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SPECIATION IN NORTH AMERICAN BLACK BASSES, *MICROPTERUS* (ACTINOPTERYGII: CENTRARCHIDAE)

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Abstract.—The Pleistocene Epoch has been frequently cited as a period of intense speciation for a significant portion of temperate continental biotas. To critically assess the role of Pleistocene glaciations on the evolution of the freshwater fish clade *Micropterus*, we use a phylogenetic analysis of complete gene sequences from two mitochondrial genes (cytochrome *b* and ND2), and a fossil calibration of the molecular clock to estimate ages of speciation events and rates of diversification. The absence of substantial morphological and ecological divergence together with endemism of five of the eight species in North American tributaries of the Gulf of Mexico may be interpreted as the result of a recent Pleistocene origin for these species. Speciation dates in *Micropterus* range from 1.01 ± 0.32 to 11.17 ± 1.02 million years ago. Only one speciation event is dated to the Pleistocene, and rates of diversification are not significantly variable in *Micropterus*. The premise that the Pleistocene was an exceptional period of speciation in *Micropterus* is not supported. Instead, a Gulf Coast allopatric speciation model is proposed, and predicts periods of dynamic speciation driven by sea level fluctuations in the Late Miocene and Pliocene. The Pleistocene, however, was a period of significant intraspecific mitochondrial lineage diversification. The application of the Gulf Coast allopatric speciation model to the remaining aquatic fauna of the Gulf of Mexico coast in North America will rely on robust phylogenetic hypotheses and accurate age estimations of speciation events.

Key words.—Centrarchidae, *Micropterus*, mitochondrial DNA, molecular clock, phylogeny, speciation.

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The cycles of glaciation and climatic oscillation during the Pleistocene epoch have been proposed as causative agents in the process of allopatric speciation for a significant portion of extant species of North American freshwater fishes (Miller 1965). However, the timing of speciation in these fishes and its correlation with the Pleistocene is unclear (Strange and Burr 1997; Near et al. 2001). The Pleistocene speciation model proposes that the geographic ranges of widespread species became fragmented and isolated in nonglaciated areas and available glacial refugia. Reproductive isolation among fragmented populations resulted from processes of allopatric speciation (Bermingham et al. 1992; Zink and Slowinski 1995). Current geographic ranges of widespread extant species with Pleistocene origins may reflect postspeciational range expansion (Riddle 1996; Bernatchez and Wilson 1998). The expectations from this model are: (1) most sister species pairs will share a common ancestor less than 2.5 million years ago (mya; Klicka and Zink 1997; Moritz et al. 2000), and (2) the net rate of speciation in a clade will increase and durations between speciation events will decrease during the Pleistocene (Zink and Slowinski 1995).

Many studies using phylogenetic methods have challenged the Pleistocene speciation model from several lines of evidence, including the synthesis of paleogeographical data to extant species relationships and distributions (Wiley and Mayden 1985, Cracraft and Prum 1988; Mayden 1988), comparisons of molecular clock estimated times of divergence between sister species (Klicka and Zink 1997; Strange and Burr 1997; Avise et al. 1998; Moritz et al. 2000), and assessment of the rate of diversification within several lineages (Zink and Slowinski 1995). Essentially, when divergence times among sister species of birds (Klicka and Zink 1997), reptiles, amphibians, and freshwater fishes (Avise et al. 1998) were estimated, most sister species pairs exhibited divergences that predated the Pleistocene. With regard to rates of diversification, lineage-through-time plots of several North American passerine bird lineages exhibited decelerated rates of speciation during the Pleistocene, the opposite pattern expected from the Pleistocene speciation model (Zink and Slowinski 1995).

Several potential problems exist in the methodologies used in assessing the effects of the Pleistocene on the history of speciation in continental lineages. A major issue involves the actual calculation of divergence times among sister species. All published studies investigating Pleistocene effects on speciation have used pairwise sequence differences to estimate divergence times (Klicka and Zink 1997; Strange and Burr 1997; Avise et al. 1998; Moritz et al. 2000). The problem of rate heterogeneity of nucleotide substitution among lineages is all but ignored in most of these studies (Arbogast and Slowinski 1998). For several reasons, including problems of combining multiple statistical comparisons, the use of tree-based methods instead of pairwise approaches is preferred in the assessment of rate heterogeneity among lineages and estimation of nucleotide substitution rates (Sanderson 1998). As an extension, there are robust methods to estimate divergence times in the absence of rate homogeneity; however, they rely on a tree-based strategy (Sanderson 1997, 1998). In addition, most studies (Klicka and Zink 1997, 1999) have relied on universal rates of sequence evolution to calibrate the molecular clock (e.g., 2% sequence divergence per mil-

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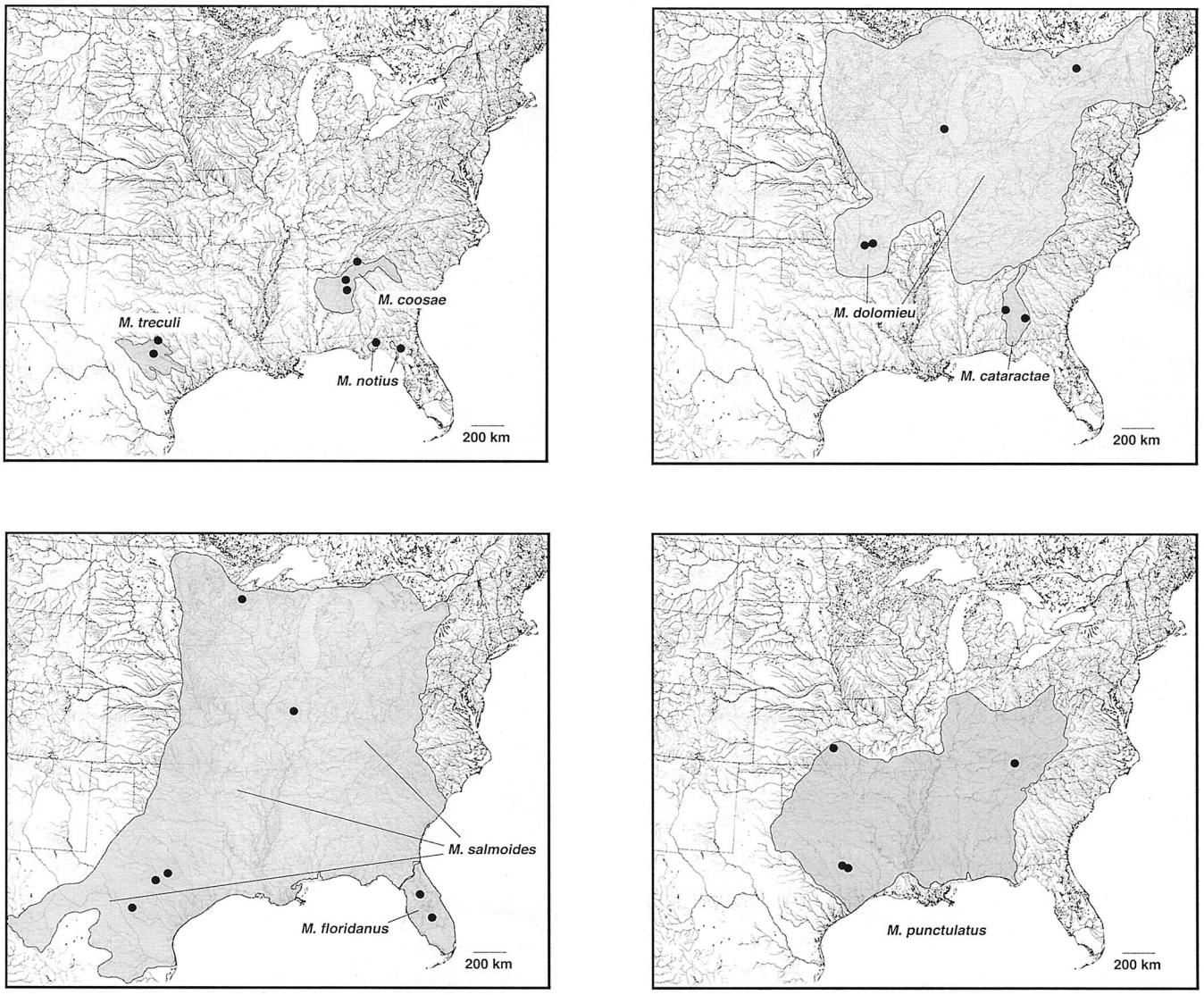

