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Molecular Phylogenetics and Evolution of the Cytochrome *b* Gene in the Cyprinid Genus *Lythrurus* (Actinopterygii: Cypriniformes)

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Cytochrome *b* (cyt *b*) sequences for eight species of the North American cyprinid genus *Lythrurus* and two outgroups were analyzed phylogenetically. Species of *Lythrurus* differed by an average of 10.3% in cyt *b* nucleotide sequence and exhibited a transition to transversion ratio of 5.44 in pairwise comparisons. Maximum-parsimony and maximum-likelihood analyses support a sister-group relationship between a clade comprised of (*L. umbratilis* (*L. ardens, L. lirus*)) and a clade comprised of (*L. snelsoni* (*L. bellus* (*L. atrapiculus* (*L. fumeus, L. roseipinnis*)))). The phylogeny derived from cyt *b* sequences has similarities to hypotheses based on morphological evidence but differs substantially in relationships of *L. fumeus*. The phylogeny indicates a biogeographic scenario that differs from those previously inferred for *Lythrurus* by suggesting an ancestral divergence between clades that currently occupy largely "northern" and "southern" geographic distributions in the central and southeastern United States. The Ouachita Mountain shiner (*L. snelsoni*) is sister to species of *Lythrurus* that now inhabit the Gulf Coastal Plain. This is consistent with earlier hypotheses that Ouachita Highland fauna are at least partially comprised of Gulf Coastal Plain derivatives.

THE cyprinid genus *Lythrurus* consists of at least eight species distributed primarily in drainages of the eastern Gulf Coast, the Mississippi Valley, and the Piedmont region of the Atlantic seaboard (Fig. 1). Species of *Lythrurus* are found in small streams, are less that 70 mm in total length, and often possess bright red breeding colors (Snelson, 1972). Monophyly of *Lythrurus* is supported by both morphological and chromosomal characters (Snelson, 1972; Amemiya and Gold, 1988; Mayden, 1989). Relationships within *Lythrurus* have been studied extensively, and three hypotheses of relationships, based primarily on morphological evidence, have been proposed (Fig. 2; Snelson, 1972; Mayden, 1989; Wiley and Siegel-Causey, 1994). These studies have not resulted in a consensus opinion of relationships among species of *Lythrurus*.

Use of cytochrome *b* (cyt *b*) sequences in phylogenetic inference has blossomed since Kocher et al. (1989) published sequences of a primer set for polymerase chain reaction (PCR) amplification of a portion of the gene. Phylogenetic hypotheses based on sequences of the cyt *b* gene range from close to distantly related taxa (e.g., Meyer and Wilson, 1990; Meyer et al., 1990; Smith and Patton, 1991). In general, cyt *b* sequences have proven more useful in resolving relationships among closely related taxa (Meyer, 1993), largely because nucleotide sequence variation is less saturated by multiple substitutions. In this paper, we employ nucleotide sequences of cyt *b* to infer a hypothesis of phylogenetic relationships among species of *Lythrurus*. We also characterize evolution of cyt *b* in *Lythrurus*.

MATERIALS AND METHODS

The complete cyt *b* gene of one to two individuals was sequenced from each of eight species of *Lythrurus* (see Materials Examined). Cyt *b* sequences from six additional cyprinid taxa were examined for possible use as outgroups. Cyt *b* sequences from *Notemigonus crysoleucas* and *Opsopoeodus emiliae* (one individual each) were acquired in our laboratory; cyt *b* sequences from *Pimephales notatus, Cyprinella spiloptera, Luxilus coccogenis,* and *L. zonatus* were provided by T. Dowling and are available from GenBank (see Materials Examined).

Whole fish were captured with minnow seines, placed in labeled cryopreservation tubes, transported in liquid nitrogen, and stored at −80 C until processed. DNAs were obtained by phenol:chloroform extraction and ethanol precipitation (Sambrook et al., 1989) of tissue samples ground in liquid nitrogen. DNA preparations were used for amplification of target DNA sequences via polymerase chain reaction (PCR). PCR reaction mixtures contained an unbalanced (asymmetric) ratio of primer concentrations for acquisition of single-stranded DNA (Allard et al., 1991). Reaction conditions typically consisted of 94 C denaturation for 1 min, 43–50 C primer annealing for 1 min, and 72 C *Taq* polymerase extension for 45 sec. This reaction profile was repeated for 35 cycles.

