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Molecular Phylogenetics and Evolution 32 (2004) 344–357
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Investigating phylogenetic relationships of sunfishes and black basses (Actinopterygii: Centrarchidae) using DNA sequences from mitochondrial and nuclear genes

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Received 27 September 2003; revised 17 December 2003
Available online 28 February 2004

Abstract

The 32 species of the Centrarchidae are ecologically important components of the diverse fish communities that characterize North American freshwater ecosystems. In spite of a rich history of systematic investigations of centrarchid fishes there is extensive conflict among previous hypotheses that may be due to restricted taxon or character sampling. We present the first phylogenetic analysis of the Centrarchidae that combines DNA sequence data from both the mitochondrial and nuclear genomes and includes all described species. Gene sequence data were collected from a complete mtDNA protein coding gene (NADH subunit 2), a nuclear DNA intron (S7 ribosomal protein intron 1), and a portion of a nuclear DNA protein-coding region (*Tmo-4C4*). Phylogenetic trees generated from analysis of the three-gene dataset were used to test alternative hypotheses of centrarchid relationships that were gathered from the literature. Four major centrarchid lineages are present in trees generated in maximum parsimony (MP) and Bayesian maximum likelihood analyses (BML). These lineages are *Acantharchus pomotis*, *Micropterus*, *Lepomis*, and a clade containing *Ambloplites*, *Archoplites*, *Centrarchus*, *Enneacanthus*, and *Pomoxis*. Phylogenetic trees resulting from MP and BML analyses are highly consistent but differ with regard to the placement of *A. pomotis*. Significant phylogenetic incongruence between mtDNA and nuclear genes appears to result from different placement of *Micropterus treculi*, and is not characteristic of relationships in all other parts of the centrarchid phylogeny. Slightly more than half of the 27 previously proposed hypotheses of centrarchid relationships were rejected based on the Shomodaira–Hasegawa test.

Keywords: Black bass; Sunfish; *Micropterus*; *Lepomis*; mtDNA; Nuclear gene phylogeny

1. Introduction

The Centrarchidae (black basses, rock basses, crappies, and sunfishes) is an ecologically prominent radiation of North American freshwater fishes that are often the dominant top-level predators in the diverse fish communities found in North American warm water lakes and rivers. Although all centrarchids are carnivores, there is marked trophic diversity in the group with the dominant prey of species ranging from zooplankton to insects, crayfish, mollusks, and other fishes (Wainwright and Lauder, 1992). There are 32 extant species of centrarchids and all but one are endemic to eastern North America (Kassler et al., 2002; Page and Burr, 1991). The centrarchid fossil record is fairly extensive and the earliest known fossils are from the Eocene in Montana (Cavender, 1986).

Investigation of centrarchid systematics has a long history (Bailey, 1938), but the overall picture is one of considerable incongruence among previous hypotheses. Several types of comparative data have been used to assess centrarchid relationships, including allozymes (Avise and Smith, 1977; Avise et al., 1977), discretely coded morphological characters (Chang, 1988; Mabee, 1989, 1993), and most recently mtDNA sequences (Roe et al., 2002). These approaches have yielded conflicting results. For instance, analyses of allozyme variation result in

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doi:10.1016/j.ympev.2003.12.010

Lepomis and *Micropterus* as sister lineages (Avise and Smith, 1977; Avise et al., 1977), while maximum parsimony (MP) and maximum likelihood (ML) analyses of mtDNA sequence data resulted in two different phylogenetic hypotheses for the relationship between *Lepomis* and *Micropterus* (Roe et al., 2002). Studies that have used modern phylogenetic methods to estimate centrarchid relationships have resulted in either limited phylogenetic resolution through the recovery of several equi-parsimonious trees (e.g., Mabee, 1993), or provide hypotheses that have fairly weak node support in bootstrap pseudoreplicate analyses (e.g., Roe et al., 2002).

It is unclear if the inability to resolve relationships of centrarchid species is the result of previous strategies of character and taxon sampling, or if this pattern reflects the true history of evolutionary diversification in the Centrarchidae. If cladogenesis was rapid near the ancestral nodes connecting the major centrarchid lineages, then one would expect a pattern of very short branches with poor support from phylogenetic analysis (Kraus and Miyamoto, 1991; Shaffer et al., 1997). Alternatively, low resolution could arise as a consequence of inadequate character or taxonomic sampling for a particular phylogenetic problem (Jackman et al., 1999; Slowinski, 2001), rather than reflecting an actual pattern of evolutionary diversification.

The goals of this investigation are to provide the first assessment of relationships among all described centrarchid species using DNA sequences from both mtDNA and nuclear genes, and to test the wealth of previous hypotheses of centrarchid relationships (Table 1). Phylogenetic inferences are based on analyses of a single mtDNA protein coding gene and two nuclear encoded loci, one intron and a portion of a protein-coding region. Genes were sampled from both genomes to assess congruence among unlinked gene regions. The recovery of congruent phylogenetic hypotheses from mitochondrial and nuclear genes would bolster confidence that phylogenetically resolved gene trees are more likely to represent the evolutionary relationships of centrarchid species, and

Table 1
Alternative hypotheses proposed for phylogenetic relationships of the Centrarchidae

Hypothesis	Data type	Method of analysis	Taxonomic focus
Schlaikjer (1937)	External morphology	PC[a]	Genera
Bailey (1938)	External morphology	PC[a]	Genera
Smith and Bailey (1961)	Dorsal-fin support morphology	PC[a]	Genera
Branson and Moore (1962, Fig. 11)	Lateralis system, osteology	PC[a]	Genera
Avise et al. (1977)	Allozyme variation	UPGMA[b]	Genera
Mok (1981, Fig. 5)	Kidney morphology	MP[c]	Genera
Mok (1981, Fig. 6)	Anal spine and olfactory morphology	MP[c]	Genera
Parker et al. (1985)	Allozyme variation	DW[d]	Genera
Lauder (1986)	Osteology	MP[c]	Genera, species of *Lepomis*
Chang (1988)	Osteology, external morphology	MP[c]	Genera
Wainwright and Lauder (1992)	Osteology	MP[c]	Genera, species of *Lepomis*
Mabee (1993, Fig. 2A)	Osteology, external morphology	MP, OGP[e]	All species
Mabee (1993, Fig. 2B)	Osteology, external morphology	MP, OntP[f]	All species
Mabee (1993, Fig. 2C)	Osteology, external morphology	MP, RevOntP[g]	All species
Roe et al. (2002, Fig. 2)	mtDNA sequences (cytochrome *b*)	MP[c]	Genera
Roe et al. (2002, Fig. 4)	mtDNA sequences (cytochrome *b*)	ML[h]	Genera
Bailey (1938)	External morphology	PC[a]	Species of *Lepomis*
Branson and Moore (1962, Fig. 14)	Lateralis system, osteology	PC[a]	Species of *Lepomis*
Avise and Smith (1977, Fig. 5)	Allozyme variation	UPGMA[b]	Species of *Lepomis*
Hubbs and Bailey (1940)	External morphology	PC[a]	Species of *Micropterus*
Branson and Moore (1962, Fig. 15)	Lateralis system, osteology	PC[a]	Species of *Micropterus*
Ramsey (1975)	External morphology	PC[a]	Species of *Micropterus*
Johnson et al. (2001)	RFLP of mtDNA genome	UPGMA[b]	Species of *Micropterus*
Kassler et al. (2002, Fig. 3)	mtDNA sequences (cytochrome *b* and NADH subunit 2)	MP[c]	Species of *Micropterus*
Kassler et al. (2002, Fig. 5)	mtDNA sequences (cytochrome *b* and NADH subunit 2)	ML[h]	Species of *Micropterus*
Near et al. (2003)	mtDNA sequences (cytochrome *b* and NADH subunit 2)	ML[h]	Species of *Micropterus*

[a] Precladistic.
[b] Unweighted pair-group method.
[c] Maximum parsimony.
[d] Distance Wagner.
[e] Maximum parsimony, outgroup polarization.
[f] Maximum parsimony, ontogenetic polarization.
[g] Maximum parsimony, reverse ontogenetic polarization.
[h] Maximum likelihood.

phylogenetic inferences are not complicated by processes such as introgression or ancestral polymorphism (Doyle, 1997; Moore, 1995). The result is a dataset that provides appreciable resolution of the relationships among all described centrarchid species, discriminates among many of the alternate phylogenetic hypotheses, and provides a novel phylogenetic hypothesis that can serve as the basis for future comparative studies in this group that has a rich history of attracting interest from workers interested in their ecology, functional morphology, and patterns of speciation.

