204	0.206	0.177	——	0.358 (67)	0.108 (74)	0.156 (64)
punctatus	0.119	0.143	0.141	0.130	0.291	0.353	0.319	0.070	——	0.061 (65)	0.196 (51)
macrochirus (genotype "A")	0.130	0.270	0.252	0.297	0.144	0.203	0.206	0.266	0.298	——	0.322 (62)
macrochirus (genotype "B")	0.105	0.155	0.133	0.146	0.151	0.340	0.338	0.124	0.108	0.075	——

Above diagonal: total proportion of shared restriction fragments in digestion profiles for 13 enzymes (and, in parentheses, total number of fragments scored, $N_x + N_y$); below diagonal: sequence divergence, p, calculated by NEI and LI's (1979) approach and weighted by the numbers of fragments produced by five- and six-base enzymes.

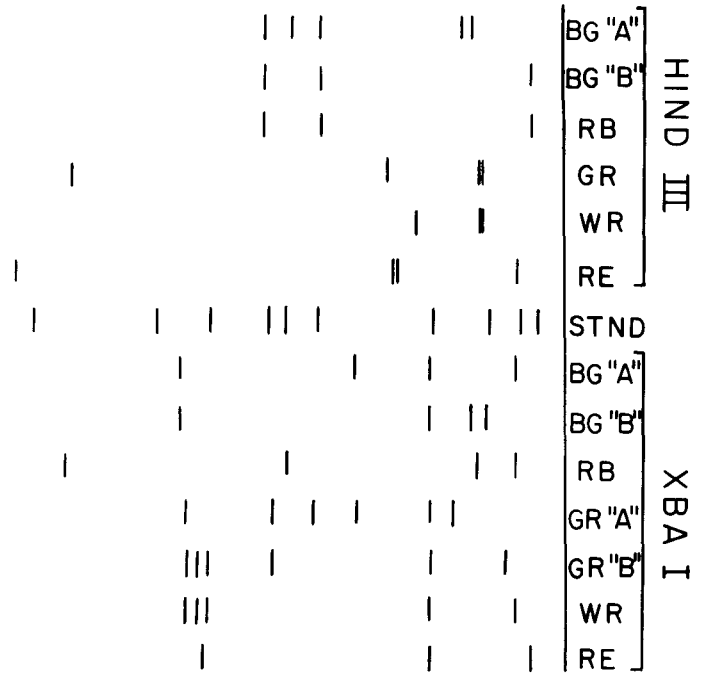

FIGURE 3.—Diagrammatic representation of the *Hin*dIII and *Xba*I mtDNA digestion profiles for the five species of Lepomis involved in hybridization at the study sites in north Georgia. BG, *macrochirus* (bluegill, genome types "A" and "B"); RB, *auritus* (redbreast); GR, *cyanellus* (green, genome types "A" and "B"); WR, *gulosus* (warmouth); RE, *microlophus* (redear). Bands of the molecular weight standard (lane 7) have the following sizes (in kilobases), from top to bottom: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 1.8, 1.4, 1.1, 0.6.

The mtDNA gel patterns of Lepomis, in which a few bands remain relatively constant against a background of otherwise changing digestion patterns, is consistent with this view.

An alternative possibility to account for the changing mtDNA digestion profiles among Lepomis is that nucleotide sequences are generally conserved, but that most of the fragment differences reflect numerous large-scale additions, deletions or rearrangements scattered about the molecule. Arguing against this possibility are the following considerations: (1) the fragment profile differences between two highly distinct mtDNA genomes in *macrochirus*, where sites have been mapped (AVISE *et al.* 1984), can all be accounted for by gains or losses of individual restriction sites, without additions, deletions or rearrangements affecting more than about 50–250 bp; (2) occasional polymorphisms observed within other Lepomis species could usually be accounted for by single-site gains or losses (for example, two low-frequency variant *Ava*I profiles in *auritus* appear to differ from the common *auritus* pattern by single-site losses, see Figure 2); (3) the multiband profiles shared by a few pairs of species (see preceding data) indicate that genome sizes for these species must be very close; (4) the general mode of mtDNA evolution in other vertebrates appears to be primarily through base substitutions, particularly transitions (reviews in AVISE and LANS-

MAN 1983; BROWN 1983). Nonetheless, from our restriction fragment data on Lepomis, we cannot eliminate the possibility that some of the digestion profile differences result from changes in genome size or arrangement; conventional restriction site mapping would still not distinguish base substitution changes from small addition/deletion changes of the sort described by CANN and WILSON (1983) in human mtDNA.