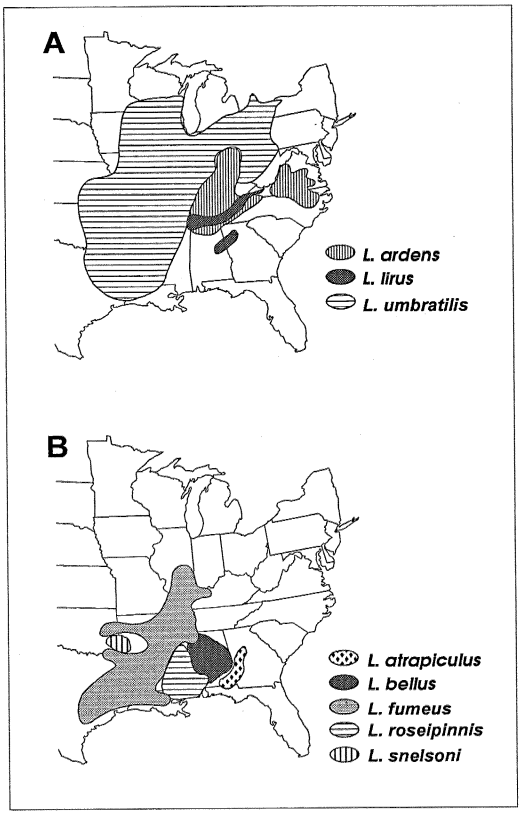


Fig. 1. Distributions of (A) *Lythrurus umbratilis, L. ardens,* and *L. lirus* and (B) *L. snelsoni, L. bellus, L. atrapiculus, L. roseipinnis,* and *L. fumeus.* Range shown for *L. ardens* includes those of two, recently elevated species (*L. fasciolaris* and *L. matutinus*) of the *L. ardens* species complex (Dimmick et al., 1996).

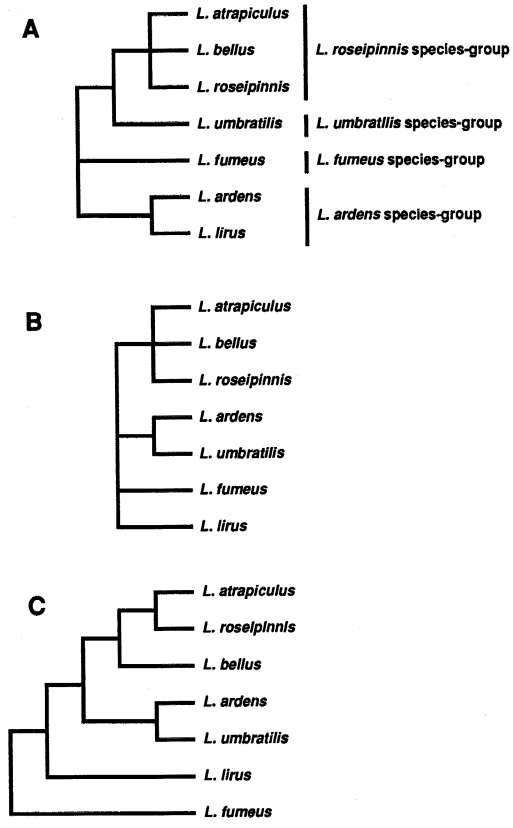
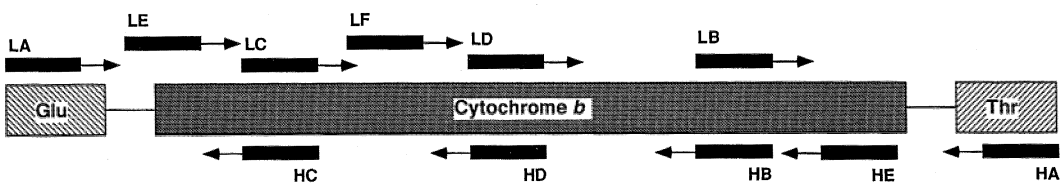