2. Materials and methods

2.1. Specimen collection and DNA sequencing

Specimens of all recognized centrarchid species were collected from native populations (Appendix A). There is no clear consensus on the closest outgroup taxa for the Centrarchidae (Mabee, 1993; Roe et al., 2002). Previous hypotheses have proposed that the Elassomatidae represents the sister taxon of the Centrarchidae, but mtDNA datasets have been conflicting (Jones and Quattro, 1999; Roe et al., 2002). The relationship of centrarchids to other perciform lineages, including the Elassomatidae, is not addressed in this study. In this study two species each from the Percidae (*Perca flavescens* and *Percina maculata*) and Nototheniidae (*Dissostichus mawsoni* and *Notothenia rossii*) were used as outgroup taxa in all analyses. Outgroup species were chosen to represent at least two lineages within the Perciformes, as well as the ability of available PCR primers to amplify all three target genes used in these analyses.

Total nucleic acids were isolated from muscle or liver tissues using proteinase-K digestion followed by phenol–chloroform extraction and ethanol precipitation. The complete coding region of the mitochondrial-encoded NADH subunit 2 (ND2) gene and the nuclear encoded S7 ribosomal protein intron 1 were PCR amplified with previously published primer sequences (Chow and Hazama, 1998; Kocher et al., 1995). Primers used to PCR amplify the nuclear encoded *Tmo-4C4* protein coding region were TMO-F2 (5′gAK TgT TTg AAA ATg ACT CgC TA-3′) and TMO-R2 (5′AAA CAT CYA AMg ATA TgA TCA TgC-3′). The final volume of PCR was 50 μl and contained 0.8 mM of dNTP, 2.5 mM $MgCl_2$, 0.5 μM of each primer, and 2.5 U of *Taq* DNA polymerase in a reaction buffer containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0), and 0.1% Triton X-100. Template DNA ranged from 100 to 300 ng. Thermal cycling conditions for the ND2 gene were an initial denaturation step of 94 °C for 3 min. followed by 30 cycles of denaturation at 94 °C (30 s), primer annealing at 55 °C (30 s), and extension at 72 °C (1.5 min). A final incubation of 72 °C for 5 min was added at the end of the cycle to ensure complete extension of amplified products. Thermal cycling conditions for both the S7 ribosomal protein intron and *Tmo-4C4* were identical to ND2, except only 25 cycles were used and the annealing temperatures were set to 60 °C.

Two primers designed to anneal at internal portions of the ND2 gene were used in conjunction with the PCR primers to sequence both strands of the PCR amplified products. Primers were designed for different groups of centrarchid species. Internal primers used to sequence species of *Micropterus* were previously published (Near et al., 2003). The reverse internal primer used for all non-*Micropterus* centrarchid species was CENT-ND2R2 (5′-AgR TgB gCR ATD gAK gAR TAR gC-3′). The forward internal sequencing primer used for all centrarchids except *Micropterus* and *Lepomis* species was CENT-ND2F1 (5′-AAY CAR ACN CAR CTY CgA AAR AT-3′). The internal forward primer used to sequence *Lepomis cyanellus*, *Lepomis gulosus*, *Lepomis humilis*, and *Lepomis macrochirus* was LEPO-ND2F1 (5′-gCY CCY TTY gCC CTN CTC CTT CAA-3′), and LEPO-ND2F2 (5′-ARC TYg CCC CCT TYg CCC THC TCC T-3′) was used to sequence *Lepomis auritus*, *Lepomis gibbosus*, *Lepomis marginatus*, *Lepomis megalotis*, *Lepomis microlophus*, *Lepomis miniatus*, *Lepomis punctatus*, and *Lepomis symmetricus*. Internal sequencing primers were not needed to obtain near complete overlap in sequenced forward and reverse strands of the PCR products for the two nuclear genes. Prior to sequencing, PCR products were cleaned of excess primers and nucleotides by digesting with 1.0 U of Exonuclease I and shrimp alkaline phosphatase, and incubated for 15 min at 37 °C and 20 min at 80 °C. Cleaned PCR products were used as template for Big Dye (Applied Biosystems) cycle sequencing, and sequencing reactions were read using an ABI 3100 automated sequencer at the Division of Biological Sciences Automated DNA Sequencing Facility at the University of California, Davis. Individual sequence files were edited using EditView version 1.0.1 and complete sequence overlaps were constructed from edited sequence files using the program Sequencher version 4.0 (Gene Codes, Ann Arbor, MI).

2.2. Phylogenetic analyses

Both the mitochondrial ND2 and nuclear *Tmo4C4* sequences were aligned by eye using the infered amino acid sequences as a guide. The S7 ribosomal protein intron sequences were aligned using Clustal X (Thompson et al., 1997). Initially, all centrarchid sequences and the four outgroup species were aligned separately using Clustal with default gap penalty settings. After the creation of two alignments, the profile alignment option in Clustal was used to align the out-

group species alignment to the centrarchid species alignment. The alignment was checked and adjusted by eye using MacClade 4.03 (Maddison and Maddison, 2000). The presence of saturation, or multiple substitutions was investigated for each of the three genes sequenced in this study. Absolute numbers of pairwise transitions were plotted against absolute numbers of pairwise transversions; similar plots were constructed for each codon position in ND2 and *Tmo-4C4*.

Both maximum parsimony (MP) and Bayesian maximum likelihood (BML) analyses were used to generate phylogenetic hypotheses from the DNA sequence data. The computer program PAUP* 4.0 (Swofford, 2001) was used for all MP analyses. The three separate gene regions were concatenated into a single data matrix. The most-parsimonious tree was found using a heuristic tree search with 1000 random addition sequence replicates and TBR branch swapping. The robustness of inferred nodes was assessed using a nonparametric bootstrap analysis with 2000 pseudoreplicates. The null hypothesis that each of the three gene regions represent a random partition of the entire pool of nucleotide sites into three subsets was tested using the incongruence length difference (ILD) test (Farris et al., 1994) in PAUP* with 1000 replications. In order to qualitatively assess phylogenetic resolution provided by of each of the three gene regions, each gene partition was analyzed separately using MP followed with a bootstrap analysis with 2000 pseudoreplicates.