FIG. 1. Geographic distribution of *Micropterus* species. Approximate sampling localities are indicated with black dots.

lion years). To resolve the pervasive lineage-specific rate heterogeneity of nucleotide substitution in mitochondrial genes, a taxon-specific calibration of the molecular clock based on robust fossil or biogeographic evidence is preferred.

Among temperate regions of the world, continental North America contains the most diverse freshwater fish fauna (Briggs 1986). The role of the Pleistocene on the origin of this diverse fauna has not been investigated using fossil-based calibrations of the molecular clock to estimate divergence times of sister species. An ecologically important component of this diversity is the genus *Micropterus*, the black basses. *Micropterus* is composed of eight species in the freshwater fish family Centrarchidae and is endemic to North America east of the Rocky Mountains (Fig. 1). In their lacustrine and riverine habitats, all species of *Micropterus* are carnivorous top-level predators (Etnier and Starnes 1993). The aggressive nature and relatively large body size of black basses have contributed to the group's status as one of the world's most popular freshwater sport fishes. Historically, the diversity of *Micropterus* was underestimated; only two species were recognized for more than 100 years (Hubbs and Bailey 1940). The remaining six species have been described in the last 75 years, and the most recently described species (*M. cataractae*) was not formally recognized until the close of the twentieth century (Williams and Burgess 1999). Relationships among *Micropterus* have been proposed (Ramsey 1975), but until recently no hypotheses have been developed using phylogenetic methods or characters amenable to cladistic analysis (Kassler et al. 2002).

The relatively slight morphological (Mabee 1993) and ecological divergence among *Micropterus* species (Miller 1975), and the endemic distribution of five species in tributaries of the Gulf of Mexico that are south of the glacial front (Fig. 1), might be interpreted as evidence for a relatively recent Pleistocene origin of the species in this clade. Absence of age estimates between sister species prevents rejection of alternative hypotheses with regard to the age of speciation events relative to the Pleistocene. Although sea level fluc-

TABLE 1. Collection localities of specimens sequenced. Localities for *Micropterus* specimens are plotted on Figure 1. Number of specimens examined and museum catalog numbers for voucher specimens (if available) are given in parentheses after locality. INHS, Illinois Natural History Survey Fish Collection.

Species	Locality
Micropterus cataractae	Little Uchee Creek, Russell County, Alabama (2)
	Flint River, Crisp County, Georgia (3)
M. coosae	Terrapin Creek, Cherokee County, Alabama (3)
	Conasauga River, Polk County, Tennessee (1, INHS 41809)
	Tallapoosa River, Chambers County, Alabama (2)
M. dolomieu	Mississippi River, Frontenac County, Ontario, Canada (4)
	Fox River, Kenosha County, Wisconsin (2)
	Big Sugar Creek, MacDonald County, Missouri (2)
	Shoal Creek, Barry County, Missouri (2)
M. floridanus	Lake Eustis, Lake County, Florida (3)
	Lake Istokpoga, Highlands County, Florida (3)
M. notius	Wacissa River, Jefferson County, Florida (2)
	Santa Fe River, Alachua County, Florida (2)
M. punctulatus	Chase Lake, Chase County, Kansas (3)
	Norris Lake, Union County, Tennessee (2)
	Cedron Creek, Bosque County, Texas (1)
	Lake Whitney, Hill County, Texas (1)
M. salmoides	Lipset Lake, Burnett County, Wisconsin (2)
	Lake Shelbyville, Moultrie County, Illinois (2)
	Nueces River, Uvalde County, Texas (4)
	Pedrenales River, Gillespie County, Texas (1)
	Guadalupe River, Kerr County, Texas (1)
M. treculi	Lake Buchanan, Burnet County, Texas (2)
Lepomis macrochirus	Blue River, Crawford County, Indiana (1, INHS 41396)
L. miniatus	Conasauga River, Bradley County, Tennessee (1)
Ambloplites rupestris	Lake Andrusia, Beltrami County, Minnesota (1)
Archoplites interruptus	Hume Lake, Fresno County, California (1, INHS 59069)
Enneacanthus obesus	West Branch Sopchoppy River, Wakulla County, Florida (1, INHS 38762)
Centrarchus macropterus	Mud Creek, Hardin County, Tennessee (1, INHS 38384)
Outgroup Species	
Ophiodon elongatus	Pacific Ocean, San Mateo County, California (1, INHS 45400)
Aplodinotus grunniens	Mississippi River, Jo Daviess County, Illinois (1, INHS 43068)
Perca flavescens	Lake Andrusia, Beltrami County, Minnesota (1, INHS 39058)
Percina roanoka	Blackwater River, Franklin County, Virginia (1, INHS 64395)

tuations may have acted as the mechanism of vicariance, isolating wide-spread ancestral species of *Micropterus* among individual tributaries of the Gulf of Mexico, sea level fluctuations in the Gulf of Mexico have occurred repeatedly through the Oligocene, Miocene, Pliocene, and Pleistocene (Riggs 1984; Haq et al. 1987). Without estimates of divergence times between sister species, it is impossible to discriminate which particular periods of sea level fluctuation might have been a factor in the diversification of *Micropterus*.

In an attempt to test predictions of the Pleistocene speciation model in relation to the diversification of *Micropterus*, we calibrated the mtDNA molecular clock in *Micropterus* using the centrarchid fossil record. Estimated divergence times and calculated diversification rates were compared to predictions of the Pleistocene speciation model. We used these results to construct a Gulf Coast allopatric speciation model that provides a hypothesis explaining the distribution of other clades of North American freshwater fishes inhabiting tributaries of the Gulf of Mexico.