Fig. 2. Phylogenetic hypotheses of relationships among species of *Lythrurus.* (A) Snelson (1972); (B) Mayden (1989); and (C) Wiley and Siegel-Causey (1994). Not included in these hypotheses are *L. snelsoni,* a species restricted to upland streams of the Little River system in southwestern Arkansas and southeastern Oklahoma and whose phylogenetic relationships have been problematic (Robison, 1985); and *L. fasciolaris* and *L. matutinus,* two species of the *L. ardens* species complex that were elevated recently by Dimmick et al. (1996).

Amplified DNA was purified with either Centricon 30 tubes or phenol:chloroform extraction. Sequencing of target DNA sequences followed standard dideoxy chain-termination protocols (Sanger et al., 1977). Sequences were aligned by hand without inferring gaps.

Primers.—Eleven primers were designed for PCR-amplification and sequencing of the entire cyt *b* gene (Fig. 3). The external primers (LA and HA) were derived from sequences in tRNA genes (Glutamine and Threonine) adjacent to the cyt *b* gene. These primers are modifications of previously described primers (Irwin et al., 1991) and were designed by using sequences of other vertebrates, including white sturgeon and carp (Araya et al., 1984; Brown et al., 1989). Sequence data acquired using external primers allowed construction of additional primers which permitted PCR-amplification and sequencing of smaller fragments within the cyt *b* gene.

Molecular evolutionary analysis.—Transition to transversion ratios, estimates of nucleotide sequence divergence, and percent nucleotide composition were calculated using the computer program MEGA (S. Kumar, K. Tamura, and M. Nei, MEGA: molecular evolutionary genetics analysis, Pennsylvania State Univ., University Park, 1993, unpubl.). Maximum-likelihood corrections for multiple substitutions were generated according to the F84 model (Felsenstein and Churchill, 1996) by using the DNAdist program of PHYLIP (Felsenstein, 1989). The F84 model parameters were set as follows: (1) transition/transversion ratio set to maximum observed value among the ingroup (6.84); (2) empirical estimates of base frequencies; and (3)



LA (15058) - 5' - GTG ACT TGA AAA ACC ACC GTT G - 3'
LB (16028) - 5' - ACC TCA AAA CAA CGA GGA CTA AC - 3'
LC (15344) - 5' - ATA CAT GCC AAC GGA GCA TC - 3'
LD (15656) - 5' - CCA TTC GTC ATC GCC GGT GC - 3'
LE (15152) - 5'- GCA TTA GTC GAC CTT CCA ACA CC - 3'
LF (15521) - 5' - TTC TGA GGT GCC ACC GTT ATT AC - 3'
HA (16249) - 5' - CAA CGA TCT CCG GTT TAC AAG AC - 3'
HB (16002) - 5' - AGT CCT CGT TGT TTT GAG GTG TG - 3'
HC (15287) - 5' - CCA TAG TTG ACG TCA CGG CA - 3'
HD (15680) - 5' - GGG TTG TTT GAT CCT GTT TCT GT - 3'
HE (16097) - 5' - ATA TAT GGG TGT TCT ACA GGT ATG CC - 3'

Fig. 3. Map of the cytochrome *b* gene and adjacent tRNA genes, showing spatial arrangement and sequence of 11 PCR- amplification and sequencing primers. Black boxes indicate approximate locations of primers. Arrows indicate directions of both *Taq* polymerase extension and sequencing. Numbers next to primer sequences are position of the 3′ base relative to the complete carp mitochondrial genome (Chang et al., 1994).

three rate constants with relative rates estimated from the proportion of total variation observed at each codon position. Patterns of substitutional saturation were investigated graphically by plotting observed counts of transitional and transversional substitutions at first and third codon positions against F84-corrected genetic distances. Patterns of substitution at second codon positions were not graphed, because only 11 substitutions (three within *Lythrurus*) were observed. Homogeneity of nucleotide composition among taxa was tested by using chi-square contingency tests of tables of nucleotide counts. Tests were performed for ingroup and outgroup taxa on each codon position, with the test among outgroup taxa including a single representative (*L. ardens*) of *Lythrurus*.