The Bayesian maximum likelihood (BML) method of phylogenetic inference, using Markov chain Monte Carlo (MCMC) to estimate posterior probabilities, was selected for ML analysis (Huelsenbeck et al., 2001; Larget and Simon, 1999). Bayesian maximum likelihood analyses were executed on a dataset that consisted of a combination of all three gene regions. A three-step strategy was used to incorporate appropriate DNA substitution models for each data partition. First, data partitions were identified from the perspective of separate gene regions as well as character classes within gene regions. A total of seven data partitions were designated, three codon positions for each of the two protein coding genes (ND2 and *Tmo-4C4*), and a single partition for the S7 ribosomal protein intron. Second, the optimal model of sequence evolution for each data partition was determined from a total of 56 progressively complex models by using a hierarchical likelihood ratio test (LRT) (Huelsenbeck and Crandall, 1997). The computer program Modeltest 3.0 was used to calculate ML scores for each model and execute the LRTs (Posada and Crandall, 1998). Third, the different models of sequence evolution selected for each data partition were used in the computer program Mr. Bayes 3.0 (Ronquist and Huelsenbeck, 2003) with the APPLYTO command, and appropriate model parameter values were estimated for each data partition using UNLINK commands. The models used for the separate partitions in a simultaneous analysis differed by no more than three parameters and each model had a unique substitution model (one, two, or six parameter) corresponding to F81, HKY or K80, and GTR models of DNA sequence evolution, distribution of among-site rate variation (equal versus Γ distributed rates), and whether or not the presence of invariant sites was modeled. Mr. Bayes was run with 10^6 generations to ensure the MCMC algorithm was run for an appropriate number of iterations, providing convergence in the estimations of the tree topology with the best ML posterior probability, branch lengths, the parameter values of the DNA substitution models, and posterior probability estimates of node support. Four chains were run simultaneously in each analysis and the analysis was repeated four separate times. The burn-in period of the MCMC analysis was determined by graphically tracking the ML scores at each generation to determine the point where generations and the ML values reach a plateau. Trees and parameter values resulting from generations prior to the burn-in were discarded. The frequency that a particular clade occurs within the collection of trees after the burn-in was interpreted as a measure of clade support.

2.3. Testing alternative phylogenetic hypotheses of the Centrarchidae

Alternative phylogenetic hypotheses of centrarchids were compared to the best tree that resulted from BML analyses. The best ML tree that represented a particular alternative phylogenetic hypothesis was found using constraint tree searches in PAUP*. The Shimodaira–Hasegawa (SH) test was executed in PAUP* to test the null hypothesis that all trees in the set of probable tree topologies are equally good explanations of the data. The set of all probable trees was defined as the tree resulting from BML analysis of the DNA sequence data, as well as all other trees resulting from constraint tree searches that represent the pool of alternative phylogenetic hypotheses. A total of 27 alternative hypotheses were identified from the literature and are summarized in Table 1.

Effort was made to include all possible alternative phylogenetic hypotheses since reducing the number of tree topologies can inflate the statistical significance of the SH test (Goldman et al., 2000). There exists in the literature a wealth of hypotheses concerning the relationships among species of centrarchid fishes (Table 1), thus simultaneously testing all hypotheses potentially avoids over inflation of statistical significance of differences between the phylogenetic hypothesis inferred from mitochondrial and nuclear gene sequences and any given previous phylogenetic hypothesis. Since many hypotheses predate the development of modern phylogenetic systematics, some were distinctly more verbal and had to

be converted to a branching dendrogram (e.g., Ramsey, 1975).

A significant challenge in testing many of the previous phylogenetic hypotheses is a lack of complete sampling of centrarchid species. There were two types of alternative phylogenetic hypotheses with missing species. First, some studies were concerned with relationships in a particular presumed monophyletic group of centrarchids. For example, Hubbs and Bailey (1940) and Johnson et al. (2001) proposed relationships only for species in *Micropterus*. Second, other hypotheses attempted to assess relationships among all major centrarchid lineages, but did not include all species (e.g., Roe et al., 2002). When alternative hypotheses did not include all recognized centrarchid species backbone constraints were constructed. Using backbone constraint tree searches allows optimal placement of missing species without penalizing a particular hypothesis for missing taxa. More importantly, this strategy permits a simultaneous SH test of all previous hypotheses despite variation among previous investigators with regard to taxonomic sampling.

3. Results

The aligned dataset of all three genes for all 32 centrarchid species and outgroup taxa contained 2272 nucleotide sites. There were a total of 1014 phylogenetically informative sites, and about half of these sites were from the mitochondrial ND2 gene, the other half were found in the two nuclear genes (Table 2). The range of observed uncorrected pairwise sequence divergence for ND2 was 1.3% (*Micropterus punctatus–Micropterus dolomieu*) to 25.6% (*Pomoxis nigromaculatus–L. miniatus*). The ranges of uncorrected pairwise sequence divergence in S7 was 0.4% (*M. dolomieu–Micropterus cataractae*) to 15.6% (*L. symmetricus–Acantharchus pomotis*), and *Tmo-4C4* uncorrected pairwise sequence divergence ranged between no observed changes (*M. treculi–M. punctatus*, *Ambloplites constellatus–Ambloplites ariommus*, and *Micropterus notius–M. cataractae*) to 7.2% (*P. nigromaculatus–Micropterus floridanus* and *A. pomotis–M. floridanus*). Between the two nuclear genes, the S7 ribosomal protein

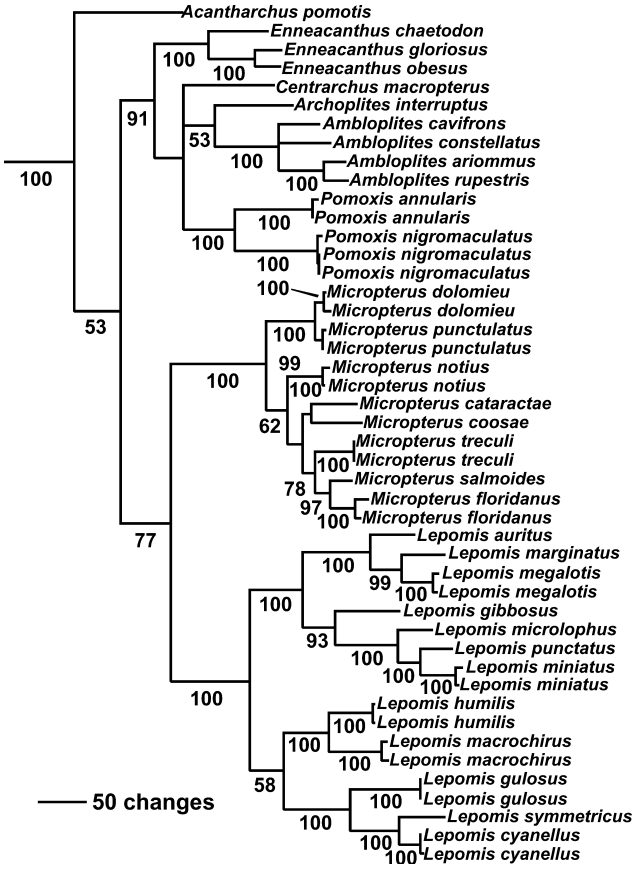

Fig. 1. Strict consensus of four trees resulting from maximum parsimony analysis of the three-gene dataset. Branches are scaled to the number of optimized changes. Numbers at nodes represent percent recovery in bootstrap analysis (2000 pseudoreplicates). See Table 3 for tree statistics.

