Early quaternary sea level changes and rapid diversification in the North American

minnow genus *Nocomis* (Ostariophysi: Cyprinidae)

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INTRODUCTION

Evolutionary biologists have long sought to characterize processes that result in the formation of evolutionarily independent lineages. The combination of genealogical and spatial patterns of variation across populations with independent lines of geologic evidence provides a powerful approach to examining the historical processes that lead to diversification (Kozak et al. 2006, Berendzen et al. 2004, Egge and Simons 2006, Hollingsworth and Near 2009). This approach is particularly useful in aquatic systems where populations may become isolated as drainage morphologies evolve, resulting in coincident biologic and geologic evolution (Avise 2000).

The rivers of eastern North America are home to the most diverse assemblage of temperate freshwater fishes in the world (Briggs 1986). This riverine system has been repeatedly subjected to dramatic geologic and climatologic changes through evolutionary time that have configured the connectivity, character, and spatial arrangement of aquatic habitats (Thornbury 1965, Wiley and Mayden 1985). Cycles of glaciation dramatically altered sea levels, river flow, major drainage patterns, and riverine habitats through topographical leveling and sediment deposition (Fisk 1944, Thornbury 1965, Haq et al. 1987, Mayden 1988). These alterations directly impacted patterns of genetic exchange across these habitats, allowing for processes such as isolation, divergence and ultimately, speciation.

This system and the diversity it harbors have drawn the focus of intense biogeographic, demographic, and systematic study (Wiley and Mayden 1985, Burr and Page 1986, Mayden 1988, Strange and Burr 1997, Near et al. 2001). As these and other studies sought to explain the derivation, distribution and diversity of the North American

ichthyofauna, competing hypotheses emerged as to the timing of diversification. One hypothesis proposes that the primary causative agents of diversification were glaciations and climatologic oscillations that occurred during the Pleistocene epoch (Schmidt 1986, Robison 1986, Burr and Page 1986). This hypothesis suggests a recent origin of North American fish diversity, while a competing hypothesis proposes that the diversity of eastern North American fishes existed prior to the Pleistocene, and that recent glacial processes resulted in extirpation, extinction and fragmentation of geographic distributions within a diverse and established fauna (Mayden 1987b, Mayden 1988, Wiley and Mayden 1985).

Advances in molecular phylogenetic and computational methods have resulted in dramatic increases in the accuracy of divergence date estimates from genetic datasets, especially through the use of tree-based approaches that may account for rate heterogeneity and nucleotide substitution rates across lineages (Welch and Bromham 2005, Sanderson 1997, 1998). These advances allow for the direct temporal correlation of biological diversification and geologic and climatologic events in history. Recent studies of North American fishes have employed large genetic data sets to identify processes that have shaped the diversity and distribution of the fauna (Berendzen et al. 2003, 2008a, 2008b, Egge and Simons 2006, Near et al. 2001, 2003, Near and Benard 2004). These studies have demonstrated that a single hypothesis is inadequate to characterize the derivation of the entire North American ichthyofauna, as some lineages exhibit recent diversification (*Percina,* Near and Benard 2004, *Hybopsis,* Berendzen et al. 2008b), while others demonstrate diversification that predate Pleistocene events (*Notropis rubellus* species group, Berendzen et al. 2008a, *Erimystax,* Simons 2004). In a

study of the black basses of the genus *Micropterus,* Near et al. (2003) not only detected pre-Pleistocene diversification, but also implicated ancient sea level fluctuations as a factor driving allopatric speciation among coastal drainages.