Relative rate tests.—Evaluation of rate homogeneity was performed using two different approaches: (1) relative rate tests of sequence evolution according to the Tajima (1993) 1D method, with sequential Bonferroni correction for multiple testing (Rice, 1989); and (2) simulation of a null distribution specific to the sampled gene and taxa (e.g., Adell and Dopazo, 1994). We employed the latter to obtain a sampling distribution for the null hypothesis of a molecular clock. The single most-parsimonious tree produced from analysis of all ingroup and outgroup taxa was used as the model tree. Branch lengths were estimated by fitting F84-corrected genetic distances to the tree and by assuming a molecular clock (KITSCH program of PHYLIP). This tree served as a template for 200 simulations of character evolution according to the F84 model (model parameters were derived from cyt *b* sequences as described earlier). Simulations were conducted using the computer program Seq-Gen (Rambaut and Grassly, 1997). Random variation expected under a molecular clock was estimated by measuring the difference in least-squares estimates of the fit of F84 distances assuming a clock (KITSCH) or no clock (FITCH) program in PHYLIP for each of 200 simulations (delta). The observed delta for *Lythrurus* was compared with the null distribution to estimate the probability that it could be a product of a clocklike process (*P*-value). To allow branch-specific tests, the distribution of differences between KITSCH and FITCH estimates of each branch length also were computed from each of the 200 simulated datasets.

Phylogenetic analysis.—Maximum-parsimony analysis (Branch and Bound search) was carried out using PAUP version 3.1.1 (D. L. Swofford, PAUP: phylogenetic analysis using parsimony, Smithsonian Institution, Washington, DC, 1993, unpubl.). Interpretations of relative support for individual nodes were based on 1000 bootstrap replications (Felsenstein, 1985), the Bremer support index (Bremer, 1988), and enumeration of the number of unreversed (CI = 1.0) synapomorphies. Maximum-likelihood analysis was carried out using the DNAML program in PHYLIP. The F84 model was employed, with model parameters derived from cyt *b* sequences as described earlier. Statistical tests of alternate

hypotheses were conducted using the Kishino and Hasegawa test (1989), as implemented in the DNAML program of PHYLIP.

Results and Discussion

Sequence variation.—Nucleotide sequence of cyt *b* from eight species of *Lythrurus, N. crysoleucas,* and *O. emiliae* are deposited in the GenBank Database (accession numbers U01318, U17268–U17275). There were 259 variable bases within *Lythrurus,* and 144 bases were phylogenetically informative under parsimony. Nucleotide sequence variation within *Lythrurus* was predominantly synonymous (95.3%), mostly at the third codon position (88.4%). At the first codon position, 81.5% of the variation was at sixfold degenerate leucine codons. Inferred amino acid sequences were nearly identical among species of *Lythrurus,* with a mean difference among pairwise comparisons of 0.94 ± 0.08% (mean ± SD).

Outgroup selection.—Six cyprinid taxa were examined for possible use as outgroups. *Notemigonus crysoleucas* was sampled because it is placed in the Leucisini, the sister group to the Phoxinini where all other cyprinids native to North America are currently placed (Cavender and Coburn, 1992). *Pimephales notatus* and *Opsopoeodus emiliae* were sampled because they were placed by Coburn and Cavender (1992) with *Cyprinella* in a clade that was sister to *Lythrurus.* Mayden (1989), alternatively, placed *Pimephales* in a polytomy basal to a larger clade that included *Cyprinella, Luxilus, Lythrurus,* and others. Both *Luxilus* and *Cyprinella* were hypothesized to be close relatives of *Lythrurus* (Coburn and Cavender, 1992; Mayden, 1989) and therefore were sampled for possible use as outgroups. In general, the outgroup method should utilize dense sampling of close relatives of the ingroup (Nixon and Carpenter, 1993). We were sensitive, however, to heterogeneous patterns of variation among ingroup and outgroup taxa that could produce inconsistency in tree estimation (Wheeler, 1990; Kuhner and Felsenstein, 1994; Lockhart et al., 1994). Consequently, we examined patterns of cyt *b* evolution among both ingroup and outgroup taxa before selection of outgroups for phylogenetic analysis.