Table 2
Summary of variation within each gene and structural categories or character classes in each gene

Gene	Structural category or character class	No. aligned sites (% of All)	No. variable sites (% of aligned sites in class)	No. informative sites (% of aligned sites in class)
ND2				
	1st Codon	349 (15.4)	168 (48.1)	144 (41.3)
	2nd Codon	349 (15.4)	71 (20.3)	62 (17.8)
	3rd Codon	349 (15.4)	344 (98.6)	339 (97.7)
	All	1047 (46.1)	583 (55.7)	545 (52.1)
S7 ribosomal protein				
	Intron	769 (33.8)	310 (40.3)	203 (26.4)
Tmo-4C4				
	1st Codon	152 (6.7)	22 (14.5)	13 (8.6)
	2nd Codon	152 (6.7)	22 (14.5)	13 (8.6)
	3rd Codon	152 (6.7)	85 (55.9)	66 (43.4)
	All	456 (20.1)	129 (28.3)	92 (20.2)

Table 3
Summary of maximum parsimony analyses for each individual gene region and the combined three gene dataset

Gene region	No. trees	Tree length	CI[a]	Percent nodes resolved[b]
ND2	1	3018	0.343	100.0
S7 intron 1	265,140	918	0.737	71.1
Tmo-4C4	649	204	0.721	42.2
All genes combined	4	4174	0.434	95.6

[a] Consistency index, excluding phylogenetically uninformative characters.
[b] Percentage of all possible nodes within the Centrarchidae (including intraspecific nodes), a fully resolved tree will have $n - 1$ nodes, where n is the number of taxa in the analysis.

intron displayed a faster relative rate of nucleotide substitution (Table 2). Evidence for multiple substitutions was detected in ND2 first and third codon positions using plots of the absolute numbers of transitions versus transversions (plots not shown). Similar plots for the other two genes did not exhibit a pattern suggestive of multiple substitutions.

Maximum parsimony analysis of the concatenated three-gene dataset resulted in four most parsimonious trees. The consensus of these three trees was a well-resolved phylogeny with high bootstrap support at many nodes (Fig. 1, Table 3). All of the polytypic genera (*Ambloplites*, *Enneacanthus*, *Lepomis*, *Micropterus*, and *Pomoxis*) were monophyletic and supported with 100% bootstrap scores (Fig. 1), and *Micropterus* and *Lepomis* were sister lineages (Fig. 1). Relationships among species in *Lepomis* were well resolved with the majority of interspecific nodes present in 93–100% of the bootstrap pseudoreplicates (Fig. 1). Relationships within *Micropterus* were less resolved when compared to *Lepomis*, as only three of seven interspecific nodes were supported with bootstrap scores greater than 78% (Fig. 1).

Analyses of each gene partition separately using MP revealed areas of phylogenetic incongruence between the

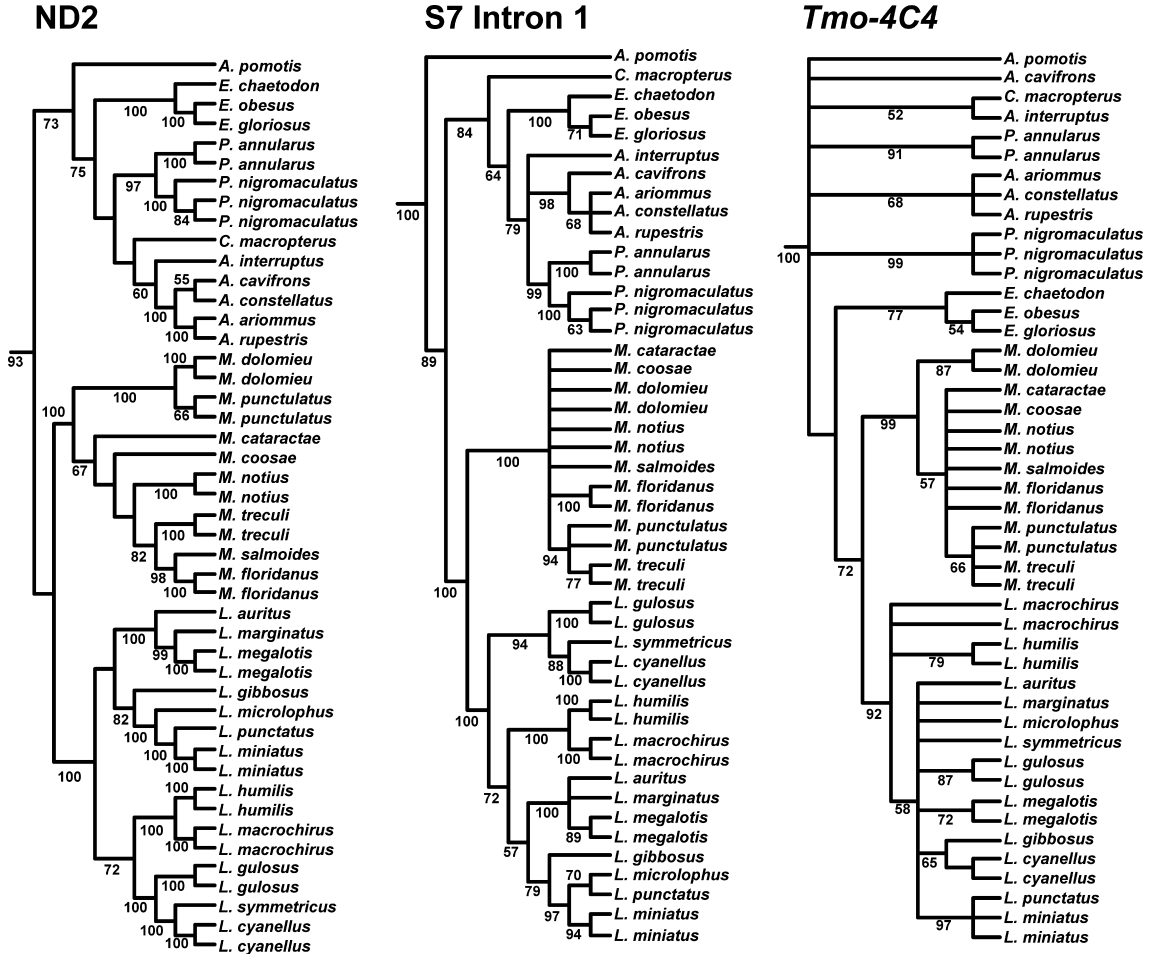

Fig. 2. Trees resulting from maximum parsimony analysis of each gene in the three-gene dataset. Numbers at nodes represent percent recovery in bootstrap analysis (2000 pseudoreplicates). See Table 3 for tree statistics.

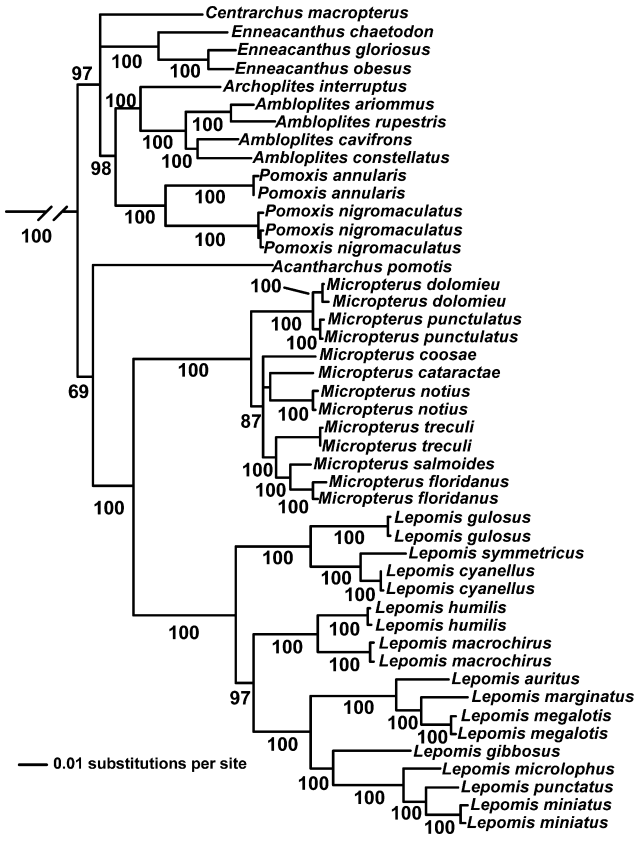

Fig. 3. Tree resulting from Bayesian maximum likelihood analysis of the three-gene dataset. Branches are scaled to the numbers of substitutions per site. Numbers at nodes represent Bayesian posterior probabilities.