Herein, the timing of diversification in a North American clade of riverine fishes is examined. Species of the nest building river chub genus *Nocomis* are abundant in highland character streams throughout their range and are important components of stream communities, as several species of cyprinid fishes are known nest associates of *Nocomis* (Johnston 1991). *Nocomis* species are widely distributed in Eastern North America, occurring throughout the Mississippi River basin as well as the Atlantic and Gulf coastal slopes (Figure 1). Regions within this distribution have distinct geologic histories and are physiographically varied, providing an excellent model to examine diversification in a temperate, riverine system containing both inland and coastal drainages. The goals of the present study are to: (1) use multiple nuclear and mitochrondrial gene loci to generate a robust phylogenetic hypothesis of relationships among the nine described taxa in *Nocomis*; (2) use tree-based methods and diversification statistics to estimate the timing of diversification among *Nocomis* lineages; (3) interpret the timing of *Nocomis* diversification in the context of the geologic and climatologic histories of the region.

The current taxonomy of *Nocomis* recognizes nine distinct taxa. As this group has not been the subject of an explicitly phylogenetic treatment, relationships among species are unknown. Early systematic work on *Nocomis* primarily employed meristics and patterns of tuberculation in nuptial males as the principal means of inferring relationships and identifying and delineating species. In a series of papers examining *Nocomis* diversity and evolution, (Lachner and Jenkins 1967, 1971a, 1971b, Jenkins and Lachner 1971, Lachner and Wiley 1971) these authors described five of the nine recognized taxa and erected three species groups that were hypothesized to share close evolutionary affinity (Figure 1): the *N. biguttatus* species group, consisting of *N. biguttatus, N. effusus,* and *N. asper*; the *N. micropogon* group, containing *N. micropogon, N. raneyi,* and *N. platyrhynchus*; and the *N. leptocephalus* group, which contains the three described subspecies, *N. l. leptocephalus, N. l. bellicus,* and *N. l. interocularis.*

Species of the *N. biguttatus* group are characterized by the presence of nuptial tubercles on the body, antrose tubercle orientation, a prominent post-ocular spot during spawning, and 1,4-4,1 pharyngeal dentition. Species of the *N. micropogon* group differ from the *N. biguttatus* group in pharyngeal dentition, the orientation, number and position of nuptial tubercles, and the presence of cephalic swelling in nuptial males. Individuals of the *N. leptocephalus* group are characterized by enlarged nuptial tubercles, an elongate, whorled gut, and the presence of prominent cephalic crests in nuptial males. Variation

within number, distribution and size of nuptial tubercles was observed among populations within *N. leptocephalus* such that three sub-specific forms were recognized and assigned to the *N. leptocephalus* species group: *N.l. leptocephalus*, *N. l. interocularis*, and *N. l. bellicus* (Lachner and Wiley 1971).

While not presented in explicitly phylogenetic terms, these studies hypothesized that species of the *N. biguttatus* group represented the most "primitive" species in the genus and that *N. effusus* was the sister taxon to *N. asper*. The *N. micropogon* group was considered to represent a more derived group of species that "arose from *N. biguttatus* stock". They further hypothesized that the *N. leptocephalus* group shared phylogenetic affinity with the *N. micropogon* group and represented the "most derived" species in the genus.

Mayden (1987a) represents the first explicitly phylogenetic review of *Nocomis* systematics. Using six morphological characters, the same three species groups were recognized as in previous studies, however, the relationships differed. *Nocomis effusus* was recovered as the sister to a clade containing *N. asper* plus *N. biguttatus*. The *N. biguttatus* species group was sister to a clade containing the *N. micropogon* group plus the *N. leptocephalus* group. Relationships within these latter groups were not examined.

MATERIALS AND METHODS

Taxon sampling

Ingroup sampling for this study included multiple individuals from each of the nine described taxa within the genus *Nocomis* (n > 5) for a total of 82 individuals. Individuals were sampled from river drainages across the range of each taxon to assess the monophyly of widespread taxa and to address relationships in a geographic context. See materials examined for a complete list of localities and specimens.

Outgroup taxa were selected based on putative phylogenetic affinity to *Nocomis* inferred from both morphological and molecular studies of cyprinid phylogeny (Mayden 1989; Coburn and Cavender 1992; Simons et al. 2003; Simons and Mayden 1999). The cyprinid species *Campostoma pauciradii*, *C. anomalum*, *C. oligolepis*, *Erimystax dissimilis*, *Exoglossum laurae*, *Phenacobius catostomus*, *Pogonichthys macrolepidotus*, *Macrhybopsis aestivalis*, *Rhinichhys atratulus* and *R. cataractae* were included as outgroups in all analyses.