If certain assumptions are met (that fragment changes arise from base substitution, that distributions of cleavage sites are similar to those expected in random sequences of same-base composition and that nonhomologous fragments are not scored as identical), then values of F can be converted to estimates of sequence divergence, p (NEI and LI 1979; UPHOLT 1977). For Lepomis, p values range from 0.07 to 1.0, with median equal to 0.206 (Table 3) (the mean is strongly affected by the presence of outlying points at $p = 1.0$, attributable to the J-shaped mathematical relationship between F and p). Median p for Lepomis interspecific comparisons is close to the observed 25–30% "plateau" of mtDNA differentiation observed between other vertebrate species thought to have been separated for about 15–80 million yr (C. F. AQUADRO, N. KAPLAN and K. J. RISKO 1984; BROWN, GEORGE and WILSON 1979; BROWN 1983).

Whatever the true molecular basis of the fragment profile differences among Lepomis, a variety of readily scorable mtDNA markers is available for analysis of hybridization and introgression. Digestion profiles produced by the restriction enzymes (*Ava*I, *Hin*dIII and *Xba*I) used in the survey of the hybridizing populations are shown in Figures 2 and 3. With one exception (*Hin*dIII in *auritus* and *macrochirus*), the marker profiles for each species are distinct and usually appear to differ from one another by multiple restriction site changes. Hence, the possibility of gross misclassification of mtDNA genotype for an individual fish (through simultaneous convergent mutations) is negligible.

As expected for a uniparentally transmitted, nonrecombining genome such as mtDNA, the disequilibrium among genetic markers in the 277 fish from Lake Oglethorpe and the Oconee River was complete (for example, any individual exhibiting an *Xba*I "*auritus*" pattern also exhibited the "*auritus*" pattern for *Ava*I or *Hin*dIII). Furthermore, for the 263 fish tentatively identified to species by gross morphology, the agreement between mtDNA and morphological identification was perfect (Table 2).

One final feature of mtDNA differentiation in our present Lepomis collection is of note. Apart from the two highly divergent ($p \simeq 0.08$) mtDNA genomes in *macrochirus* (described previously in AVISE *et al.* 1984), the major within-species mtDNA polymorphism involved the green sunfish, *cyanellus*. Two mtDNA genomes (labeled *cyanellus* "A" and "B", Table 3) were consistently distinguishable by multiple-fragment differences in *Xba*I (Figure 3), *Bgl*I and *Ava*I digests. Between *cyanellus* types A and B, $F = 0.788$ and estimated $p = 0.014$. All 5 assayed *cyanellus* from the Oconee River population exhibited the "A" genome, as did four of nine fish in the Lake Oglethorpe sample.

Allozyme markers: Electromorph frequencies in the Lake Oglethorpe and Oconee River samples of Lepomis are shown in Table 4. Between three and seven loci completely distinguish (exhibit fixed allele differences between) any

TABLE 4

Allele frequencies at nine "marker" allozyme loci in populations of Lepomis from the Oconee River and Lake Oglethorpe