mitochondrial and nuclear genes, as well as illustrating differing degrees of phylogenetic resolution provided by each gene region (Fig. 3, Table 3). For example, *A. pomotis* was the sister taxon of a clade containing *Enneacanthus*, *Pomoxis*, *Centrarchus*, *Archoplites*, and *Ambloplites* with a 73% bootstrap in the analysis of ND2. However, the two nuclear genes placed *A. pomotis* as either the sister taxon of all other centrarchids with strong bootstrap support (S7), or in an unresolved basal polytomy (*Tmo-4C4*). Another region of incongruence between the mitochondrial ND2 and the two nuclear genes involves relationships within *Micropterus*. *M. treculi* placed was the sister species of *Micropterus salmoides* + *M. floridanus* in the MP analysis of ND2; however, *M. treculi* was in an unresolved clade with *M. punctulatus* in the analysis of each nuclear gene (Fig. 2). Reflecting this incongruence was the rejection of the null hypothesis that the different gene regions represent a random subsample of the entire pool of nucleotide sites in an ILD test ($P = 0.02$). We explored the cause of a significant ILD test result by excluding particular species involved with the observed incongruence between the mitochondrial and nuclear genes in MP analyses (Fig. 2). Excluding *A. pomotis* did not alter the significant result ($P = 0.02$); however, excluding *M. treculi* resulted in a nonsignificant ILD test ($P = 0.48$).

A different ML model was selected for all but two of the seven data partitions using LRTs (Table 4). Four different substitution models were selected with various combinations of parameters for invariant sites and distribution of among-site substitution rates. The most complex model was selected for the ND2 third codon positions, which also exhibited the greatest percentage of polymorphic DNA sites. The least complex model was selected for the *Tmo-4C4* second codon, which was the character class with the lowest percentage of polymorphic DNA sites (Table 2). Overall, there was a correlation between the amount of polymorphism observed at a given data partition, and the complexity of the ML model selected using LRTs.

Bayesian ML analysis was run four times for 1×10^6 generations with nearly identical results from each run. The first of the four runs was used for assessing centrarchid relationships. The MCMC algorithm converged on a stable likelihood score after approximately 60,000 generations in each run and the first 70,000 generations were discarded as the burn-in. The mean ML scores from the 9300 optimal trees ranged between −22526.97

Table 4
Summary of models of DNA substitution selected for data partitions using maximum likelihood ratio tests

Data partition	DNA substitution model	No substitution types	Invariant sites?	Substitution rates[a]
ND2 1st Codon	HKY85[b]	2	No	Γ distributed
ND2 2nd Codon	HKY85[b]	2	Yes	Γ distributed
ND2 3rd Codon	GTR[c]	6	Yes	Γ distributed
S7 Intron 1	HKY85[b]	2	No	Γ distributed
Tmo-4C4 1st Codon	F81[d]	1	No	Γ distributed
Tmo-4C4 2nd Codon	F81	1	No	Equal
Tmo-4C4 3rd Codon	K2P[e]	2	No	Γ distributed

[a] Among-site rate variation.
[b] Hasegawa-Kishino-Yano 1985 model.
[c] General time reversible model.
[d] Felsenstein 1981 model.
[e] Kimura-2-parameter model.

Table 5
Shimodaira–Hasegawa test of alternative phylogenetic hypotheses of centrarchid fishes

Hypothesis	ln L	Difference in ln L	P
Centrarchidae genera			
Bayesian ML (Fig. 3)	−22387.72	Best	—
Schlaikjer (1937)	−22851.49	463.77	<0.001*
Bailey (1938)	−22585.99	198.27	<0.001*
Smith and Bailey (1961)	−22585.99	198.27	<0.001*
Branson and Moore (1962, Fig. 11)	−22624.29	236.57	<0.001*
Avise et al. (1977)	−22438.73	51.01	0.452
Mok (1981; Fig. 5)	−22562.16	174.44	<0.001*
Mok (1981; Fig. 6)	−22519.38	131.66	<0.001*
Parker et al. (1985)	−22600.72	213.00	0.002*
Lauder (1986)	−22405.01	17.29	0.912
Chang (1988)	−22436.97	49.25	0.472
Wainwright and Lauder (1992)	−23358.48	970.76	<0.001*
Mabee (1993, Fig. 2A)	−23330.84	943.12	<0.001*
Mabee (1993, Fig. 2B)	−23345.87	958.15	<0.001*
Mabee (1993, Fig. 2C)	−23341.71	953.99	<0.001*
Roe et al. (2002, Fig. 2)	−22412.05	24.33	0.808
Roe et al. (2002, Fig. 3)	−22392.74	5.02	0.991
Roe et al. (2002, Fig. 4)	−22401.65	13.93	0.922
Lepomis			
Bailey (1938)	−22606.41	218.69	<0.001*
Branson and Moore (1962, Fig. 14)	−22483.56	95.84	0.089
Avise and Smith (1977, Fig. 5)	−22921.43	533.71	<0.001*
Micropterus			
Hubbs and Bailey (1940)	−22537.06	149.34	0.014*
Branson and Moore (1962, Fig. 15)	−22624.71	236.99	<0.001*
Ramsey (1975)	−22631.29	243.57	<0.001*
Johnson et al. (2001)	−22402.61	14.89	0.900
Kassler et al. (2002, Fig. 3)	−22394.51	6.79	0.986
Kassler et al. (2002, Fig. 5)	−22391.41	3.69	0.994
Near et al. (2003)	−22392.61	4.89	0.989

Hypotheses are listed chronologically. Significant results are presented with an asterisk.

and −22467.19 with a mean equal to -22493.79 ± 8.22. The 50% majority rule consensus tree of the 9300 post burn-in trees was well-resolved with most nodes supported by a significant (>95%) posterior probability (Fig. 3). Very similar to the MP analysis of the combined data (Fig. 1), all polytypic genera were monophyletic. Also, *Micropterus* and *Lepomis* were sister lineages, and relationships of species in *Lepomis* were more resolved than *Micropterus*. In the BML tree, nodes with a posterior probability less than 95% were considered as not significantly supported by the data. This is not a measure of accuracy of convergence to the true phylogenetic relationships, but rather a metric of how well the hypothesized model and the given data supported the estimated parameters, including the tree topology.

Constraint tree searches resulted in a single tree for each of the 27 alternative phylogenetic hypotheses examined (Table 1). The SH test rejected 16 of the alternative hypotheses (Table 5). Among the 17 hypotheses tested that addressed relationships among centrarchid genera, 11 were rejected. Two of three hypotheses of relationships in *Lepomis*, and three of seven hypotheses of relationships among species of *Micropterus* were rejected (Table 5).

4. Discussion

Despite appreciable interest in centrarchid fishes conflicts among previously proposed phylogenetic hypotheses and the absence of comparative DNA sequence datasets sampled for all species in the clade has significantly restricted interpretations of centrarchid evolution. This investigation represents the first attempt to resolve the phylogenetic relationships of all species in a family of North American freshwater fishes using DNA sequences sampled from both mitochondrial and nuclear genes. The analyses of this dataset result in considerable phylogenetic resolution of centrarchid relationships (Figs. 1–3), and the ability to discriminate and reject many of the alternative hypotheses presented in previous studies (Table 5).