Character sampling

An approach similar to the character sampling strategy outlined by Weins et al. (2005) was adopted to resolve both species and population level relationships simultaneously. In this approach, a dataset is comprised of slowly evolving characters scored for a limited set of taxa to resolve deeper divergences within the group and a faster evolving marker scored for many taxa to provide resolution at the population level. Despite an inherent preponderance of missing data cells in a matrix assembled in this

manner, recent studies demonstrate accurate phylogenetic placement of highly incomplete taxa in both simulated (Weins 2003; Phillipe et al. 2004; Weins 1998a) and empirical datasets (Egge and Simons 2009; Driskell et al. 2004; Weins et al. 2005).

The best estimate of phylogeny is yielded through combined analyses of all available data, so long as areas of strong incongruence between datasets are taken into account (Weins and Reeder 1997; Weins 1998b). In the absence of strong conflict among datasets derived from distinct markers, each dataset shares the same phylogenetic history; the most accurate hypothesis of phylogenetic relationships will be provided by the combined analysis of a large number of independently evolving markers (Weins et al. 2005)

Sequences of two single copy, protein coding nuclear markers, rhodopsin and interphotoreceptor retinol binding protein (IRBP) were obtained for 30 individuals of *Nocomis* and all outgroup taxa. Sequences of the mitochondrially encoded cytochrome *b* (cyt *b*) gene were obtained for 78 individuals of *Nocomis* and all outgroups (Table 3).

Acquisition of DNA sequence data

Fishes were collected in the field with a 6' x 10' x 1/8" pole seine, a 6' x 20' x 1/8" bag seine and a Smith Root model 12-B backpack electroshocker (See materials examined for a list of localities), anesthetized in MS222, and immediately frozen whole in liquid nitrogen. Genomic DNA extractions were performed in the laboratory following the manufacturer's instructions using DNeasy tissue kits (Qiagen Inc.). Amplification of target regions of DNA was performed using the polymerase chain reaction (PCR). A total volume of 25μl was used for all PCR reactions containing 1.0μg of genomic DNA and

the following concentrations of reagents: 1.2 µM of both primers, 1X *Taq* salts, 4mM

MgCl$_2$, 0.4 µM dNTPs, and 1.25 units of *Taq* DNA polymerase. See Table 1 for all

primer sequences.

Unwanted reagents were removed from PCR products with Exonuclease 1 and

Shrimp Alkaline Phosphatase (SAP). Automated sequencing reactions were performed at

the Advanced Genetics Analysis Center, University of Minnesota, using Big Dye (Perkin

Elmer) terminator cycle sequencing on an ABI 3700. Sequences were obtained for both

heavy and light strands to ensure accuracy of sequence data. All sequences were

assembled, checked for accurate base determination and aligned with the software

package Sequencher 4.0 (Gene Codes Corp.).

See Tables 1 and 3 for PCR and sequencing primers, and numbers of individuals

sequenced for each locus.

Partitioning Scheme

A multi-locus DNA dataset is a composite of sequences from disparate regions of

the genome. Within such a dataset, regions of sequence that have evolved under differing

models of DNA sequence evolution may be identified and partitioned. Partitions may be

between different genes or within the same coding region (codon positions).   By

identifying these partitions, multiple models may be chosen to accommodate these

differences and more accurately reflect the evolution of each sequence region than is

possible through the application of a single model to the entire dataset.

Multiple partitioning schemes were evaluated to determine the optimal

partitioning scheme for the multi-gene dataset. The following five sets of partitions were

evaluated: P1) a single partition for all three genes, P2) two partitions (mtDNA, nuclear

DNA), P3) three partitions (1st, 2nd, 3rd codon positions), P4) three partitions (cyt *b*, rhodopsin, IRBP), and P5) nine partitions (1st, 2nd, 3rd codon positions within each locus, cyt *b*, rhodopsin, and IRBP).