Species (by morphological identification)	N	Locus								
		Got-2	*Es-1*	*Mdh-1*	*Mdh-2*	*Pgi-1*	*Pgi-2*	*Pgm-1*	*Pgd-1*	*Ipo-1*
macrochirus										
Oconee River	20	100 (0.98) 58 (0.02)	100 (0.98) 96 (0.02)	100	100	100	100	100 (0.98) 90 (0.02)	100	100
Lake Oglethorpe	151	100 (0.47) 58 (0.53)	100	100 (0.997) 90 (0.003)	100	100	100	100 (0.96) 90 (0.04)	100	
auritus										
Oconee River	30	58	96	116	110	95 (0.98) 81 (0.02)	100 (0.32) 130 (0.02) 62 (0.66)	110	100 (0.91) 92 (0.07) 105 (0.02)	100
Lake Oglethorpe	32	58	96	116	110	95 (0.92) 90 (0.08)	100 (0.56) 130 (0.03) 62 (0.41)	110	100 (0.97) 92 (0.03)	100
cyanellus										
Oconee River	15	58	100	116	110	95	130	105 (0.90) 110 (0.10)	92 (0.53) 88 (0.37) 78 (0.10)	122
Lake Oglethorpe	9	58	100	116	110	95	130	105 (0.56) 110 (0.44)	92	122
gulosus										
Oconee River	6	58	100	116	100	95	198	110	100	100
microlophus[a]	4	109	96	116	110	90 (0.70) 81 (0.30)	100 (0.97) 62 (0.03)	110	87	122 (0.58) 96 (0.42)

Electromorphs are labeled by mobility relatively to the common *macrochirus* allelic product designated 100. Frequencies were 1.0 except where noted by parentheses. Locus designations are described in the text.

[a] Since no "pure" *microlophus* individuals were assayed from the Oconee or Oglethorpe sites, electromorph frequencies were taken from our previous work with other populations (AVISE and SMITH 1974).

of the possible ten pairwise combinations of species. Hence, we refer to these as "marker" loci. Some other loci are imperfect markers distinguishing various pairs of species. For example, the *Pgi-2* "100" allele is monomorphic in *macrochirus* but present in mean frequency 0.44 in *auritus*. Whether such allelic sharing between species can be attributed to past introgression is problematic and will be discussed later. Several of these surveyed marker loci are known to be unlinked in Lepomis (*Pgi-1* and *Pgi-2*; *Mdh-1*, *Mdh-2*, *Ipo-1*, *Pgd-1*) (WHEAT, WHITT and CHILDERS 1972, 1973; WHITT *et al.* 1976).

Genotypic characterization of hybrids: Upon gross morphological inspection, a total of 14 fish (1.3% of the collections) from Lake Oglethorpe and the Oconee River appeared to be hybrid Lepomis. Although the physical appearance of each fish provided clues to its parentage, by morphological criteria alone we were unable to ascertain the particular parental species involved in any cross or whether an individual represented an F_1, later-generation or backcross hybrid. The mtDNA and allozyme genotypes of these hybrid fish are shown in Table 5.

As indicated in Table 6, the male and female parentage of each presumptive hybrid could be unambiguously determined by the combination of mtDNA and allozyme markers. For example, hybrid individual 1 was heterozygous at each of the eight allozyme marker loci distinguishing *macrochirus* from *cyanellus*, and it possessed an mtDNA genome characteristic of *cyanellus*. All five Lepomis species (*macrochirus*, *auritus*, *gulosus*, *cyanellus* and *microlophus*) were involved in the production of various hybrids, and each hybrid had an allozyme genotype expected of an F_1 generation.

Further information about hybrid brood composition can be made by reference to data on size class (Table 5), collection localities and directions of cross (Table 6). For example, since hybrid individual 6 was collected from the same locale as was individual 1, was of the same approximate size (and hence age class) and had the same species percentage, these two hybrids are very likely broodmates. Altogether, the 14 collected hybrids represent at least six and probably seven to nine separate hybridization events (the only unanswered questions are whether the *macrochirus-cyanellus* hybrids from sites 1 and 6 in the Oconee River, which were about 5 km apart, belong to a common brood and whether the three *macrochirus-microlophus* hybrids with *microlophus* mothers from Lake Oglethorpe represent products of a single spawn).