MATERIALS AND METHODS

Collection of Specimens and DNA Sequences

Specimens of all species of *Micropterus* and six other centrarchid species were collected from native populations (Fig. 1, Table 1) using several techniques (angling, seine net, and electrofishing). Based on analyses of morphology, mtDNA, and allozyme variation, we followed the recommendation of Kassler et al. (2002), and recognized the subspecies *M. salmoides floridanus* as a species, *M. floridanus*. Nucleic acids were isolated using standard phenol-chloroform extraction and ethanol precipitation protocols. Polymerase chain reaction (PCR) was used to amplify complete gene regions of cytochrome *b* (cyt*b*) and ND2 using previously published primer sequences (Kocher et al. 1995; Song et al. 1998). Successful PCR amplifications were purified of excess nucleotides and primers using the Qiagen (Valencia, CA) PCR clean-up kit. Purified PCR products were used as template for Big Dye terminator (Applied Biosystems, Foster City, CA) cycle sequencing reactions. In addition to the two PCR primers, two internal primers were used to sequence both strands for both cyt*b* (basscytbf1 CAC CCC TAC TTC TCC TAC AAA GA; basscytbr1 AAG GCR AAG AAG CGG GTG AGG G) and ND2 (bassnd2-f1 TRA ACC AAA CCC ARC TCC GRA AAA T; bassnd2-r1 ATT GTA AGA TGA GRA TTA TTC). Sequencing reactions were cleaned of excess nucleotides by centrifugation on Sephadex (Princeton Separations, Adelphia, NJ) columns. Sequences were read with an ABI 377 automated sequencer (Applied Biosystems) at the W. M. Keck Center for Comparative and Functional Gen-

omics at the University of Illinois, Urbana-Champaign, and 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 contigs were constructed using the program Sequencher 3.0 (Gene Codes, Ann Arbor, MI).

Phylogenetic Analyses

Alignments of complete cyt*b* and ND2 gene sequences were guided by inferred amino acid sequences. PAUP* (Swofford 2000) was used to tabulate pairwise values of polymorphic sites between individual sequences and estimate phylogenetic relationships using maximum likelihood (ML). Despite extensive evidence for monophyly of the Centrarchidae, different phylogenetic analyses of relationships of centrarchid species have not resulted in congruent hypotheses (Avise et al. 1977; Mabee 1993). The choice of appropriate outgroups for the Centrarchidae is difficult. Centrarchid fishes are in classified in the teleost order Perciformes. This diverse taxonomic group is the largest order of vertebrates and contains 9293 species classified into 148 families (Nelson 1994). Because no concerted efforts have been able to resolve the phylogenetic relationships in this diverse group, no sister taxon of the Centrarchidae has been clearly identified. Outgroup choice, at this stage, is rather arbitrary, with the criteria for this study being one representative species outside the Perciformes, but in the superorder Percomorpha, and inclusion of taxa from more than one other family of Perciformes. The final criterion is the ability of the existing PCR primers to successfully amplify the target genes in these taxa. Based on these criteria, four species were chosen for the outgroup pool, (1) *Ophiodon elongatus*, family Hexagrammidae, order Scorpaeniformes; (2) *Aplodinotus grunniens*, family Sciaenidae, order Perciformes; and two species in the family Percidae, order Perciformes, (3) *Perca flavescens* and (4) *Percina roanoka*.

Initially, gene regions (cyt*b* and ND2) were partitioned and analyzed individually and phylogenetic incongruence among gene regions was determined with the maximum parsimony (MP) incongruence-length difference method (Farris et al. 1994), as implemented by the partition-homogeneity test in PAUP* (Swofford 2000). The optimal model of nucleotide sequence evolution for each gene was determined using hierarchical likelihood-ratio (LR) tests as implemented in the computer program Modeltest 3.0 (Posada and Crandall 1998). The parameters of the optimal model for each gene were estimated using parsimony inferred tree topologies. Maximum-likelihood analyses were executed using optimal model parameters of sequence evolution separately for each gene. In addition, a model of sequence evolution that allowed three different rates of substitution, corresponding to the three-codon positions, was investigated with a combined cyt*b*-ND2 dataset. Likelihood scores from the site-specific model were compared with optimal gene-specific models using the Akaike information criterion (AIC), which allows comparisons of non-nested models of nucleotide evolution. Nucleotide frequencies, among-site rate variation, and proportion of invariable sites were estimated from combined analysis MP topologies. Maximum-likelihood tree searches were performed using heuristic searches with 10 random-sequence addition replicates and tree-bisection and reconnection branch-swapping. Support values of recovered nodes were provided by bootstrap pseudoreplicate analysis executed in PAUP* with heuristic tree searches (subtree-pruning and regrafting branch-swapping and 10 sequence addition replicates) and 100 pseudoreplicates.

Estimation of Rate Heterogeneity and Calibration of the Molecular Clock

Lineage ages were estimated using a ML methodology that relied on fossil calibration of nucleotide substitution rates. The earliest centrarchid fossils are from the Eocene in Montana (Cavender 1986), and fossils assignable to extant centrarchid genera extend from the Miocene to the late Pleistocene (Uyeno and Miller 1963; Smith 1981). To circumvent problems associated with using a single calibration point to calculate rates of nucleotide substitution and subsequent estimation of divergence times, minimal ages using the fossil record were assigned to two nodes on the centrarchid phylogeny. Assignment of fossils to appropriate nodes followed strategies and guidelines discussed in Doyle and Donoghue (1993) and Magallon and Sanderson (2001). The oldest *Micropterus* fossil is an articular from the Toledo Bend site on the Sabine River, Texas (Albright 1994). The site and its constituent fauna is dated in the middle Arikareean of the Early Miocene (Albright 1994), corresponding to approximately 23.0 mya. (Berggren et al. 1995; Prothero 1998). There are several fossil *Micropterus* specimens later in the Miocene, dating from 16.5 to 10.0 mya (Smith et al. 1975; Smith 1981). The age of the Toledo Bend *Micropterus* fossil (23.0 mya) was used as the minimal age estimate for *Micropterus*. It is important to point out that this fossil is used to estimate the minimal age of the stem group that includes the most recent common ancestor (MRCA) of *Micropterus* and its sister taxon (see Magallon and Sanderson 2001). We are not dating the crown group of *Micropterus* with this fossil because it is not assigned to a particular *Micropterus* species or to an internal node on the phylogeny. The second node calibrated with the fossil record is the MRCA of the two centrarchid genera *Archoplites* and *Ambloplites*. These two lineages are strongly supported as sister taxa in a phylogenetic dataset that includes three mitochondrial and five nuclear genes sampled from all 32 recognized centrarchid species (T. Near, D. Bolnick, and P. Wainwright, unpubl. data). The oldest fossil assignable to this node is the extinct *Archoplites clarki*, from the Miocene Clarkia Lake Beds in Idaho (Smith and Miller 1985). The Clarkia fossil bed is dated at 15.5 mya (Wing 1998), and was used as the minimal age estimate for the node representing the MRCA of *Archoplites* and *Ambloplites*. Prior to estimating rates of nucleotide substitution with this fossil based calibrations, heterogeneity of substitution rates was investigated using ML. A tree-wide LR test was used to directly compare rate-variable and rate-constant models of sequence evolution for all three datasets (cyt*b*, ND2, and combined). The LR test statistic was compared to a chi-square distribution with $s - 2$ degrees of freedom, where s equals the number of sequences in the analysis (Huelsenbeck and Crandall 1997).