To select the model that best fit the data for each partition within each dataset, the computer program Mr. Modeltest (Nylander 2004) was employed to compare hierarchical hypothesis testing of alternate models of DNA sequence evolution. The best fitting models, as determined by the Akaike Information Criterion (AIC), were identified for each partition in each dataset in independent analyses (Table 4).

Two million generations of Markov chain Monte Carlo (MCMC), implemented in Mr. Bayes version 3.1.2 (Ronquist and Huelsenbeck 2003), were performed independently for each set of partitions (P1-P5) under the determined models using a random starting topology with trees sampled every 100 generations. Log likelihood scores of sampled trees were plotted against generation time and all trees sampled from generations prior to a stationary likelihood value were discarded as burnin.

The relative tenability of each partitioning scheme was evaluated through the use of Bayes factors. Bayes factors are determined through pairwise comparisons of mean harmonic likelihood values from each independent Bayesian analysis of each partitioned dataset (P1-P5), and may be interpreted as the relative success of each alternative partitioning scheme at predicting the data (Kass and Raftery 1995; Nylander et al. 2004).

Assume that one wishes to evaluate the efficacy of two competing partitioning schemes, P_0 and P_1, at predicting the data set D. The Bayes factor that favors partition scheme 1 over partition scheme 0, B_{10}, may be calculated as the ratio of mean harmonic likelihood scores from independent analyses $f(D \mid P_i)$:

$$B_{10} = (f(D \mid P_1)) / (f(D \mid P_0))$$

The Bayes factor is not employed to determine whether to reject or accept alternative hypotheses given a subjective cutoff value, as in a typical statistical test, but rather it compares the relative efficacy of the two competing partition schemes and the interpretation of this comparison is left to the researcher (Nylander et al. 2004). Guidelines for interpretation of Bayes factors were presented by Jeffreys (1961), and have been modified by subsequent investigators. This study uses the version given by Kass and Raftery (1995) (Table 2).

Phylogenetic analysis

A phylogenetic hypothesis of relationships within *Nocomis* was generated with Bayesian methods implemented in Mr. Bayes version 3.1.2 for the optimal partitioning scheme (Ronquist and Huelsenbeck 2003). Ten million generations of Markov chain Monte Carlo (MCMC) were performed using a random starting topology with trees sampled every 100 generations. Log likelihood scores of sampled trees were plotted against generation time and all trees sampled from generations prior to a stationary likelihood value were discarded as burnin. A 50% majority rule consensus tree was constructed from retained trees, and the percentage of times a particular node was

recovered was interpreted as the posterior probability of the occurrence of that node (Huelsenbeck et al. 2001).

The multi-gene dataset was also analyzed under a maximum likelihood optimality criterion as implemented in the software GARLI (Zwickl 2006). As this program does not allow for data partitions, the dataset was analyzed under the GTR + I+Γ model determined by MrModeltest to be the best-fitting model for the single partition dataset. The analysis was run until 10,000 generations were completed without significant improvement (lnL increase of 0.01) to the topology. Node support was evaluated through 100 bootstrap replicates in which each repetition terminated after 1,000 generations were completed without topological improvements.

Estimation of Divergence Dates and Diversification Rates

The software package BEAST (Drummond and Rambault 2007) was used to estimate divergence times among *Nocomis* lineages under a Bayesian MCMC framework. In this manner, the set of nucleotide sequences may be used to generate sample-based estimates of evolutionary parameters and model the rate of molecular evolution on each branch of the tree. Model parameters included a GTR+I+G nucleotide substitution model and a coalescent Bayesian skyline plot tree prior. Five independent MCMC runs were performed for 10 million generations each. The resulting trees and log files were pooled using the program LogCombiner in the BEAST package. The 95% highest posterior density (HPD) of divergence times were plotted onto each node of the phylogeny using the program TreeAnnotator in the BEAST package.