Phylogenetic analysis of the combined dataset containing all of the mitochondrial and nuclear genes sampled in this study identified four major clades of

centrarchid fishes (Figs. 1 and 3). Within each of the major monophyletic clades the degree of phylogenetic resolution and node support varied. Relationships of species in the clade containing *Ambloplites*, *Archoplites*, *Centrarchus*, *Enneacanthus*, and *Pomoxis* were the least resolved in MP analysis, as indicated by the presence of two polytomies in the strict consensus tree (Fig. 2). *Enneacanthus* was monophyletic with *Enneacanthus chaetodon* strongly supported as the sister taxon of *Enneacanthus gloriosus* + *Enneacanthus obesus* (Figs. 1 and 3), supporting previous classifications that included *E. chaetodon* in the monotypic genus *Mesogonistius* (Bailey, 1938; Schlaikjer, 1937). Both MP and BML analyses resulted in *Archoplites interruptus* as the sister species of *Ambloplites*. Within *Ambloplites* there was a basal polytomy in the MP analysis (Fig. 1), but relationships were completely resolved and well supported in BML analyses (Fig. 3).

Relationships among *Micropterus* were not completely resolved in the MP analysis, but five of seven possible interspecific nodes were strongly supported with bootstrap pseudoreplicate scores (Fig. 2). Nodes that were strongly supported in *Micropterus* include the most recent common ancestor (MRCA) of all *Micropterus* species, the sister species relationship between *M. floridanus* and *M. salmoides*, and *M. treculi* as the sister species of *M. salmoides* + *M. floridanus*. Perhaps the most surprising result is the strongly supported sister species relationship between *M. dolomieu* and *M. punctulatus*. These two species differ substantially in pigmentation relative to other *Micropterus* species, exhibit partial geographic range overlap (Ramsey, 1975), and a very recent age since their MRCA (Near et al., 2003). Trees resulting from MP and BML analyses of this dataset were similar to recent analyses of *Micropterus* relationships using mtDNA gene sequences (Kassler et al., 2002; Near et al., 2003).

Phylogenetic relationships among species of *Lepomis* are the best resolved and strongly supported among the three polytypic major clades of centrarchids (Figs. 1 and 3). The only incongruence between the MP and BML analyses in *Lepomis* involves the relationships of the clade (*L. gulosus* (*L. symmetricus* + *L. cyanellus*)). In the MP analysis this clade is the sister taxon of *L. humilis* + *L. macrochirus* with poor bootstrap support, but in the BML analysis this clade is the sister taxon of all other *Lepomis* species with a significant Bayesian posterior probability (Figs. 1 and 3). Even with this incongruence at a relatively basal position of the *Lepomis* phylogeny, all species are placed into monophyletic clades that are strongly supported in both analyses.

There are three interesting results from the phylogenetic analyses regarding patterns of ecological and morphological diversity among *Lepomis* species. First, two sister species pairs (*L. marginatus* + *L. megalotis* and *L. miniatus* + *L. punctatus*) in both MP and BML inferred trees were expected based on previous systematic analyses of external morphological variation and allozyme allele frequency data (Avise and Smith, 1977; Bailey, 1938; Warren, 1992). Relative to other sister species pairs in *Lepomis*; these are the most similar with regard to morphological and ecological divergence. Considering this similarity it is interesting to note that these species pairs exhibit limited, or no geographic range overlap (Lee et al., 1980; Warren, 1992).

Second, two sister species pairs were proposed from pre-cladistic analysis of morphology, *L. cyanellus* + *L. symmetricus*, and *L. macrochirus* + *L. humilis* (Bailey, 1938; Branson and Moore, 1962). These species pairs include both the largest and smallest species in *Lepomis*. For instance, *L. macrochirus* is the largest species and *L. humilis* is the second smallest, and *L. cyanellus* is the second largest species and *L. symmetricus* is the smallest (Page and Burr, 1991). Both of these sister species pairs were found with very strong support in the MP and BML analyses (Figs. 1 and 3). It appears that there is the potential for substantial body size disparity to have been an important factor in ecological diversification and speciation within *Lepomis*. Also, unlike the species pairs *L. marginatus* + *L. megalotis* and *L. miniatus* + *L. punctatus*, there is near complete overlap in the geographic ranges of species in each of these two sister species pairs that differ substantially in body size (Lee et al., 1980).

Third, *L. microlophus* and *L. gibbosus* are the only *Lepomis* species with specialized diets, feeding primarily on snails. Both species exhibit behavioral and morphological specializations that function in crushing snail shells. The specializations include expanded tooth areas on the upper and lower pharyngeal jaws, hypertrophied pharyngeal jaw muscles, and a specialized muscle motor pattern (Lauder, 1983, 1986; Wainwright and Lauder, 1992). Previous morphology-based phylogenetic hypotheses proposed *L. microlophus* and *L. gibbosus* as sister species, indicating that these specializations for molluscivory have had a single evolutionary origin (Bailey, 1938; Mabee, 1993). However, both MP and BML analyses do not recover this relationship, but instead result in a set of strongly supported nodes with *L. gibbosus* as the sister species to the clade (*L. microlophus* (*L. punctatus* and *L. miniatus*)) (Figs. 1 and 3). *L. miniatus* and *L. punctatus* are not molluscivores and do not exhibit the specialized behavior or morphology observed in *L. gibbosus* and *L. microlophus* (Lauder, 1983, 1986). The result of this phylogenetic analysis indicates that ecological diversification between generalist and specialist strategies may have a higher frequency of change in the course of the evolutionary history of *Lepomis* than previously hypothesized.

The SH test was able to discriminate between the BML tree and many of the 27 alternative phylogenetic hypotheses of centrarchid relationships (Table 5).

The failure to reject almost half of these alternative hypotheses demonstrates a degree of congruence among several previous phylogenetic hypotheses; however, some recent phylogenetic hypotheses that have been used in comparative studies were rejected in the SH test (Mabee, 1993; Wainwright and Lauder, 1992). The use of backbone constraints permitted a simultaneous comparison of all hypotheses despite the incomplete taxon sampling that characterizes the majority of these previous studies. This approach prevented inflation of statistical significance of the SH test since all probable hypotheses were included in the analysis (Goldman and Whelan, 2000). Several of the hypotheses regarding relationships among centrarchid genera may have been rejected in the SH test because of the proposal that *L. gulosus* is not most closely related to other *Lepomis* species, and represents a monotypic genus *Chaenobryttus* (Branson and Moore, 1962; Mabee, 1993; Schlaikjer, 1937; Wainwright and Lauder, 1992). Also, several of the genus-level hypotheses may have been rejected in the SH test because they propose that *Enneacanthus* is nested within *Lepomis* (Mabee, 1993; Schlaikjer, 1937; Wainwright and Lauder, 1992), or has a close phylogenetic affinity with *Lepomis* and *Micropterus* (Bailey, 1938; Branson and Moore, 1962; Smith and Bailey, 1961). Mok (1981) proposed two very different hypotheses of centrarchid relationships, one using kidney morphology and the other using number of anal spines and olfactory sac morphology. Both were rejected in the SH test (Table 5); however, the second of these two hypotheses differed from the Bayesian ML tree by placing *A. pomotis* in a clade also containing *Amblopliles*, *Archoplites*, *Centrarchus*, and *Pomoxis* (Mok, 1981, Fig. 5). Two genus-level hypotheses of centrarchid phylogenetic relationships were based on analyses of allozyme allelic variation. The tree presented in Parker et al. (1985) placed *L. macrochirus* as the sister taxon of *Micropterus*, and was rejected in the SH test (Table 5). The other allozyme-inferred phylogenetic hypothesis is similar to the BML tree, except *Pomoxis* is the sister taxon of *Lepomis* + *Micropterus* (Avise et al., 1977). This tree was not rejected in the SH test (Table 5). Three additional genus-level hypotheses were not rejected in the SH test. Two were similar in general tree topology, but the likelihood for incongruence with the BML tree is small since both efforts had limited taxon sampling (Lauder, 1986; Roe et al., 2002). The hypothesis presented in Chang (1988) could not be rejected despite the fact it depicts *Micropterus* as the sister taxon to all other centrarchids, a result that appears quite different from trees resulting from both MP and BML analyses (Figs. 1 and 3). This result indicates that the strong bootstrap and Bayesian posterior probability support for this node in the MP and BML trees rely on a relatively low number of characters, a proposition that is supported when bootstrap support for this node is compared between the mitochondrial ND2 and the two nuclear genes (Fig. 2).