After discovery of lineage rate constancy, lineage ages were estimated using the Langley-Fitch (LF) method (Langley and Fitch 1974) as implemented in the program r8s version 1.50 (Sanderson 1997). The LF method assumes a molecular clock and uses ML to reconstruct divergence times. A global substitution rate across all nodes was estimated from nodes dated with the fossil record and divergence times were estimated for all other nodes. The tree used for these analyses was a ML inferred topology using a GTR+SS+G model, with branch lengths estimated without enforcing a molecular clock. The sensitivity of divergence times estimated from mtDNA sequence data to particular fossil minimal age estimate calibration points was investigated by comparing age estimates calculated using each of the two fossil calibration points independently. Divergence time estimates were compared to determine whether the two different calibration points converged upon similar age estimates. Fossil estimates of node ages were implemented in r8s using the ''fixage'' command. In the analyses using both fossil calibrations, both nodes were fixed using fixage in r8s.

Error contribution from data sampling was estimated by using a nonparametric bootstrap procedure, as outlined in Baldwin and Sanderson (1998). One hundred bootstrap replicate datasets were generated using the seqboot program in Phylip (Felsenstein 1993), replicates were imported into PAUP*, and branch lengths were calculated on the ML tree using the optimal model of sequence evolution, and the distribution of divergence times at each node was assessed using r8s. The central 95% of the distribution of bootstrap age estimates provided the confidence interval for each node (Baldwin and Sanderson 1998; Sanderson 1998).

Estimating Rates of Diversification

Lineages-through-time plot and estimates of diversification rate were used to test the predictions of the Pleistocene speciation model for an increased rate of speciation and a decrease in the duration between speciation events. A lineages-through-time plot (Harvey et al. 1994) was constructed by plotting the log-transformed number of lineages against the age of the node (as estimated above). Given a birth-death model of diversification, if rates of speciation are uniform throughout the history of the clade, the lineages-through-time plot is expected to be linear. An increase in the rate of speciation or a constant rate of background extinction will cause an upturn in the plot, giving a steeper slope. Alternatively, a decrease in the rate of speciation or an increase in the rate of extinction will cause a leveling of the slope (Nee et al. 1994). Distinguishing between speciation and extinction using lineages-through-times plots is often difficult, if not impossible (Kubo and Iwasa 1995). However, we used the pattern of the lineages-through-time plot to qualitatively assess whether there is the appearance of an increased rate of speciation during the Pleistocene.

The rate of diversification for the entire *Micropterus* clade was estimated using a Yule birth diversification model that accounts for speciation, but not extinction. Based on previous studies, computer simulations recover high variance and bias in estimates of speciation and extinction for a birth-death model relative to estimates for the Yule model (Baldwin and Sanderson 1998). For purposes of calculating diversification rates, we assumed the Yule model and used the Kendall-Moran estimator (Baldwin and Sanderson 1998),

$$S = (N - 2)/B, \quad (1)$$

where N is the number of extant lineages and B is the summed times of all branches descended from the most recent common ancestor. Two estimates of the variance of S are based on different assumptions regarding the stochastic processes involved with the diversification rate (Baldwin and Sanderson 1998; Nee 2001). We calculated 95% confidence intervals for the Kendall-Moran estimator using the observed variance in nucleotide substitution rate from 100 bootstrap datasets.

To test the expectation of an increased rate of diversification in *Micropterus* during the Pleistocene, we used the methodologies described by Paradis (1997; 1998a,b). Essentially, divergence times within a clade are assessed with survival models. Using divergence times, a rate of diversification (δ) is estimated using a maximum-likelihood approach (Paradis 1997). For each node in the phylogeny, an instantaneous diversification rate $\delta(t)$, with components of instantaneous speciation $\sigma(t)$ and instantaneous extinction $\epsilon(t)$ is assumed. Another assumption is that speciation and extinction rates cannot be separately estimated. Using estimated divergence times, the presence of variation in the rate of diversification within *Micropterus* was tested. The computer program Diversi version 0.2 (E. Paradis, Univ. of Montpellier, France, http://www.isem.univ-montp2.fr/ppp/phylogenie/ParadisHom.php) allows the fitting of three models using ML. Model A assumes a constant rate of speciation throughout time, model B allows δ to vary through time, and model C assumes two different rates of diversification before and after a breakpoint in time (Paradis 1997). Model B assumes a gradual change in δ through time, and the critical parameter is β. If $\beta > 1$, then the δ is decreasing; if $\beta < 1$, δ is increasing through time in the clade (Paradis 1997). Diversi 0.2 provides likelihood and AIC for the fit of the divergence times to each of the three models. Because model A is nested in models B and C, they can be compared using a LR test. Models B and C are not nested, therefore AIC can be used to reject one model over the other. The model with the lowest AIC is the model selected as the one best describing the temporal variation of divergence times. Based on the expectation of the Pleistocene speciation model, we set the breakpoint for model C to 2.5 mya. If an increase in the rate of diversification during the Pleistocene is projected, rejection of model A in favor of model B or model C is expected. If any increase in the rate of diversification occurred after 2.5 mya, AIC should favor model C over model B.

RESULTS

A total of 50 individuals sampled from all eight species of *Micropterus* were sequenced. Sequences were deposited on Genbank (AY225661–AY225778, AF386597, AF386600). Table 2 summarizes nucleotide variation by codon position observed in both cyt*b* and ND2. Uncorrected genetic distances for both genes combined ranged from 3.75% to 11.25% among species comparisons. Intraspecific uncorrected genetic distances for both genes combined ranged from 0

TABLE 2. Summary of nucleotide variation by gene in *Micropterus.*

Gene	Total variable sites	First codon variable sites	Second codon variable sites	Third codon variable sites	Percent parsimony informative	Number of most-parsimonious trees
cyt*b*	415 (36.4%)	75 (19.7%)	30 (7.9%)	310 (81.6%)	63.6%	8
ND2	416 (39.7%)	93 (26.6%)	39 (11.2%)	284 (81.4%)	65.0%	36

to 3.0%. Proportion of variable sites and proportion of all variable sites that were informative for parsimony were very similar for both genes; however, ND2 exhibited a greater proportion of variation at first and second codon positions when compared to cyt*b* (Table 2). Maximum-parsimony analysis of ND2 recovered a much larger number of most-parsimonious trees than the analysis using cyt*b* (Table 2). Partition homogeneity test did not detect significant incongruence between the cyt*b* and ND2 datasets (sum of partitions = 1542, $P = 0.67$).