Fossil calibration of substitution rates was not possible for the *Nocomis* phylogeny due to an absence of closely-related taxa in the fossil record. The literature provides a range of fossil-calibrated substitution rates for cytochrome *b* in other teleost fishes and divergence dates were calculated from these substitution rates under a molecular clock (Zardoya and Doadrio 1999; Machordom and Doadrio 2001; Dowling et al. 2002; Webb 1998; Perdices and Doadrio 2001; Near and Benard 2004). These independent studies found substitution rates for cyt *b* ranging from 0.76 % per million years in European cyprinids (Zardoya and Doadrio 1999) to 2.2 % per million years in North American logperches (Near and Benard 2004). Divergence times for lineages of *Nocomis* were estimated under a uniform prior that spanned this range of substitution rates (Berendzen et al. 2008b). Whereas the combined analysis of all available data provides the best hypothesis of phylogenetic relationships, branch lengths were estimated from the cytochrome *b* nucleotide sequences on the topology from the combined analyses for the BEAST analysis.

The software package R (R core-development team, 2006) was used to implement diversification statistics for *Nocomis*, employing functions in the packages APE (Paradis et al. 2004) and Geiger (Harmon et al. 2008). A Kendal- Moran estimate of a global diversification rate (r) for *Nocomis* was calculated based on a pure-birth process (Nee 2001, Baldwin and Sanderson 1998). A novel function (Brock unpublished, see Dornburg et al. 2008) was employed to calculate rates of cladogenesis during specific time intervals and to test whether they differed significantly from the global rate. This test accounts for the effect that extinction may have on the distribution of waiting times, or the "pull of the present" (Pybus and Harvey 2000), as well as incomplete taxon sampling. 20,000 birth-

death trees were simulated under the global estimate of *Nocomis* diversification to generate a null distribution for comparison with observed values of r for each interval.

RESULTS

Phylogenetic Analysis

Alignment of nucleotide sequence data from three target loci yielded a dataset of 2861 aligned base pairs for 30 individuals of *Nocomis* and all outgroups. An additional 48 ingroup taxa were scored for 1140 of these characters (cyt *b* only) resulting in a dataset of 88 taxa. See Table 3 for complete list of sequences generated for each taxon, at each locus. See Table 4 for a list of AIC-selected models for each partition within each partitioning strategy. See Table 5 for a list of mean harmonic likelihood scores and Bayes factor comparisons.

The partitioning scheme, P3 (three partitions, by codon position) yielded a superior harmonic mean likelihood score and was favored over schemes P1, P2, P4 and P5 through comparison of Bayes factors. Although scheme P3 was favored over scheme P5 (nine partitions, by gene, by codon position) in Bayes factor comparison, it was not considered to be significantly better than scheme P5 following Jeffreys' (1961) guidelines for interpretation of the Bayes factor. To further evaluate the performance of the alternative partitioning schemes P3 and P5, subsequent Bayesian analyses were run on each dataset for 10 million generations to ensure that stationarity had been achieved and was not a bias in the calculation of mean harmonic likelihood scores. Subsequent Bayes factor comparisons strongly favored scheme P3 over P5 (Table 6).

Partitioning schemes P1-P4, yielded congruent topologies, while P5 differed from schemes P1-P4 in the phylogenetic position of individuals from the Mobile Basin. To determine if any of the selected markers exhibited conflicting phylogenetic signals, Bayesian analyses (as described above) were performed on each individual dataset for each of the three loci (results not shown). Few strongly supported conflicts were observed and nearly all nodes recovered were congruent across all analyses. Minor differences were noted among the placement of some taxa within phylogroups. Trees generated from Rhodopsin and IRBP sequence data exhibited congruent topologies. Incongruence was observed between two nodes in the cytochrome b gene tree and the congruent gene trees from the two nuclear loci. Bayesian analysis was performed on a dataset containing only individuals that were scored for all characters (n=38). The observed topology was congruent with the independent nuclear gene trees and very well supported at the nodes that differed in the cyt b gene tree. Maximum likelihood analyses performed in GARLI (Zwickle 2006) yielded a tree that was entirely congruent with the Bayesian topology to the population level. In all analyses of combined and individual datasets, a well-supported monophyletic *Nocomis* was recovered, and in all cases, *Campostoma* was resolved as its sister taxon.