DISCUSSION

Analysis of possible introgression: An important empirical question in evolutionary biology is whether gene flow between hybridizing species provides a significant avenue for interspecific exchange of genetic adaptations. As provocatively reasoned by ANDERSON (1949), "A trickle of genes so slight . . . might still be many times more important than mutation in keeping up the basic variability of the parental species. . . . In a variable environment, species that through introgression are able to achieve a great increase in genic variability should be at a selective advantage." But what kinds of genetic data can be taken as definitive evidence of low-level introgression? Suppose species B

TABLE 5

Allozyme and mtDNA genotypes in hybrid Lepomis from the Oconee River and Lake Oglethorpe collections

Location	Hybrid individual	Standard length (mm)	Allozyme genotype									mtDNA genotype	
			Got-2	*Es-1*	*Mdh-1*	*Mdh-2*	*Pgi-1*	*Pgi-2*	*Pgm-1*	*Pdg-1*	*Ipo-1*	*Xba*I	*Ava*I
Oconee River													
	1	125	100/58	100	116/100	110/100	100/95	130/100	105/100	100/88	122/100	GR	GR
	2	74	100/58	100	116/100	110/100	100/95	130/100	105/100	100/88	122/100	GR	GR
	3	110	100/58	100	116/100	110/100	100/95	130/100	105/90	100/88	122/100	GR	GR
	4	110	100/58	100	116/100	110/100	100/95	130/100	105/100	100/92	122/100	GR	GR
	5	96	100/58	100	116/100	110/100	100/95	130/100	105/100	100/92	122/100	GR	GR
	6	126	100/58	100	116/100	110/100	100/95	130/100	105/90	100/88	122/100	GR	GR
	7	115	100/58	100	116/100	110/100	100/95	130/100	105/100	100/88	122/100	GR	GR
	8	110	100/58	100	116/100	100	100/95	198/100	110/90	100	100	WR	WR
	9	116	100/58	100	116/100	100	100/95	198/100	110/100	100	100	WR	WR
Lake Oglethorpe													
	10	158	109/100		116/100	110/100	100/81	100/62	110/100	100/87		RE	RE
	11	130	109/100		116/100	110/100	100/81	100/62	110/100	100/87		RE	RE
	12	118	109/100		116/100	110/100	100/81	100/62	110/100	100/87		RE	RE
	13	80	109/100		116/100	110/100	100/90	100/62	110/100	100/87	100/96	BG	BG
	14	180	58	100/96	116	110	95	130/100	110/105	100/92	122/100	RB	RB

Single numbers for an allozyme genotype indicate homozygosity. BG, bluegill (*macrochirus*) type; GR, green (*cyanellus*); WR, warmouth (*gulosus*); RE, redear (*microlophus*); RB, redbreast (*auritus*).

TABLE 6

Genetic identification of hybrid Lepomis from the Oconee River and Lake Oglethorpe collections

Location	Hybrid individuals (Table 5)	Probable hybrid generation	Parent		Comments
			Male	Female	
Oconee River					
Site 1	1, 6	F_1	*macrochirus*	*cyanellus*	Probable broodmates
Site 6	3, 4, 5, 7	F_1	*macrochirus*	*cyanellus*	Probable broodmates
Site 5	2	F_1	*macrochirus*	*cyanellus*	Not broodmate to above
Site 4	8, 9	F_1	*macrochirus*	*gulosus*	Probable broodmates
Lake Oglethorpe					
	10, 11, 12	F_1	*macrochirus*	*microlophus*	Possible broodmates
	13	F_1	*microlophus*	*macrochirus*	
	14	F_1	*cyanellus*	*auritus*	

possesses in low frequency a genetic character normally characteristic of species A. Three explanations for this distribution are possible: (1) past introgression from A; (2) symplesiomorphy, *i.e.*, the retention by A and B of the genetic character since time of separation from common ancestral stock; or (3) convergence, *i.e.*, the independent acquisition of the character in one or both species by mutation. To adequately confirm introgression, possibilities (2) and (3) must be eliminated. The likelihood that this can be accomplished is in large part a function of the nature of the genetic markers available for study. If an apparent individual of species B possesses *simultaneously* many genetic markers (or a trait with a complex genetic basis) normally characteristic of potential hybridizer A, the probability that this situation can be attributed to convergence or symplesiomorphy is minimized.