Plots of transversions at first and third codon positions exhibited no indications of saturation (Fig. 4 top). Plots of transitions, however, showed evidence of saturation at approximately 20% (F84-corrected) sequence divergence (Fig. 4 bottom). Pairwise comparisons of sequence

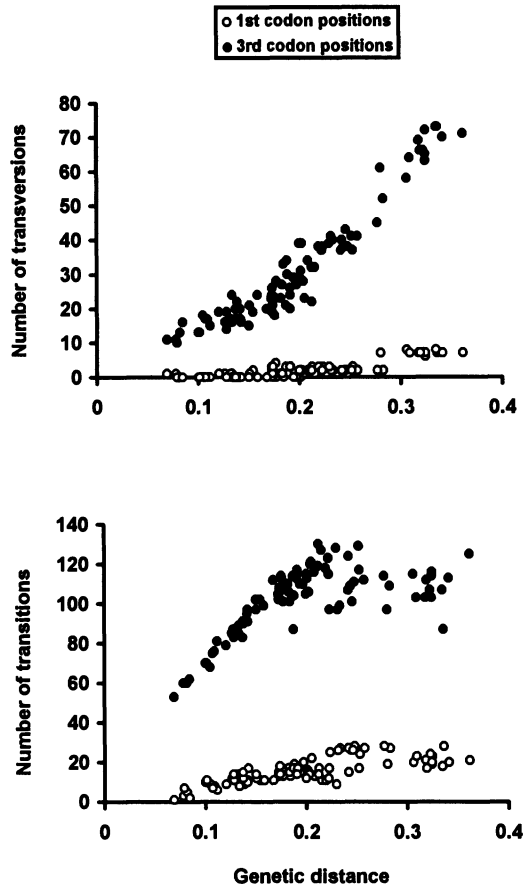


Fig. 4. Plots of (top) number of transversions and (bottom) number of transitions at first and third codon positions. Plots are relative to nucleotide sequence divergence estimated using F84-correction (Felsenstein and Churchill, 1996) for multiple substitutions.

divergence among species of *Lythrurus* and *Luxilus* appeared largely linear (14 ± 3.7%; mean ± SD), whereas comparisons that included *N. crysoleucas, P. notatus,* and *O. emiliae* indicated that transitional substitutions might be randomized with respect to *Lythrurus* (26 ± 5.8%; mean ± SD; Fig. 4 bottom). The latter could constrain use of *N. crysoleucas, P. notatus,* or *O. emiliae* as outgroups for *Lythrurus,* either because of random placement of the root or because of "long-branch attraction" (Hendy and Penny, 1989; Wheeler, 1990).

Nucleotide composition in *Lythrurus* is similar to that in other bony fish and vertebrate taxa, although there appear to be differences among sampled cyprinids at third codon positions (Table 1). Nucleotide counts within *Lythrurus* were homogeneous at all three codon positions (first positions, $\chi^2 = 0.480$, 21 df, $P > 0.05$; second

TABLE 1. PERCENT NUCLEOTIDE COMPOSITION OF CYTOCHROME *b* GENE AT ALL CODON POSITIONS AND AT THIRD CODON POSITIONS. Values for cyprinid Genera and other vertebrates are averages across species.

