

2003

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Recommended Citation

Johnson, Ronald L. and Williams, K. Ashlei (2003) "Genetic Relationships of Some Common Arkansas Freshwater Sunfishes (Centrarchidae: Lepomis) Inferred From Restriction Endonuclease Analysis of Mitochondrial DNA," *Journal of the Arkansas Academy of Science*: Vol. 57 , Article 14.

Available at: <http://scholarworks.uark.edu/jaas/vol57/iss1/14>

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Genetic Relationships of Some Common Arkansas Freshwater Sunfishes (Centrarchidae: *Lepomis*) Inferred From Restriction Endonuclease Analysis of Mitochondrial DNA

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Abstract

Geographic isolation and habitat specialization has aided in the evolution and maintenance of genetic integrity of the lepomid sunfishes (*Lepomis*: Centrarchidae) of North America. Our goal was to measure genetic distances between four of the eleven extant sunfish species by using mitochondrial DNA analysis. Mitochondrial DNA restriction fragment length polymorphisms (RFLPs) were examined in bluegill (*L. macrochirus*), redear sunfish (*L. microlophus*), longear sunfish (*L. megalotis*), and green sunfish (*L. cyanellus*) using 15 restriction endonucleases. The largemouth bass (*Micropterus salmoides*) was used as an outgroup. The phylogeny inferred from Dollo parsimony cladistic analysis largely concurred with published results from allozyme analyses and the fossil record, yet was inconsistent with published anatomical analyses. Genetic distances between species ranged from 0.1627 to 0.3328. The green sunfish was the basal member of the genus, whereas the bluegill was the most diverged from the largemouth bass. These four species diverged over a broad time frame, with estimated times of speciation occurring during Miocene (8.14 - 16.64 mya).

Introduction

The family Centrarchidae originated within the Mississippi River system of North America. Included among the Centrarchidae are the sunfishes, genus *Lepomis*, which are the most diverse group of this family, with eleven described species (Avisé and Smith, 1977). Several attempts have been made to classify the phylogenetic relationships of the sunfishes, yet there have been inconsistencies between proposed phylogenies. Bluegill (*L. macrochirus* Rafinesque) have been suggested as a more recently derived species within the genus based upon the fossil record (Miller, 1965; Mabee, 1988) and allozyme analysis (Avisé and Smith, 1977). However, Branson and Moore (1962) proposed that the longear sunfish (*L. megalotis* Rafinesque) was more recently diverged based on comparisons of the acoustico-lateralis system. Each of the studies above places the green sunfish as the basal member of the genus.

The research reported here focused on four species common to Arkansas and throughout much of the Mississippi river drainage system: green sunfish (*L. cyanellus* Rafinesque), bluegill sunfish, longear sunfish, and redear sunfish (*L. microlophus* Gunther). Our goal was to assess the concordance of mitochondrial DNA sequence divergence to previous models developed from anatomic and genetic evidences.

Materials and Methods

Single populations of bluegill (n = 18), redear sunfish (n = 12) and green sunfish (n = 14) were studied from private farm ponds near the Arkansas State University campus, and longear sunfish (n = 10) were collected from the South Fork of the Spring River. Mitochondria and mitochondrial DNA (mtDNA) were isolated and analyzed from liver tissue using techniques described by Johnson et al. (2002). Purified mtDNA was digested for 7 h at 37° C using 15 restriction endonucleases (*Bam*HI, *Bgl*I, *Bgl*II, *Csp*45I, *Dra*I, *Eco*RI, *Eco*RV, *Him*DIII, *Mlu*I, *Pst*I, *Pvu*II, *Sal*I, *Sca*I, *Xba*I, *Xho*I), under conditions recommended by the supplier (Promega Corp.). Fragment sizes generated were determined through the use of a least-squares fit program (Schaeffer and Sederoff, 1981). Variables measured included genome size, percentage genome analyzed, nucleon diversity (Nei and Tajima, 1981) and nucleotide sequence divergence (Nei and Li, 1979). Times of divergence were estimated from observed levels of sequence divergence using a divergence rate of 2% per million years (Brown et al., 1979). A phenogram was constructed by the unweighted pair group method (UPGMA; Sokal and Sneath, 1963) using matrices of distance values with NTSYS-PC: Numerical Taxonomy and Multivariate Analysis System (Rohlf, 1990) as well as an inferred phylogenetic tree using the Dollo parsimony algorithm in Phylogeny Inference Package (PHYLIP; Felsenstein, 1993). The dominant haplotype of the northern

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Table 1. MtDNA genetic analysis for sunfishes of the genus *Lepomis*.