In the Oconee River and Lake Oglethorpe populations of Lepomis, morphologically recognizable hybrids (which proved to be F_1's) are present in low frequency, less than 2%. STEBBINS (1971) gives three reasons why naturally occurring F_1 hybrids are generally more likely to form progeny by backcrossing than by mating with one another: (1) the F_1 hybrids are far fewer than parentals; (2) the low fertility of hybrids results in a far greater number of parental gametes available for hybrid fertilization; and (3) backcross progeny will contain genes derived principally from one parental species and, hence, may be more likely to survive in habitats to which parentals are already well adapted. For this third reason as well, later-generation backcross hybrids should become increasingly difficult to distinguish from "pure" parental species by either morphological or genetic criteria. If various kinds of genetic markers are used, what is the probability of correct recognition of those true backcross progeny that do exist?

Consider Figure 4. For sake of argument, assume that two species are completely distinct in allelic composition at $m = 4$ neutral nuclear loci (A–D) and at four neutral mtDNA markers distributed along the two distinct mtDNA

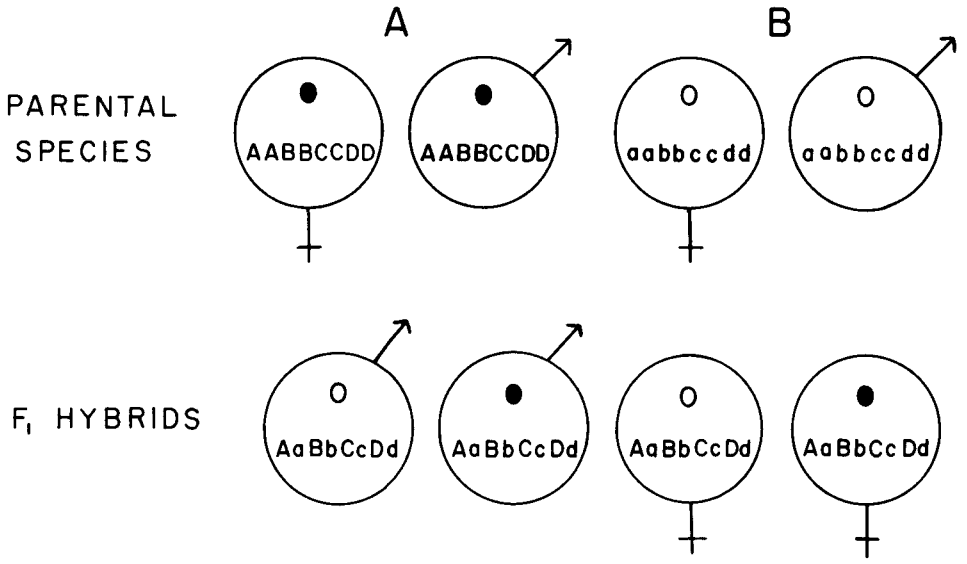

FIGURE 4.—Possible genotype/sex classes of F_1 hybrids between two parental species (A and B) which can be distinguished by four nuclear gene markers (indicated by upper- and lower-case letters) and by mtDNA genotype (indicated by solid *vs.* open circles).

genomes (indicated by closed *vs.* open circles). Among the F_1 hybrids from such a cross the four sex/genotype classes are shown. *If all such classes backcross to a given parental species with equal likelihood*, the frequency distributions of numbers of heterospecific genetic markers per individual (or the proportions of backcross hybrids exhibiting various numbers of genetic markers) are shown in Figure 5. For example, due to random segregation and independent assortment of unlinked nuclear markers, only 6.25% of first-generation backcross progency (BC1) will exhibit (in heterozygous condition) all four markers; by BC5, the total proportion of true backcross progeny recognizable by two or more unlinked nuclear markers is far less than 1%. In general, for any m, the *mean* number ($\bar{m}$) of nuclear markers per backcross individual is halved in each successive backcross generation.