	All codon positions				Third codon positions			
	A	T	C	G	A	T	C	G
North American cyprinids								
Lythrurus (8 species)	25	29	29	17	32	21	36	12
Luxilus (2 species)	25	28	29	18	31	19	37	13
Cyprinella spiloptera	23	29	29	19	27	20	36	17
Pimephales notatus	27	29	28	16	36	23	32	09
Opsopoeodus emiliae	28	29	28	15	37	22	34	07
Notemigonus crysoleucas	27	29	28	16	37	20	34	09
Other vertebrates								
Sharks	27	31	29	12	34	23	40	02
Bony fishes	27	27	31	15	35	17	42	06
Birds	28	25	35	12	36	12	48	04
Mammals	31	25	29	13	43	16	37	04

Sources for bony fishes: Chang et al., 1994; Tzeng et al., 1992; Whitmore et al., 1994; Brown et al., 1989; for birds: Desjardins and Morais, 1990; Korenegay et al., 1994; for mammals: Irwin et al., 1991; and for sharks: Martin and Palumbi, 1994.

positions, $\chi^2 = 0.134$, 21 df, $P > 0.05$; third positions, $\chi^2 = 9.436$, df 21, $P > 0.05$), whereas nucleotide counts among cyprinid outgroups exhibited significant heterogeneity at third codon positions (first positions, $\chi^2 = 1.981$, df 18, $P > 0.05$; second positions, $\chi^2 = 0.282$, df 18, $P > 0.05$; third positions, $\chi^2 = 39.049$, df 18, $P < 0.005$). Of all cyprinids sampled as possible outgroups, nucleotide counts of species of *Luxilus* were closest (nearly identical) to those of *Lythrurus* (Table 1). Comparisons restricted to *Luxilus* and *Lythrurus* suggest homogeneity at all three codon positions (first positions, $\chi^2 = 0.921$, df 27, $P > 0.05$; second positions, $\chi^2 = 0.157$, df 27, $P > 0.05$; third positions, $\chi^2 = 13.382$, df 27, $P > 0.05$;).

Relative rate tests (Tajima, 1993) that employed *N. crysoleucas* as outgroup for all possible pairwise comparisons of taxa generated five significant results, none of which were significant after sequential Bonferroni correction (Rice, 1989). However, the 17 largest chi-square values invariably involved either *P. notatus* or *C spiloptera*, suggesting that the power of the test may be low. The rate test based upon the null distribution specific to cyt *b* and the sampled taxa indicated significant deviation from rate homogeneity within the phylogeny when all possible outgroup taxa were considered (delta = 2.03, $P < 0.001$). Branch length tests suggested significant changes in substitution rates associated with the following outgroup taxa: *N. crysoleucas* (delta = 0.0085, $P < 0.01$); *P. notatus* (delta = 0.0274, $P < 0.0001$); *O. emiliae* (delta = 0.0274, $P < 0.0001$); and *C. spiloptera* (delta = 0.0297, $P < 0.0001$). The inability of the Tajima (1993) 1D test to detect deviations from rate homogeneity could be due to the very long branch connecting *N. crysoleucas* to the remaining taxa.

Based on the foregoing analyses, we chose to employ only the two species of *Luxilus* as outgroup taxa for phylogenetic analysis of *Lythrurus*. Substitutional saturation, base compositional heterogeneity, and significant differences in rate of nucleotide substitution are all known to negatively impact consistency of tree estimation (Wheeler, 1990; Kuhner and Felsenstein, 1994; Lockhart et al., 1994). Comparisons of the two species of *Luxilus* with the species of *Lythrurus* revealed no evidence of substitutional saturation or base composition differences at third codon positions nor significant differences in rates of nucleotide substitution. Comparisons with the remaining four "outgroup" taxa (i.e., *N. crysoleucas, P. notatus, O. emiliae*, and *C. spiloptera*) revealed the opposite, indicating that use of these four taxa as outgroups could compromise tree estimation. This was confirmed by jackknifing the six outgroup taxa and conducting both maximum-parsimony and maximum-likelihood analyses. Tree topologies involving the species of *Lythrurus* varied depending on the outgroup(s) employed, and relative support for individual trees (measured by percent concordant clades across 1000 bootstrap replications) varied from 37.5% to 78.7%.