For the combined gene data a GTR+SS (site-specific) model was preferred using AIC. This model was used in a tree search that resulted in the recovery of a single tree (Fig. 2). Bootstrap pseudoreplicate scores were low for two of the internal nodes, but most intraspecific groupings of haplotypes were recovered in the vast majority of bootstrap pseudoreplications (Fig. 2). The exception was the set of haplotypes from *M. punctulatus*, which were recovered as monophyletic in less than half of the bootstrap pseudoreplications.

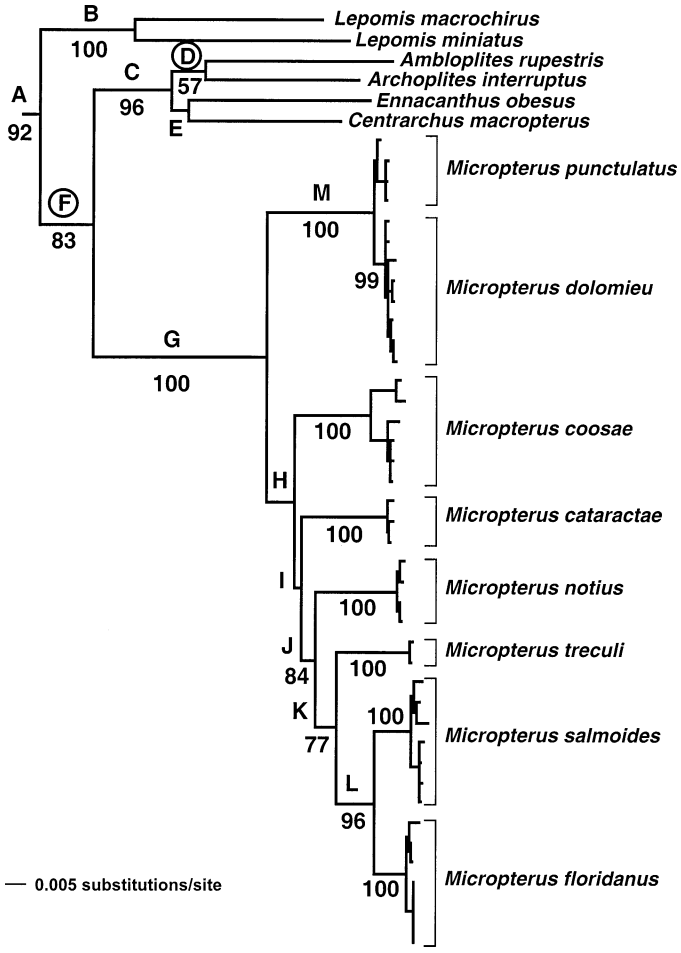

FIG. 2. Maximum likelihood phylogram showing relationships among *Micropterus* mitochondrial lineages (complete cyt*b* and ND2). The model used in the analysis was GTR+SS. The logL of the topology was −20,163.77. Bootstrap values (100 pseudoreplicates) are listed below nodes. Letters are used to label nodes for discussion of divergence time estimations. Circled letters mark nodes calibrated with fossil age estimations.

Within *Micropterus* there are two major monophyletic lineages, the first containing *M. dolomieu* and *M. punctulatus* and the second containing all other species. There are some rather surprising and well-supported relationships in the ML tree (Fig. 2). First, a sister species relationship between *M. punctulatus* and *M. dolomieu* has never been proposed. This relationship is interesting because these two species exhibit appreciable geographic overlap in their respective native ranges (MacCrimmon and Robbins 1975; Lee et al. 1980). Second, all species of *Micropterus* endemic to Gulf of Mexico tributaries are found in a monophyletic clade that includes *M. salmoides*, which extends north of the Gulf of Mexico drainages. Third, *M. treculi* had long been considered a subspecies of *M. punctulatus* (Hubbs 1942); however, it is recovered as the sister species to a clade containing *M. salmoides* and *M. floridanus*, and relative to species of *Micropterus* is only distantly related to *M. punctulatus*.

Estimation of Divergence Times and Rates of Diversification

A total of 10 individuals from *M. salmoides*, *M. cataractae*, *M. dolomieu*, and *M. punctulatus* were removed to eliminate all terminal zero branch lengths from the ML tree (Fig. 2). The ML tree search was rerun without these individuals and a tree topology identical to Figure 2 was recovered. We used this dataset to determine the simplest model of sequence evolution that provided an optimal fit to the data, using LR tests on ML-inferred trees. The log-likelihood scores for the rate-constant molecular clock model (GTR+SS+Gc; −20,195.50) and the rate-variable model where the molecular clock was relaxed (GTR+SS+G; −20,163.77) were used in a LR test, which failed to reject the molecular clock model ($\chi^2 = 63.46$, df = 49, $P > 0.05$). As a result, methods of estimating divergence times that assume rate homogeneity among lineages are appropriate for the *Micropterus* dataset.

Three sets of divergence times were estimated for all nodes in the phylogeny. The first two used either of the two fossil calibration points, and the third used both calibration points in combination to estimate the divergence times. All analyses converged on very similar divergence time estimates (Fig. 3). When the calibration point assigned to the stem group node of *Micropterus*, dated at 23.0 mya (Albright 1994), is used to estimate the age of the MRCA node of *Archoplites* and *Ambloplites*, the estimated age and confidence intervals are within the 15.5 mya estimate from the fossil record (14.20

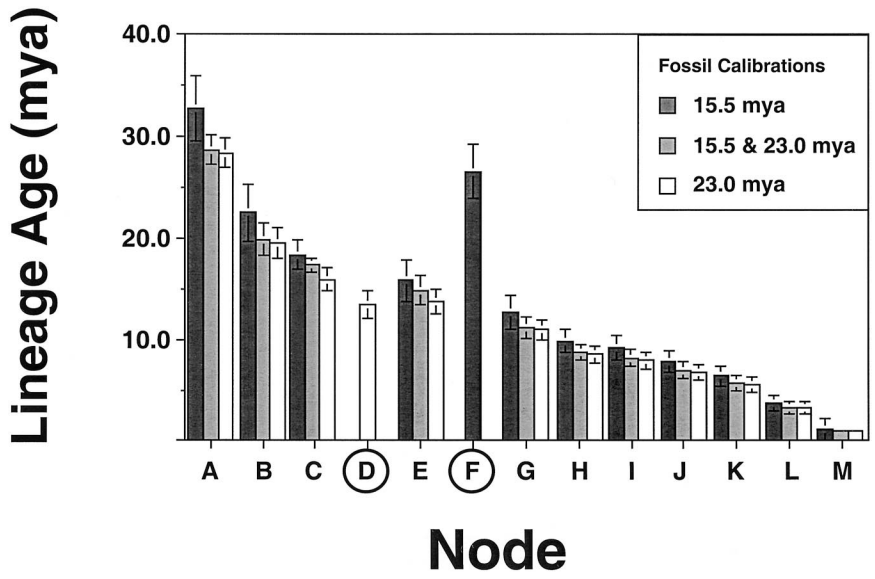

FIG. 3. Lineage ages estimated from three different analyses for a given node in the phylogeny (see Fig. 2). The analyses differ in using one of either calibration points at 15.5 and 23.0 million years ago (mya), and estimates using both calibration points. Error bars were estimated using bootstrap analyses. Circled letters mark nodes calibrated with fossil age estimations.