For completely linked nuclear markers (second column, Figure 5), the mean proportion of heterospecific markers in backcross progeny decreases at the same rate, but the frequency distribution of markers remains strictly bimodal (*i.e.*, a given backcross hybrid to species B exhibits alleles from species A in heterozygous condition at either zero or four loci). For mtDNA, the linkage of markers imposed by the maternal transmission genetics also leads to a bimodal frequency distribution of heterospecific markers in recurrent backcross generations. However, because mtDNA is maternally inherited, only 25% of true BC1 progeny may be expected to exhibit the foreign mtDNA, and for a given backcross generation the mean number of mtDNA markers is one-half that for nuclear genes (Figure 5). Thus, the significance of linkage among mtDNA markers (or among physically linked nuclear genes) for detecting introgression lies in the increased potential for *simultaneous* appearance of multiple markers in a putative backcross or later-generation hybrid individual.

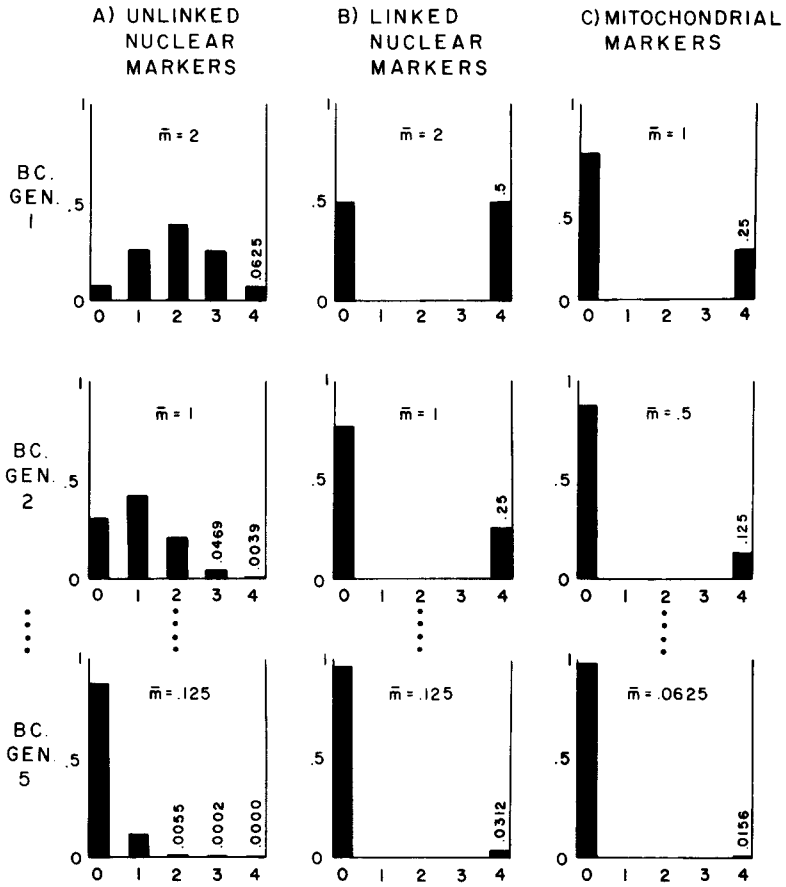

FIGURE 5.—Probability distributions of numbers of heterospecific nuclear or mtDNA genetic markers in the first five generations of unidirectional backcross progeny following hybridization of two species as shown in Figure 4. The horizontal axis gives numbers of heterospecific markers per individual, and m̄ is the mean number of such markers in a given backcross generation. See text for additional explanation.

These scenarios involve probabilities of detection of backcross hybrids produced by rare and isolated instances of backcrossing. However, for some species introgression may be a more common and recurrent phenomenon. TAKAHATA and SLATKIN (1984) have provided a mathematical model of recurrent low-level introgression and concluded that a "small amount of gene flow between the two species would be sufficient to lead to the replacement of the mitochondrial genotype of one species by that of the other," but that "only a small amount of selection against immigrant mitochondrial genotype is sufficient to prevent its establishment."