The combined dataset recovered two major clades within *Nocomis*. The first of these clades consists of *N. biguttatus*, *N. asper*, and *N. effusus*, with *N. asper* recovered as the sister taxon to *N. biguttatus* plus *N. effusus*. All nodes in this clade were well-supported with Bayesian posteriors of 0.95 or greater. Bayesian topology and support values are shown in Figure 2.

The second major clade is comprised of the remainder of *Nocomis* with *N. raneyi* sister to *N. micropogon* plus *N. leptocephalus*. *Nocomis platyrhynchus* was firmly nested among individuals of *N. micropogon* in all analyses.

While a monophyletic *N. leptocephalus* is strongly supported, none of the three described sub-species of the complex were monophyletic. Individuals from the Mississippi embayment in western Mississippi and Gulf Coast drainages west of the Mobile Basin are sister to a clade containing the remaining species of the *N. leptocephalus* complex *sensu* Lachner and Wiley (1971). Within this clade, individuals from the New and Roanoke drainages are sister to an unresolved trichotomy containing an individual from the Mobile Basin, a clade containing individuals from the Savannah River sister to an individual from the Altamaha River, and a clade containing samples from the Edisto and Santee Drainages sister to samples from the Catawba River.

Estimation of Divergence Dates and Diversification Statistics

Nocomis diverged from its sister taxon, *Campostoma,* in the late Miocene, 7.3 mya, 95% HPD: [6.2, 8.4], and the eastern and western clades diverged around the time of the Miocene-Pliocene boundary 5.3 mya, 95% HPD: [4.5, 6.1] (Figure 3). *Nocomis raneyi* diverged from the common ancestor of the *N. micropogon* complex and Gulf and Atlantic Coast populations in the late Pliocene, 3.0 mya, 95% HPD: [2.6, 3.4]. *Nocomis micropogon* diverged from Gulf and Atlantic slope populations very near the Plio-Pleistocene boundary, 2.9 mya, 95% HPD: [2.5, 3.3]. Populations west of the Mobile Basin diverged from Gulf and Atlantic Slope *Nocomis* 2.7 mya, 95% HPD:[2.3, 3.0].

Populations in the Gulf and Atlantic Slope drainages diverged from one another in rapid succession between 2.5 and 2 mya.

The global diversification rate (r_G) of 0.355 s/my (species / million years) was estimated for *Nocomis*. Kendal-Moran estimates of diversification rates across one million year intervals showed evidence of variation in diversification rates through time, however only the interval from two to three mya ($r_{2-3} = 1.43$ s/my) was significantly different from the global mean rate ($p = 0.004$). Estimates of r for each time interval are presented in Table 7.

DISCUSSION

Phylogeny and Species Limits

As the first comprehensive and explicitly phylogenetic treatment of the genus *Nocomis*, this study provides a novel, robust hypothesis of relationships. Previous systematic studies of this group relied heavily on tuberculation patterns and meristic counts to delineate boundaries among *Nocomis* species, recognizing nine described forms (Lachner and Jenkins 1967, Lachner and Wiley 1971, Lachner and Jenkins 1971a, 1971b, Mayden 1987a). Results from the present study suggest that diversity in *Nocomis* was underestimated by these methods and that some nuptial tuberculation patterns are not consistent with the genealogical history of *Nocomis*.