Phylogenetic analysis.—Maximum-parsimony and maximum-likelihood analyses (carried out using the two species of *Luxilus* as outgroups) produced identical tree topologies (Fig. 5). Bootstrap replications and Bremer indices indicated strongest support for a predominantly northern

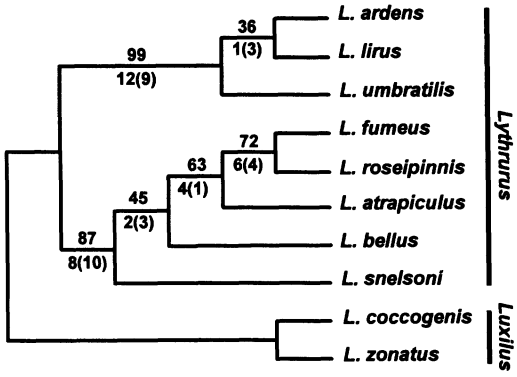


Fig. 5. Phylogenetic tree derived from maximum-parsimony and maximum-likelihood analysis of the complete cytochrome *b* gene of *Lythrurus*. Outgroup taxa are *Luxilus coccogenis* and *Luxilus zonatus*. Maximum-parsimony analysis produced a single tree of 626 steps (CI = 0.612, RI = 0.456); maximum-likelihood analysis resulted in an identical tree with a *ln*-likelihood of −4880.84. Numbers above internodes are the percentage of times that internode was supported in 1000 bootstrap replicates. Numbers below internodes are Bremer support indices and (in parentheses) synapomorphies with CI = 1.0.

clade comprised of *L. ardens, L. lirus,* and *L. umbratilis* (hereafter referred to as the *L. umbratilis* clade); a predominantly southern clade comprised of *L. snelsoni, L. bellus, L. atrapiculus, L. fumeus,* and *L. roseipinnis* (hereafter referred to as the *L. snelsoni* clade); and a sister-group relationship between *L. fumeus* and *L. roseipinnis*. Monophyly of the *L. umbratilis* and *L. snelsoni* clades was supported by nine and 10 unreversed synapomorphies (CI = 1.0), respectively. The sister-group relationship between *L. fumeus* and *L. roseipinnis* was supported by four unreversed synapomorphies.

Lesser support, as measured by bootstrap proportions and Bremer indices, was indicated for monophyly of *L. lirus* and *L. ardens* (but which was supported by three unreversed synapomorphies); a sister-group relationship between *L. atrapiculus* and the *L. roseipinnis* and *L. fumeus* clade; and monophyly of the species of *Lythrurus* inhabiting the Gulf Coastal Plain (*L. bellus, L. atrapiculus, L. fumeus,* and *L. roseipinnis*; but which was supported by three unreversed synapomorphies).

The phylogenetic hypothesis generated from cyt *b* sequences of *Lythrurus* was not wholly consistent with previously proposed hypotheses based on morphology. For example, *L. ardens, L. lirus,* and *L. umbratilis* have not been placed previously within the same clade, although *L. lirus* and *L. umbratilis* have been hypothesized to be sister to *L. ardens* (Snelson, 1980; Mayden, 1989; Wiley and Siegel-Causey, 1994). Exclusion of *L. lirus* or *L. umbratilis* from the clade including *L. ardens* increased overall cyt *b* tree length by a minimum of 16 and 17 steps, respectively. Our hypotheses also differs from those of Snelson (1972), Mayden (1989), and Wiley and Siegel-Causey (1994) in placement of *L. fumeus* within the *L. roseipinnis* species group as the sister species of *L. roseipinnis*. All previous hypotheses have placed *L. fumeus* outside of the *L. roseipinnis* species group, and Snelson (1973) suggested that *L. fumeus* might represent a transitional form between *Lythrurus* and other cyprinid species groups. Exclusion of *L. fumeus* from the *L. roseipinnis* species group increased overall cyt *b* tree length by a minimum of eight steps. Finally, previous researchers have not discerned relationships of *L. snelsoni* (Snelson, 1973; Robison 1985). Our results support placement of *L. snelsoni* as the basal member of a clade distributed primarily in the Gulf Coastal Plain.

The fully resolved hypothesis of Wiley and Siegel-Causey (1994) was compared statistically with the cyt *b*-based hypothesis by using the likelihood test of Kishino and Hasegawa (1989). More than 95% of log-likelihood differences taken across sites between the two trees were larger than zero, indicating that the Wiley and Siegel-Causey (1994) hypothesis (*ln* likelihood = −3347.75) has a significantly lower likelihood as compared with the cyt *b*-based hypothesis (*ln* likelihood = −3281.61).