Common Name	N	Restriction Fragments	Genome Size (bp) ± SE	Percent Genome Sampled	Number of Haplotypes	Nucleon Diversity
Green sunfish	14	40	16,958 ± 182	1.42%	7	0.64
Bluegill 18	58	16,642 ± 130	2.09%	9	0.67	
Longear sunfish	10	31	16,639 ± 135	1.12%	7	0.78
Redear sunfish	12	53	16,671 ± 164	1.91%	6	0.62

Table 2. Number of restriction fragments generated from mtDNA for *Lepomis* species sampled. Abbreviations are as follows: Green, green sunfish; Blue, bluegill; Long, longear sunfish; and Red, reardear sunfish. Total number of polymorphisms identified by an individual restriction endonuclease in parentheses.

Restriction Endonuclease		Common Name			
		Green	Blue	Long	Red
<i>Bam</i> HI	(4)	2	0	2	1
<i>Bgl</i> II	(7)	3-4	3-4	0-1	3
<i>Bgl</i> II	(5)	1	2	2	2
<i>Csp</i> 45I	(6)	0	2-3	3	3
<i>Dra</i> I	(5)	3	5	0	3-4
<i>Eco</i> RI	(2)	0-1	1	0-1	1
<i>Eco</i> RV	(4)	0	3	2	5
<i>Hind</i> III	(5)	3	4-5	3	4-5
<i>Mlu</i> I	(2)	1	0	0-1	0
<i>PS</i> I	(7)	2	3-4	2-3	3-4
<i>Pvu</i> II	(3)	2	2	0	0
<i>Sal</i> I	(4)	1-2	0,2	0	1
<i>Sca</i> I	(6)	4-5	3	2-3	5
<i>Xba</i> I	(6)	5	3-4	1-2	3
<i>Xho</i> I	(4)	1-2	2	0,2	2

largemouth bass (*Micropterus salmoides salmoides* Lacepede; Johnson et al., 2002), a representative of the genus most closely related to *Lepomis* (Branson and Moore, 1962; Avise and Smith, 1977), was used as an outgroup to root the tree.

Results and Discussion

The total number of restriction fragments generated using 15 restriction endonucleases was 140, with ranges of 31 for longear sunfish to 58 for bluegill (Table 1). From 1.12 to 2.09 percent of the mtDNA genome was surveyed. Seven of the restriction endonucleases did not identify recognition sequences within individual species (*Bam*HI bluegill; *Csp*45I, green sunfish; *Dra*I, longear sunfish; *Eco*RV, green sunfish; *Mlu*I, bluegill and reardear sunfishes; *Pvu*II, reardear and longear sunfishes; and *Sal*I, longear sunfish). *Bgl*II, *Csp*45I, *Dra*I, *Eco*RV, *Hind*III, *Pst*I, *Sca*I and *Xba*I generated higher numbers of polymorphisms (Table 2). The enzymes *Bgl*II and *Pst*I were particularly informative phylogenetically, with large numbers of restriction sites and high polymorphism between species (Table 2). Conversely, the restriction endonucleases *Eco*RI and *Mlu*I were less informative, with zero to one restriction site. However, no profile for an individual restriction endonuclease was shared by all species (Table 3).

Mean genome size (Table 1) ranged from 16,639 (± 135 SE) base pairs for longear sunfish to 16,958 (± 182 SE) base pairs for green sunfish. These estimates are similar to those found for bluegill (16,200 bp) by Avise et al. (1984) and for the shadow bass, *Ambloplites rupestris* (16,751 bp; Johnson and Cavanaugh, 2003), yet lower than that found for six species of *Micropterus*, another centrarchid genus (range of 17,346 to 17,779; Johnson et al., 2002).

A total of 29 haplotypes was identified among the 54 individuals (Table 1). A single haplotype was dominant for each species, with all other haplotypes found in single individuals (Table 3). Haplotype diversity was similar for all species studied, with *h* values ranging from 0.62 to 0.78 (Table 1). Nucleon diversity was similar to that found for

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Table 3. Dominant composite mtDNA haplotypes observed for *Lepomis* species studied. Order of restriction endonucleases in columns as follows: *Bam*HI, *Bgl*I, *Bgl*II, *Csp*45I, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Mlu*I, *Pst*I, *Pvu*II, *Sal*I, *Scal*, *Xba*I, and *Xho*I. Asterisks indicate restriction profiles which are invariant for that restriction endonuclease and samples. All variant haplotypes not listed were represented by single individuals.