± 1.52 mya; Fig. 3). Using the 15.5 mya calibration point to estimate the age of the *Micropterus* stem group node provides an estimate of 26.58 ± 1.34 mya, slightly older than the fossil age estimate for this node. This implies that the estimated age of the fossil assigned to the *Micropterus* stem group node (23.0 mya) may represent an underestimation of the true age of the node. There is a small degree of uncertainty in the estimation of the age of this fossil, since the fossil bearing Toledo Bend formation was derived from several different stratigraphic horizons (Albright 1994). Despite this uncertainty, the age of this node as inferred from the other fossil calibration is an underestimate of only approximately 1.50 million years. With regard to dating nodes in the phylogeny with fossils, the assumption is that the fossils represent minimal age estimates. It is encouraging that the estimated age of the *Micropterus* stem group node is younger, not older, than the estimate using the *Archoplites* 15.5 mya fossil calibration.

At all interspecific nodes in the phylogeny, there is substantial overlap of estimated divergence times and associated error between all three estimations using different calibration points. Despite uncertainty in assigning minimal age estimates to nodes in the centrarchid phylogeny using the fossil record, two calibration points independently result in very similar age estimates (Fig. 3). Because there is no significant difference in the age estimates using different fossil-calibrated nodes, the age estimates using both calibration points are used for purposes of estimating rates of diversification in *Micropterus*.

Estimation of divergence times using the LF method with both fossil calibration points yields an age estimate of 11.17 ± 1.02 mya for the common ancestor of the extant species of *Micropterus* (node G; Fig. 4). Estimates of error due to data sampling provided confidence intervals for all age estimates. The rate of nucleotide substitution was estimated as 0.0058 substitutions per site per million years per lineage, giving a pair wise rate of 1.16% per million years. Divergence times and confidence intervals for all interspecific and basal intraspecific nodes are given in Table 3. Comparison of intraspecific and interspecific divergence times reveals little overlap between these two classes of age estimates (Fig. 5). The exception is *M. coosae*, which has comparatively ancient divergence between two recovered intraspecific lineages and relatively recent speciation events between *M. dolomieu* and *M. punctulatus* (Table 3 and Fig. 4).

The lineages-through-time plot, using the estimated divergence times is shown in Figure 6. Interestingly, there is not an upturn in the slope in the plot as the Pleistocene is approached (Fig. 6). The area of the lineages-through-time plot that implies the greatest rate of speciation includes four speciation events between approximately two to five million years since the origin of *Micropterus* (Fig. 6). Using the Kendall-Moran estimator from the point on the phylogeny that includes common ancestor to the extant species of *Micropterus* (Figs. 2 and 4), we estimate the diversification rate to be $S = 0.11 \pm 0.02$ species per million years.

A model of constant lineage diversification through time (model A) could not be rejected in *Micropterus* (Table 4) when compared to alternative models that involved variation in diversification rate through time (model B), or a different rate of diversification before and after the onset of the Pleistocene (model C; Paradis 1997; 1998a,b). Despite the lack of significance, it is interesting to note that the chi-square values between models A and B were much higher than between models A and C. This result indicates that model C is very similar to model A, and model A may be more different, but not significantly so, from model B.

DISCUSSION

The significance of the Pleistocene on the evolution of the extant diversity of *Micropterus* can be critically assessed with

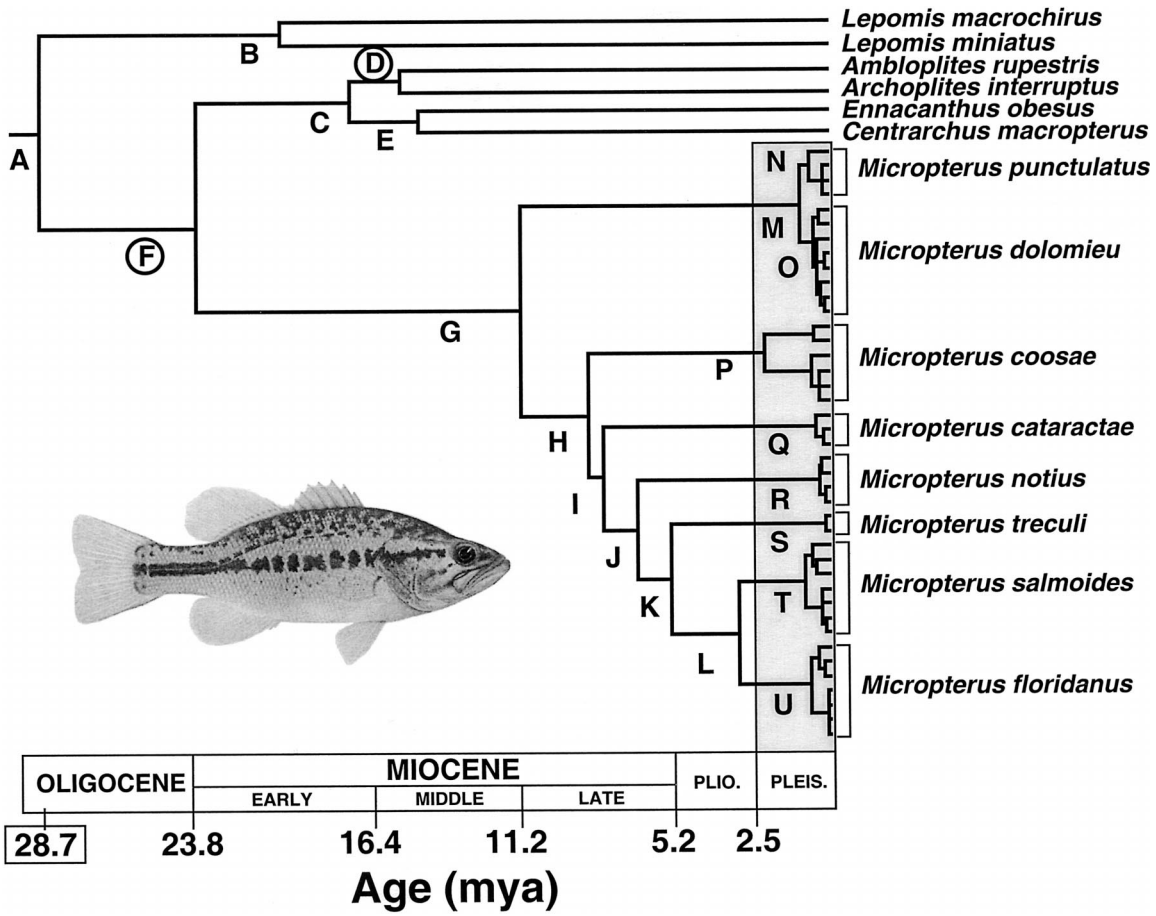

FIG. 4. Time-calibrated phylogram (chronogram) of the ML topology. Nodes calibrated with fossils are circled (node 4 = 15.5 mya and node 6 = 23.0 mya). The period corresponding to the duration of the Pleistocene is shaded. The chronogram is calibrated against the geological time scale (Berggren et al. 1995). Exact age estimates for labeled nodes are given in Table 3. Plio, Pliocene; Pleis, Pleistocene.