Additional biological considerations may of course alter the predictions of Figure 5 about detectability of introgression by mtDNA markers. In many animal groups, F_1 hybrids of the heterogametic sex are absent or sterile (Haldane's rule). If the sterile sex is male, any BC1 progeny would have a hybrid mother and a 50% probability of exhibiting the heterospecific mtDNA. In the

case of Lepomis, a strong biological bias *against* introgression of mtDNA may be present: F_1 hybrids in many (but not all) Lepomis crosses exhibit adult sex ratios strongly biased toward males (CHILDERS 1967; HUBBS and HUBBS 1933). [By cytologic evidence, it is not clear which sex in Lepomis is heterogametic (ROBERTS 1964). Systems of both male and female heterogamety are known in other fishes (GOLD 1979).] Another theoretical possibility is that nuclear-mitochondrial genotypic interactions affect organismal fitness in such a way as to favor those backcross progeny exhibiting homospecific mtDNA. This possibility is in part motivated by the observation that cultures of hybrid somatic cells tend to lose chromosomes from one or another parental species, and the mtDNA from the same parent is also lost (DEFRANCESCO, ATTARDI and CROCE 1980).

In any event, in the present study we have no mtDNA (or allozyme) evidence for introgression between species of Lepomis. The morphological, allozyme and mtDNA identifications of all 263 assayed fish were perfectly concordant (Tables 2 and 4). In these particular locations, the level of interspecific gene flow must be very low if present at all. The conclusion that absolutely no introgression has occurred must be tempered, however, by the realization that, for rare or intermittent hybridizers, only a small minority of later-generation backcross fish would likely be recognizable as such (Figure 5), even in this ideal situation in which a large number of genetic markers distinguish the parental species. Although we could discern only F_1 hybrids in our study locales, it of course remains possible that further generation hybrids between Lepomis occur in other areas of the species' distributions.

Parentage of F_1 hybrids: A more straightforward application of mtDNA and allozyme analysis of hybridization involves characterization of the parentage of F_1 hybrids. Five species of Lepomis proved to be involved in the production of the 14 F_1 hybrids discovered in our collections from the Oconee River and Lake Oglethorpe (Table 6). These hybrids represent at least six and probably seven to nine separate mating events. Two features of the crosses are of special note.

First, every assayed hybrid represented a cross between a common and a rare species. *Macrochirus* and *auritus* were the abundant species, together constituting 89.6 and 94.4% of the parental samples in the Oconee River and Lake Oglethorpe collections. If interspecific matings in these locations were strictly a function of relative species abundance, a random sample of seven crosses should have included more than five *macrochirus/auritus* pairings, but none was observed [despite that fact the *macrochirus* and *auritus* are known to be physiologically capable of hybrid production (BAILEY and LAGLER 1938; CHILDERS 1967)]. Conversely, given the rarity of *gulosus* (Table 2), a sample of more than 100 interspecific matings might have been expected necessary to include the one that was observed with a *gulosus* parent. It has previously been noted that Lepomis hybridization is often associated with great inequalities in numbers of sympatric species (KEENLEYSIDE 1967). Our data support the idea that hybridizations preferentially take place between parental species differing greatly in abundance.

A second interesting feature of the genetic results is that for six of the probable seven hybrid broods, the rare species in the cross was the female parent (Table 6); in five of these hybridizations the father was *macrochirus*. Lepomis males typically construct nests (often in colonies) to which females are attracted to spawn. Species-specific mate recognition involves a variety of cues including behavior, coloration and morphology and sound production (BREDER 1936; GERALD 1971; KEENLEYSIDE 1967). In *macrochirus*, competition among males for reproductive success is known to be severe (particularly in crowded breeding situations) and has even resulted in the evolution of a bizarre life-history strategy in which some "satellite" males mimic females (morphologically and behaviorally) and thereby gain access to nests and spawning events to which they would otherwise be excluded by territorial males (DOMINEY 1980; GROSS and CHARNOV 1980). Female Lepomis also have behavioral predispositions toward prosmiscuity, since observations indicate that "many females may visit one nest and one female may visit several nests" (BREDER 1936). Our data suggest that absence of conspecific pairing partners and mating stimuli for females of rarer species may be an important factor in increasing the likelihood of interspecific hybridization.

It remains to be determined whether the trends reported in this study will hold true for other hybridizing populations of Lepomis. Because mtDNA is maternally transmitted and can often be used to distinguish closely related species, it should offer many opportunities for novel genetic analyses of hybridization and introgression phenomena in these and other organisms.

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