Common Name	N	Haplotype															
Green sunfish	8	E*	L	B*	D*	C*	F	B*	A*	D*	D*	C*	A	D	C*	F	
Bluegill	10	F*	H	E	K	O*	F*	K*	K	B*	K	N*	C	E*	L	F*	
Longear sunfish	4	G*	E	I*	O*	B*	B	L*	N*	B*	M	B*	C*	G*	N	G	
Redear sunfish	7	B*	J*	E*	N*	A	F*	A*	M	B*	A	B*	G*	C*	A*	F*	

Table 4. Mitochondrial DNA sequence divergence (above diagonal) and estimated time of divergence in millions of years (below diagonal) for *Lepomis* species studied. Standard errors of the mean in parentheses beneath the means.

Species	1	2	3	4	5
1. Green sunfish	***	0.3328	0.3228	0.2778	0.3216
		*** (0.0012)	(0.0017)	(0.0010)	(0.0011)
2. Bluegill	16.64	***	0.2187	0.1627	0.4166
	(0.01)		*** (0.0019)	(0.0006)	(0.0003)
3. Longear sunfish	16.17	10.94	***	0.2453	0.5763
	(0.01)	(0.01)		*** (0.0010)	(0.0005)
4. Redear sunfish	13.89	8.14	12.27	***	0.3277
	(0.01)	(0.01)	(0.01)		*** (0.0067)
5. Largemouth bass	16.08	20.83	28.82	16.39	***
	(0.01)	(0.01)	(0.01)	(0.01)	

several *Micropterus* species (Johnson et al., 2002; range of 0.46 to 1.00), but the diversity was higher relative to other micropterid studies (population range of 0.07 to 0.37; Nedbal and Philipp, 1994; Snyder et al., 1996). The relatively high genetic heterogeneity values observed in the present study and in Johnson et al. (2002) may be attributed in part to the larger number of restriction endonucleases utilized (15 versus 7-8 in previous studies), which provided a better opportunity to detect genetic variability.

Estimated nucleotide sequence divergence, standard error, and time of divergence for *Lepomis* and *Micropterus* are found in Table 4. Divergence values ranged from 0.1627 for

bluegill and redear to 0.3328 for the bluegill and green sunfish. These congeneric values are similar to that found in the genus *Micropterus* (Johnson et al., 2002). Data indicated that the green sunfish is most closely related to the out-group largemouth bass (0.3216), whereas the longear was most genetically distinct from the largemouth bass (0.5763).

Estimated time of divergence for the species of *Lepomis* studied ranged from 8.14 to 16.64 million years ago (Table 4). According to our estimates, the point of divergence between *M. salmoides* and *Lepomis* was during the late Oligocene to Miocene (16-29 mya), which is more recent than observed in the fossil record, which traces both genera

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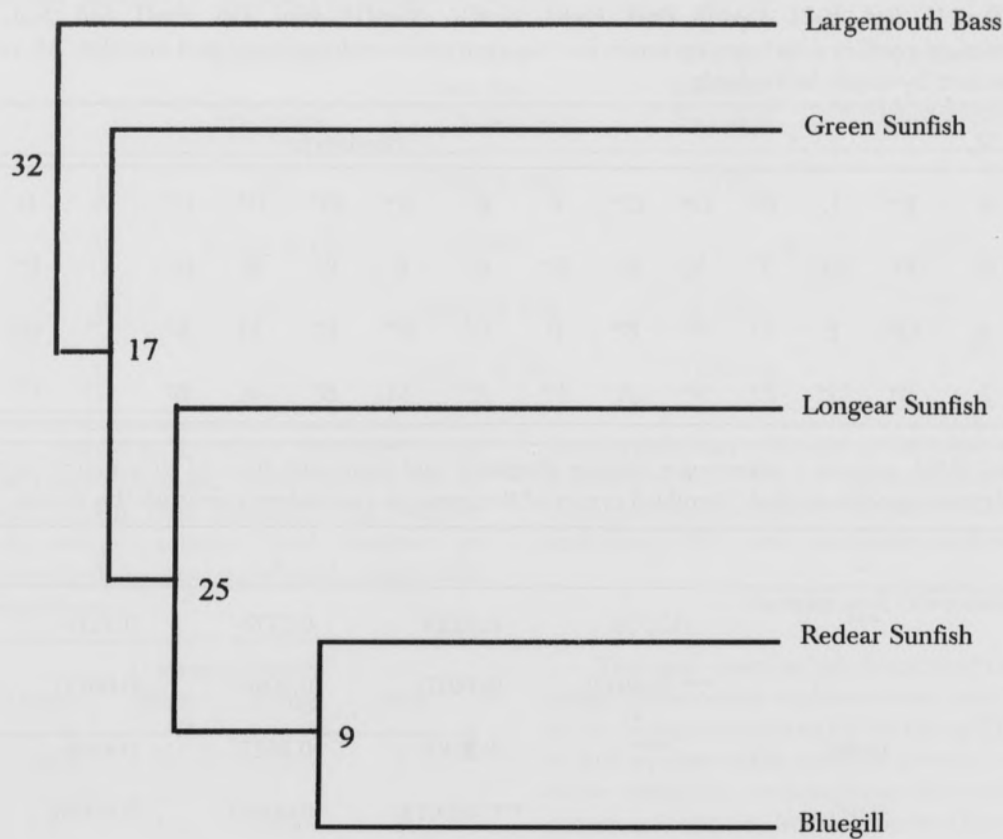