One feature of mtDNA that makes it an attractive source of characters for phylogenetic studies among closely related species is a much higher rate of nucleotide substitution relative to nuclear encoded genes (Brown et al., 1979). This results in more polymorphic DNA sites, helping to produce well-resolved phylogenetic hypotheses. The expectation of a higher rate of mtDNA nucleotide substitution rate was confirmed when observed DNA polymorphisms in the mitochondrial encoded ND2 gene were compared to either of the two sampled nuclear genes (Table 2). In addition, nuclear-encoded introns are expected to exhibit a greater nucleotide substitution rate as compared to 2-fold degenerate and nondegenerate sites in nuclear-encoded protein coding genes (Li, 1997). Our data are consistent with this expectation since the S7 ribosomal protein intron exhibited a greater percentage of polymorphic sites than the protein coding *Tmo-4C4* (Table 2). With regard to phylogenetic resolution, the two nuclear genes provided less resolution than the mitochondrial ND2 (Fig. 2, Table 3). The utility of using mtDNA genes for phylogeny inference among closely related species is demonstrated by the observation that the degree of phylogenetic resolution provided by any one of the three genes was correlated with the rate of DNA substitution of the gene (Tables 2 and 3). The mitochondrial ND2 resolves all possible nodes, followed in percent of nodes resolved by the S7 intron and then *Tmo-4C4*.

The major benefit of collecting data from both mitochondrial and nuclear genes for molecular phylogenetic investigations is to test for congruence of phylogenetic inferences from multiple unlinked loci. Incongruence between mitochondrial and nuclear genes may reflect conflict between the gene trees that are used in the phylogenetic analyses to reconstruct species relationships. In fact incongruence among unlinked nuclear encoded loci is expected. The number of generations required for alleles to become fixed in a population is inversely related to the effective population size (Pamilo and Nei, 1988). Since mtDNA genes are maternally inherited in most animals, the effective population size is one quarter of that for any nuclear locus. Therefore, ancestral mtDNA polymorphisms will become fixed faster than nuclear encoded alleles, and on average mtDNA gene trees will be more likely to reflect the true species relationships than any given individual nuclear encoded gene (Moore, 1995). Despite a higher probability of recovering the species tree, mtDNA does experience phenomena such as ancestral polymorphism, introgression, and horizontal transfer (Hudson and Turelli, 2003). However, demonstration of congruence between mitochondrial and nuclear gene phylogenies reduces the likelihood that gene trees do not reflect species relationships.

The trees resulting from MP analysis of each individual gene indicate a high degree of congruence between the mitochondrial and nuclear genes (Fig. 2). However, a significant ILD test indicated appreciable phylogenetic incongruence between these two data partitions. Although the removal of *A. pomotis* from the analysis did not reduce the inferred phylogenetic incongruence among the mitochondrial and nuclear genes, including *A. pomotis* and removing *M. treculi* did result in a non-significant ILD test. This result indicates that the different placement of *M. treculi* in mtDNA and nuclear gene trees is the main source of incongruence detected in the ILD test.

Despite the relatively frequent occurrence of natural interspecific *Lepomis* hybrids and experimental demonstration of fertility among *Lepomis* hybrids (Childers, 1967; Hubbs, 1955; Hubbs and Hubbs, 1932), no appreciable incongruence between the mitochondrial and nuclear genes was observed among these species (Fig. 2). If introgression has been widespread in *Lepomis* subsequent to speciation, incongruence between mitochondrial and nuclear gene trees would be expected, as observed for *M. treculi*. The only apparent topological incongruence between the MP analyses of mitochondrial and nuclear genes among apical *Lepomis* species relationships involves *L. microlophus*, *L. miniatus*, and *L. punctatus*. Both the mitochondrial ND2 and nuclear *Tmo-4C4* identify *L. punctatus* and *L. miniatus* as sister species. This is the expected relationship between these two species since *L. miniatus* had previously been considered a subspecies of *L. punctatus* (Warren, 1992). However, MP analysis of the nuclear encoded S7 ribosomal protein intron results in *L. punctatus* and *L. microlophus* as sister species in a clade that also contains *L. miniatus*. The failure of *L. punctatus* and *L. miniatus* to form a clade in the S7 ribosomal protein intron gene tree may be the result of ancestral polymorphism that has not coalesced to reciprocal monophyly.

The continued use of the Centrarchidae as a model system for ecological and evolutionary studies is greatly enhanced by the availability of a phylogenetic hypothesis for all species in the group. Future systematic studies could ask if the collection of additional mitochondrial and nuclear genes provides greater phylogenetic resolution and node support than the trees resulting from the three-gene dataset. In addition to systematic questions addressed in this study, the evolution of habitat and resource utilization (Werner and Hall, 1979), functional morphological diversification (Wainwright and Lauder, 1992), estimation of divergence times using fossil-calibrated DNA substitution rates (Near et al., 2003), and the evolution of reproductive isolation (Childers, 1967) will rely on well-resolved phylogenetic hypotheses. We hope that the dataset presented in this study is expanded and used to continue the rich tradition of research using centrarchid fishes.

Acknowledgments

We thank G.M. Hogue, T.W. Kassler, J.B. Koppelman, R. Lawson, M.E. Raley, and W.C. Starnes for providing tissues. J.W. Armbruster, P.A. Ceas, M. Hardman, T.W. Kassler, J.H. Knouft, J.B. Koppelman, T.M. Near, L.M. Page, J.C. Porterfield, C. Priede, S.E. Russo, M.H. Sabaj, J.M. Serb, R. Steinberg, C.A. Taylor, and F.M. Veraldi provided assistance with collection of specimens. Collection permits were granted by State of Alabama Department of Conservation, the Florida Fish and Wildlife Commission, North Carolina Wildlife Resources Commission, and Tennessee Wildlife Resources Agency. M.K. Fujita, C.D. Hulsey, and G. Pauly provided laboratory assistance. W.C. Starnes provided comments on earlier versions of this manuscript. This research was funded with a National Science Foundation Grant IBN-0076436 awarded to P.C.W. T.J.N. was supported with a postdoctoral fellowship in the Center for Population Biology at the University of California, Davis. This work was carried out in the laboratory of H.B. Shaffer at the University of California, Davis.