TABLE 3. Estimated ages and bootstrap estimates of standard error of nodes in the centrarchid chronogram (see Fig. 4). mya, millions of years ago. Ages of interspecific nodes are highlighted in bold.

Node	Age estimate (mya)	Bootstrap estimate of standard error (mya)
A	**28.69**	**±1.46**
B	**19.88**	**±1.54**
C	**17.34**	**±0.72**
D	**15.50**	**fixed**
E	**14.85**	**±1.47**
F	**23.00**	**fixed**
G	**11.17**	**±1.02**
H	**8.74**	**±0.78**
I	**8.17**	**±0.81**
J	**6.95**	**±0.80**
K	**5.69**	**±0.73**
L	**3.30**	**±0.60**
M	**1.01**	**±0.32**
N	0.66	±0.34
O	0.50	±0.19
P	2.30	±0.49
Q	0.46	±0.26
R	0.38	±0.20
S	0.11	±0.10
T	0.89	±0.32
U	0.66	±0.28

the inference of phylogenetic relationships (Fig. 2), estimation of divergence times (Figs. 4 and 5, Table 3), and estimation of diversification rates (Table 4, Fig. 6). One could argue that because only one speciation event in *Micropterus* is dated younger than 2.5 mya (Figs. 4 and 5), the Pleistocene was not a period of accelerated diversification for this clade. Supporting this conclusion is the appearance of a constant, or near-constant rate of diversification in *Micropterus* (Table 4; Fig. 6), both before and after the Pleistocene. Despite potential problems in methodologies used in assessing the significance of the Pleistocene on the diversification of continental biota, patterns from these previous studies are relevant to the results from our analyses of diversification in *Micropterus*. The majority of speciation events in *Micropterus* predate the Pleistocene (Fig. 4), and rates of diversification appear uniform throughout the history of the clade (Table 4).

Because the Pleistocene appears not to have been an exceptional time of diversification, what information does the estimation of divergence times provide for the illumination of processes of speciation in *Micropterus*? *Micropterus salmoides* has a large native geographic range that overlaps with six of the seven other *Micropterus* species (Fig. 1). However,

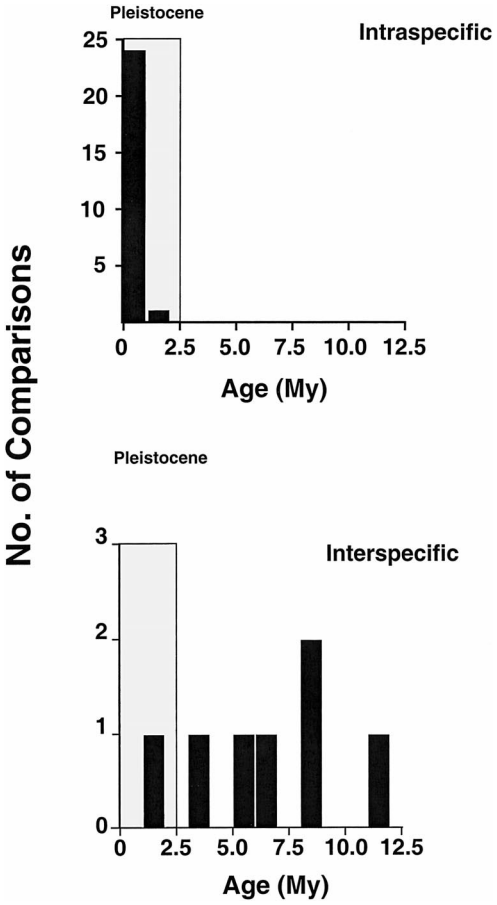

FIG. 5. Distribution of age estimates for intraspecific and interspecific clades in *Micropterus*. The period corresponding to the duration of the Pleistocene is shaded. My, million years.

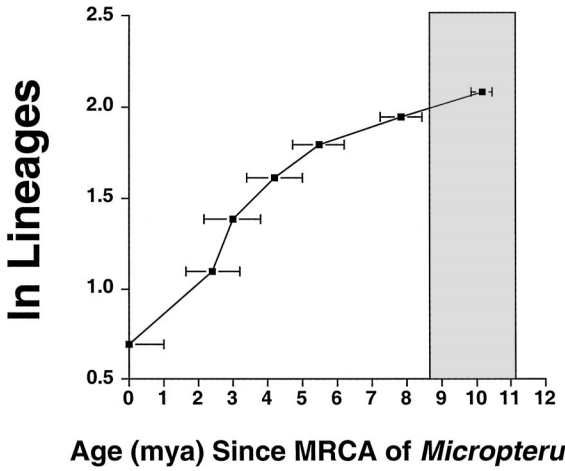

FIG. 6. Lineages-through-time plot of *Micropterus*. Estimated errors on divergence times are shown. The period corresponding to the duration of the Pleistocene is shaded.

it is allopatric with its sister species *M. floridanus*. Within *Micropterus* there is only one sister species pair (*M. dolomieu* and *M. punctulatus*) that exhibits any geographic overlap in distribution (Fig. 1), indicating that allopatric modes of speciation best explain the mechanisms of diversification in *Micropterus* (Lynch 1989; Barraclough and Vogler 2000). The estimation of divergence times provides a temporal perspective on these events, potentially allowing a more robust correlation of earth history events with hypothesized vicariant mechanisms proposed to facilitate allopatric speciation.

The dating of the diversification of the extant species of *Micropterus* at 11.17 ± 1.02 mya places the origin of this lineage in the Middle Miocene (11.0–16.5 mya; Berggren et al. 1995), this time was characterized by a dramatic rise in sea level between 80 and 100 m above the present day sea level (Riggs 1984; Haq et al. 1987). The effect of this rise was a marine incursion along the coast of the Gulf of Mexico, completely covering the areas now occupied by two and possibly four species of *Micropterus* (*M. notius*, *M. cataractae*, *M. floridanus*, and *M. treculi*; Fig. 1). Estimates of divergence times for all *Micropterus* species are placed after this marine incursion (Table 3). In the Late Miocene there was a sharp drop of 80–100 m below present sea levels, extending Gulf of Mexico tributaries further south and seaward. This southward extension of Gulf Coast rivers created connections between tributaries that were isolated during periods of higher sea level. The Late Miocene drop in sea level correlates with the estimated age of the first speciation event among the extant species of *Micropterus* (Table 3). The Pliocene (2.5–5.5 mya;, Berggren et al. 1995) was characterized by a 50–80 m rise above current sea level, but this incursion lasted for only a short time, approximately one million years (Riggs 1984). Sea levels dropped in the Late Pliocene, and during the Pleistocene there were at least three major fluctuations in sea level, none rising higher than 10–20 m above the current level (Riggs 1984; Swift et al. 1986).