Biogeography.—Results of phylogenetic analysis of cyt *b* sequences suggest a biogeographic scenario that differs from those previously inferred for species of *Lythrurus*. Most notable is the indication of an ancestral split between the largely northern *L. umbratilis* species group and largely southern *L. snelsoni* species group. Regions that these clades occupy are consistent with several other species and species groups of eastern North American fishes (Wiley and Mayden, 1985; Mayden, 1988). Previously proposed hypotheses of relationships within *Lythrurus* have not recognized this ancestral divergence. For example, the hypothesis of Wiley and Siegel-Causey (1994) suggests an initial divergence between *L. fumeus* (which occurs generally in lowland tributaries and drainages west of the Mississippi River) and other *Lythrurus*, followed by isolation of *L. lirus* (which occurs in the central highlands of Tennessee).

Historical biogeography of the Ouachita Highland fauna of southwestern Arkansas and southeastern Oklahoma has received extensive attention (Matthews and Robison, 1982; May-

den, 1985). Mayden (1985) concluded that the Ouachita Highland fauna was a montage of taxa from two sources: (1) relatives and species of the Ozarkian Highlands; and (2) relatives of groups that are widespread on the Gulf Coastal Plain. He hypothesized that Ozarkian relatives were the result of a vicariant split between the Ozark and Ouachita Highlands and that Gulf Coastal Plain derivatives were either the result of peripheral isolation (via dispersal) or a vicariant split during uplift in the early Tertiary. Our data are consistent with the hypothesis that the Ouachita Highlands are at least partially populated with Gulf Coastal Plain derivatives. The Ouachita Mountain shiner (*L. snelsoni*) is sister to members of the *L. roseipinnis* species group that occur in the Gulf Coastal Plain. This distributional pattern is nearly identical to that of the crayfish pair, *Procambarus ouachitae* and the *Procambarus spiculifer* species group (Hobbs and Robison, 1982) and to that of the salamander pair *Desmognathus brimleyorum* and *Desmognathus auriculatus* (Cook and Brown, 1974). Molecular-based investigation of these and other taxa with similar distributional patterns may discern either repeated invasion of the Ouachita Highlands by Gulf Coastal Plain fauna or a larger vicariant event, such as capture of Ouachita Highland drainages by the Red River.

Material examined.—Voucher specimens were deposited in the Texas Cooperative Wildlife Collection (TCWC) at Texas A&M University. Collection localities (drainage in parentheses) of materials examined are as follows: *Lythrurus ardens*, Roanoke River, Roanoke Co., Virginia (Roanoke River), TCWC: 6896.03; *L. atrapiculus*, Big Horse Creek, Okaloosa Co., Florida (Yellow River), TCWC: 8910.01; *L. bellus*, unnamed tributary of Mulberry Creek, Bibb Co., Alabama (Alabama River), TCWC: 7653.01; *L. fumeus*, Little Brazos River, Brazos Co., Texas (Brazos River), TCWC: 8911.01; *L. lirus*, Buck Creek, Shelby Co., Alabama (Cahaba River), TCWC: 7673.01; *L. roseipinnis*, Chickaswhay River, Clarke Co., Mississippi (Pascagoula River), TCWC: 7658.01; *L. snelsoni*, Lick Creek, Little River Co., Arkansas (Little River); *L. umbratilis*, Mill Creek, Waubunsee Co., Kansas (Kansas River), TCWC: 7666.01; and *Opsopoeodus emiliae*, Navasota River, Brazos Co., Texas, TCWC: 7246.01. A specimen of *Notemigonus crysoleucas* was acquired from a bait shop in Bryan, Texas. Cyt *b* sequences of *Pimephales notatus* (GenBank: U66606), *Cyprinella spiloptera* (GenBank: U66605), *Luxilus coccogenis* (GenBank: U66603), and *Luxilus zonatus* (GenBank: U66600), were provided by T. Dowling.

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