Appendix A. Locality, museum voucher information, and GenBank Accession Nos. for specimens sequenced in this study

Voucher specimens (if available) are deposited in the Illinois Natural History Survey (INHS), North Carolina State Museum (NCSM), or the University of Tennessee Research Fish Collection (UT). Collection localities (drainage), museum catalogue number, TJN tissue catalogue number, and GenBank Accession Nos. (ND2, S7 ribosomal protein intron 1, and *Tmo-4C4*) are as follows: *Acantharchus pomotis*, Lake Nummy Belleplain State Forest, Cape May county, New Jersey, UT 90.3357, TJN 491, (AY517726, AY517757, and AY517807); *Ambloplites ariommus*, Conasauga River at St. Rt. 74 bridge, Bradley county, Tennessee, INHS 41656, TJN 455, (AY517727, AY517758, and AY517808); *Ambloplites cavifrons*, Tar River, Franklin county, North Carolina, NCSM 30358, TJN 2099, (AY517728, AY517759, and AY517809); *Ambloplites constellatus*, North Fork White River, Douglas county, Missouri, no voucher, TJN 2310, (AY517729, AY517760, and AY517810); *Ambloplites rupestris*, Lake Andrusia, Beltrami county, Minnesota, UT 90.3358, TJN 284, (AY225723, AY517761, and AY517811); *Acantharchus interruptus*, Hume Lake, Fresno county, California, INHS 59069, TJN 1077, (AY225725, AY517762, and AY517812); *Centrarchus macropterus*, Mud Creek, Hardin county, Tennessee, INHS 38384, TJN 384, (AY225726, AY517763, and AY517813); *Enneacanthus chaetodon*, Lake Mummy Belleplain State

Park, Cape May county, New Jersey, INHS 41251, TJN 428, (AY517730, AY517764, and AY517814); *Enneacanthus gloriosus*, Wacissa River, Jefferson county, Florida, no voucher, TJN 1990, (AY517731, AY517765, and AY517815); *Enneacanthus obesus*, West Branch Sopchoppy River, Wakulla county, Florida, INHS 38726, TJN 379, (AY225724, AY517766, and AY517816); *Lepomis auritus*, Conasauga River, Bradley county, Tennessee, INHS 41665, TJN 454, (AY517732, AY517767, and AY517817); *Lepomis cyanellus*, Saline Branch, Champaign county, Illinois, UT 90.3359, TJN 378, (AY517733, AY517768, and AY517818); Embarras River, Champaign county, Illinois, UT 90.3360, TJN 500, (AY517734, AY517769, and AY517819); *Lepomis gibbosus*, Lake Andrusia, Beltrami county, Minnesota, INHS 39505, TJN 258, (AY517735, AY517770, and AY517820); *Lepomis gulosus*, Pine Hills Swamp, Union county, Illinois, INHS 42744, TJN 484, (AY517736, AY517771, and AY517821); Horseshoe Lake, Alexander county, Illinois, UT 90.3361, TJN 1509, (AY517737, AY517772, and AY517822); *Lepomis humilis*, Mississippi River, Clinton county Iowa, INHS 40071, TJN 319, (AY517738, AY517773, and AY517823); Horseshoe Lake, Alexander county, Illinois, INHS 42594, TJN 501, (AY517739, AY517774, and AY517824); *Lepomis macrochirus*, Blue River, Crawford county, Indiana, INHS 41396, TJN 424, (AY225728, AY517775, and AY517825); Sabinal River, Bandera county, Texas, no voucher, TJN 2086, (AY517740, AY517776, and AY517826); *Lepomis marginatus*, Panther Creek, Henry county, Tennessee, INHS 88971, TJN 1939, (AY517741, AY517777, and AY517827); *Lepomis megalotis*, Saline Branch, Champaign county, Illinois, UT 90.3362, TJN 380, (AY517742, AY517778, and AY517828); Horseshoe Lake, Alexander county, Illinois, INHS 52749, TJN 1502, (AY517743, AY517779, and AY517829); *Lepomis microlophus*, Wacissa River, Jefferson county, Florida, UT 90.3363, TJN 1996, (AY517744, AY517780, and AY517830); *Lepomis miniatus*, Conasauga River, Bradley county, Tennessee, UT 90.3364, TJN 444, (AY225728, AY517781, and AY517831); San Marcos River, Hays county, Texas, no voucher, TJN 2077, (AY517745, AY517782, and AY517832); *Lepomis punctatus*, Wacissa River, Jefferson county, Florida, UT 90.3365, TJN 1991, (AY517746, AY517783, and AY517833); *Lepomis symmetricus*, Pine Hills Swamp, Union county, Illinois, INHS 42745, TJN 503, (AY517747, AY517784, and AY517834); *Micropterus cataractae*, Flint River, Crisp county, Georgia, no voucher, TJN McatD, (AY225776, AY517785, and AY517835); *Micropterus coosae*, Conasauga River, Polk county, Tennessee, INHS 41809, TJN 440, (AY225728, AY517786, and AY517836); *Micropterus dolomieu*, Fox River, Kenosha county, Wisconsin, no voucher, TJN MdolD, (AY225747, AY517787, and AY517837); Big Sugar Creek, MacDonald county, Missouri; no voucher, TJN MdolJ, (AY225751, AY517788, and AY517838); *Micropterus floridanus*, Lake Eustis, Lake county, Florida, no voucher, TJN MflaG, (AY225729, AY517789, and AY517839); Lake Eustis, Lake county, Florida, no voucher, TJN MflaH, (AY225730, AY517790, and AY517840); *Micropterus notius*, Wacissa River, Jefferson county, Florida, no voucher, TJN MnotA, (AY225764, AY517791, and AY517841); Santa Fe River, Alachua county, Florida, no voucher, TJN MnotD, (AY225766, AY517792, and AY517842); *Micropterus punctulatus*, Chase Lake, Chase county, Kansas, no voucher, TJN MpunA, (AY225755, AY517793, and AY517843); Lake Whitney, Hill county, Texas, no voucher, TJN MpunK, (AY225761, AY517794, and AY517844); *Micropterus salmoides*, Lipset Lake, Burnett county, Wisconsin, no voucher, TJN MsalA, (AY225735, AY517795, and AY517845); *Micropterus treculi*, Lake Buchanan, Burnet county, Texas, no voucher, TJN MtreE, (AY225762, AY517796, and AY517846); Lake Buchanan, Burnet county, Texas, no voucher, MtreG (AY225763, AY517797, and AY517847); *Pomoxis annularis*, North Fork White River, Douglas county, Missouri, no voucher, TJN PannB, (AY517748, AY517798, and AY517848); North Fork White River, Douglas county, Missouri, no voucher, TJN PannC, (AY517749, AY517799, and AY517849); *Pomoxis nigromaculatus*, Mud Creek, Hardin county, Tennessee, INHS 38388, TJN 499, (AY517750, AY517800, and AY517850); Horseshoe Lake, Alexander county, Illinois, UT 90.3366, TJN PngrB, (AY517751, AY517801, and AY517851); Horseshoe Lake, Alexander county, Illinois, no voucher, TJN PngrC, (AY517752, AY517802, and AY517852). **Outgroups**. *Dissostichus mawsoni*, McMurdo Sound Antarctica, no voucher, TJN 1226, (AY256561, AY517753, and AY517803); *Notothenia rossii*, Palmer Peninsula Antarctica, no voucher, TJN 1803, (AY256566, AY517754, and AY517804); *Percina maculata*, Dismal Creek, Fayette county, Illinois, UT 91.6497, TJN 75, (AY517725, AY517756, and AY517806); *Perca flavescens*, Lake Andrusia, Beltrami county, Minnesota, INHS 39508, TJN 261, (AY225721, AY517755, and AY517805).

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