Four of the seven speciation events in *Micropterus* are clustered between 8.74 ± 0.78 and 5.69 ± 0.73 mya (Fig. 4 and Table 3). All of these species are endemic to Gulf of Mexico tributaries that were directly impacted by sea level fluctuations. The period of diversification in this segment of *Micropterus* is dated during the dynamic sea level fluctuations outlined above. To explain the extant diversity of *Micropterus*, based on the correlation of dates of diversification and documented earth history events associated with sea level fluctuations, we propose a model of allopatric speciation that invokes passive vicariance among tributaries of the Gulf of Mexico during periods of high sea level, and postspeciation range shifts (dispersal) between interconnected tributaries during periods of low sea level. The repeated vicariance and subsequent postspeciation dispersal were facilitated by sea level fluctuations that successively isolated and reconnected tributaries of the Gulf of Mexico.

Despite failure to reject a model of no variation in the diversification rate throughout the history of *Micropterus* (Table 5), we propose that the time between approximately 8.8

TABLE 4. Comparison of diversification models.

Model	Description	log L	χ^2	*P*
A	Constant rate	−18.94		
B	Variable rate	−17.73	1.46	>0.05
C	Variable rate before and after 2.5 million years ago	−18.72	0.05	>0.05

and 6.0 mya was a very significant period in the diversification of *Micropterus*. Each of the four successive speciation events dated to this period are not separated by much more than one million years (Figs. 2 and 5), yielding a rate of approximately 0.5 species per million years for this duration of *Micropterus* diversification. This is a remarkable rate of speciation when compared to studies of well-documented adaptive radiations. For example, Hawaiian silverswords (0.56 ± 0.17 species per million years), murid rodents (0.35 species per million years), and Neogene horses (0.50 species per million years) all exhibit rates of speciation similar or lower than observed during this critical period of *Micropterus* diversification (Stanley 1979; Hulbert 1993; Baldwin and Sanderson 1998). Because there is no appreciable morphological or physiological diversification among species associated with differential patterns of resource use, the radiation of *Micropterus* is nonadaptive, sensu Schluter (2000). The apparent burst of diversification of *Micropterus* species in the Late Miocene to Pliocene was induced by rapidly fluctuating sea levels that isolated populations among disjunct riverine systems. The speciation of *Micropterus* illustrates that independent allopatric speciation events can be clustered during a relatively short time period, if geography and climatic fluctuations are conducive to isolation of populations and the eventual evolution of reproductive isolation.

One speciation event in *Micropterus* is dated near the Pliocene-Pleistocene boundary, and a second is dated in the Pleistocene (Table 3; Fig. 4). Sea level fluctuations may have isolated the common ancestor of the *M. salmoides-M. floridanus* clade near the Florida peninsula (Fig. 1). The most recent speciation event in *Micropterus*, between *M. punctulatus* and *M. dolomieu*, is dated at 1.01 ± 0.32 mya (Table 3; Fig. 4), during the Middle Pleistocene. Interestingly, *M. dolomieu* is the only species of *Micropterus* with a distribution that is exclusively north of the Gulf of Mexico tributaries (Fig. 1), other than the Mississippi River (MacCrimmon and Robbins 1975). Among species of *Micropterus*, *M. punctulatus* and *M. dolomieu* are among the most divergent with regard to coloration and pigmentation patterns, making the recent speciation and appreciable geographic overlap between these two sister species very interesting. Hybridization among species of *Micropterus* is rare under natural conditions, but extensive introgression between *M. dolomieu* and *M. punctulatus* has been documented on two occasions when either species has been introduced into areas not naturally containing the other species (Koppelman 1994; Avise et al. 1997).

All of the intraspecific coalescent ages of mitochondrial haplotypes, or phylogroups, date to the Pleistocene (Table 3; Figs. 4 and 5). The hypothesized effects of the Pleistocene on continental biota should not be confined to species formation. A mechanism that reduces genetic variation among populations of widespread species, coupled with the potential for geographic isolation of fragmented populations during the Pleistocene, has led to the development of a model for Pleistocene-induced intraspecific phylogeographic structuring (Hewitt 1996). The model of Pleistocene intraspecific diversity involves changes in the distribution of species resulting in the formation of genetic variation among isolated populations. During the Pleistocene, distributions north of the glacial front of *Micropterus* species were potentially fluctuating dramatically with the repeated cycles of glacial advance and recession. The confinement of species to small areas may have led to reduction of genetic variability via population bottlenecks or long-term reductions in effective population size (Avise et al. 1984). Additionally, repeated recolonization of previously glaciated areas may have led to reduced genetic variation from repeated founder-flush cycles (Hewitt 1996; Bernatchez and Wilson 1998). This model explains the observation that northerly distributed continental species tend to exhibit less genetic variation than species found in more southerly, nonglaciated regions (Hewitt 1996; Bernatchez and Wilson 1998). Intraspecific divergence times within *Micropterus* fit this model. The species that exhibit the greatest phylogeographic structuring are distributed south of the glacial front. Species with exclusively northern distributions (*M. dolomieu*), or species widely distributed south and north of the glacial front (*M. salmoides*), exhibit very shallow genetic divergence in these northern areas.

Conclusion

This investigation demonstrates the advantages of using the fossil record to estimate lineage specific rates of nucleotide polymorphism and to calibrate the molecular clock. The estimation of divergence times using molecular data carries several caveats (Hillis et al. 1996). However, the methodology outlined in this investigation is preferred to uses of pairwise estimates of divergence times. The molecular clock analysis provides a robust test of the Pleistocene speciation model for a very important lineage of North American freshwater fishes. Sea level fluctuations during the Late Miocene and Pliocene provide a mechanism of vicariance that explains the diversification of *Micropterus*. The rate of speciation, though uniform overall, appears to be conspicuously clustered during this time period. We hypothesize that this apparent rapid rate of allopatric speciation is the result of a dynamic earth history that resulted in repeated periods of isolation and dispersal among tributaries of the Gulf of Mexico. These results illustrate that rapid rates of speciation should not be automatically associated with adaptive diversification. Several lineages of North American freshwater fishes exhibit Gulf of Mexico coastal distributions similar to those of *Micropterus* (Wiley and Mayden 1985; Swift et al. 1986). We predict that the application of this Gulf Coast allopatric speciation model will provide a mechanism for the diversification for these other lineages of nonrelated freshwater fishes. Also, we expect the ages of speciation events within these lineages to have occurred between 8.5 and 3.5 mya and rates of diversification in these clades will be highest between 8.5 and approximately 6.0 mya.

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