Fig. 1. Cladogram of character state matrices for presence/absence of presumptive restriction sites utilizing the Dollo parsimony algorithm. Each of the 29 equally most parsimonious trees (144 steps) were consistent to the species level.

back to the Eocene (37-58 mya, Smith, 1962; Wainwright and Lauder, 1992; Wilson and Williams, 1992). Conversely, individual species analyzed have earlier divergence times than those found in the fossil record. For example, our data indicates the green sunfish diverged from the redear sunfish 14 mya. The fossil record suggests the divergence of the green sunfish 4-6 mya (Smith, 1962). The time frames determined here also predate fossil records for redear and longear sunfishes (Smith, 1962). Additionally, the bluegill has been proposed as a more recently derived species (2-4 mya) [Miller, 1965; Mabee, 1988], which is consistent with our phylogenetic tree, but not with our estimated time of divergence (8.14 mya). It must be noted, however, that the fossil record of fish is not always complete (Wilson and Williams, 1992) and that the molecular clock of 2% per million years has not been consistent through all taxa (Grewe et al., 1990). Lastly, and most importantly, these estimates are based on single populations within each

species, and therefore the values obtained are at best estimations of the divergence periods between these particular populations rather than the species as a whole.

Construction of a cladogram generated utilizing the Dollo parsimony algorithm program (Figure 1) revealed that the green sunfish was most similar to the largemouth bass and the bluegill the most distant. A UPGMA dendrogram derived from the average genetic distances between species is consistent with the cladogram. A cophenetic correlation of 0.87 was obtained, indicative of a good fit between the tree generated and the data matrix (Rohlf, 1990).

Both the best-fit cladogram and the phenogram concur with the consensus of research, which shows the green sunfish as the basal member of the genus (Branson and Moore, 1962; Avise and Smith, 1977). However, Branson and Moore (1962) studied the acoustico-lateralis system and proposed that the longear was more recently diverged, instead of the bluegill as found in our data, allozyme

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analysis (Avisé and Smith, 1977) and the fossil record (Miller, 1965; Mabee, 1988; Wainwright and Lauder, 1992). Whereas allozyme analysis by Avisé and Smith (1977) showed the longear and redear sunfishes to be grouped with the green sunfish, our data suggest a closer relationship of these two species with the bluegill.

Future study of the genus *Lepomis* at the population, subspecies, and species levels is required to further our understanding of the historical biogeography of this genus. Additional population data may very well alter the proposed relationships of these taxa. Genetic analysis of within-species population divergence of mtDNA has only been performed on bluegill mtDNA (Avisé and Saunders, 1984; Avisé et al., 1984; Chapman, 1989). Population studies, particularly of sympatric populations, can provide insight as to possible source taxa of those species having restricted ranges.

ACKNOWLEDGMENTS.—We would like to thank C. Hutts, M. Oltmann, and C. McGrath for technical assistance and for assistance in the collection of specimens. We also thank A. Grippo, L. Hilburn, J. Loutsch, and L. Olson for their constructive criticisms of earlier drafts of this manuscript. This work was supported by grants from the Silo Undergraduate Research Foundation and Arkansas State University.

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