These novel findings are consistent, if not perfectly congruent with, some of Lachner and Jenkins' (1971a, 1971b) early hyphotheses on *Nocomis* evolution,

particularly at deeper nodes in the phylogeny. It appears that characters associated with the position, location, and orientation of nuptial tuberculation provided a somewhat accurate phylogenetic signal in the erection of the species groups (Lachner and Jenkins 1967, 1971a, 1971b; Mayden 1987a) and their relationships to one another. It does not appear in this case that number and size of tubercles provide useful historical signal at the population level. Even within populations, the number and size of tubercles are correlated with the overall size of the fish; larger individuals have larger and more numerous tubercles (Lachner and Wiley 1971). Additionally, characters such as nuptial tubercles may be heavily influenced by factors such as sexual selection, variation in male-male agonism, and environmental factors (Sabaj et al 2000). A superficial trend of larger, fewer tubercles in southern drainages giving way to smaller, more numerous tubercles in northern drainages supports the notion that environmental factors may play a greater role in tuberculation patterns than phylogeny (Lachner and Wiley 1971).

Two major clades are recovered within *Nocomis:* a western clade containing the *N. biguttatus* species group, *N. asper, N. effusus, and N. biguttatus*, and an eastern clade containing members of the *N. micropogon* and *N. leptocephalus* species groups. The recovery of the *N. biguttatus* species group represents congruence between previous morphological and meristic work and the present phylogenetic study, however, species-level relationships within the group differed among studies; Mayden (1987a) considered *N. biguttatus* and *N. asper* to be a species pair, while Lachner and Jenkins (1967), argued a sister relationship between *N. asper* and *N. effusus*. The hypothesized sister relationship between *N. biguttatus* and *N. effusus* is unique to this study.

Within the eastern clade recovered in molecular analyses, departures from the picture of *Nocomis* evolution offered by earlier investigations are observed (Lachner and Jenkins 1967, 1971a, 1971b, Lachner and Wiley 1971). Rather than being resolved with *N. micropogon* and *N. platyrhynchus*, as predicted by meristic and morphological lines of evidence, *Nocomis raneyi* is sister to a clade containing *N. micropogon* plus the *N. leptocephalus* complex. This biogeographic pattern has been observed in other clades of fishes (see below). *Nocomis platyrhynchus* (*sensu* Lachner and Jenkins 1967) was nested within populations of *N. micropogon* and was recovered as the sister to Tennessee River populations of *N. micropogon* in all analyses, in both combined (presented here) and individual analyses of gene sequence datasets (results not shown). Three monophyletic phylogroups are recovered within *N. micropogon* in all analyses; it is possible that *N. micropogon* represents a complex of three species, consisting of Ohio, Tennessee, and New River (*N. platyrhynchus*) forms, however this issue is beyond the scope of the present study. The data presented here suggest that *N. platyrhynchus* should be subsumed into *N. micropogon* until further study suggests otherwise.

The present study reveals from at least five to as many as eleven phylogenetic species within what is presently regarded as *N. leptocephalus*. The resolution of this taxonomic issue, given the short branches deep within the Gulf/Atlantic slope clade and the detailed meristic work required to describe new taxa, is beyond the scope of this study and provides ample area for subsequent investigation. To err on the side of conservatism, it is recommended that five species be recognized within *N. leptocephalus* (*sensu* Lachner and Wiley 1971) at present: *N. bellicus* occurring in the Mobile Basin, *N. interocularis* of the Savannah, Altamaha and Chatahoochee drainages; *N. leptocephalus*

in the Pee Dee, Catawba, Santee, and Edisto drainages; an undescribed form of *Nocomis* in the Roanoke and New River systems; and an undescribed *Nocomis* species occupying the rivers west of the Mobile Basin to the Mississippi River. Morphological characters in support of some of these taxa are described by Lachner and Wiley (1971), who noted two distinct coloration patterns among northern and southern populations of what they referred to as *N. l. leptocephalus*. The northern, undescribed *Nocomis sp*. (this study) is found in the Roanoke, New, James and Neuse drainages. Breeding males of this form exhibit orange to brassy pigmentation on the lateral surfaces of the head, lateral band, and to varying degrees in fins. Additionally, the orange lateral band abruptly changes to olive-yellow caudally at the height of nuptial coloration. The southern form, *N. leptocephalus* (this study), occurs in the Pee Dee, Cape Fear and Santee drainages and differs from the northern form in having blue pigment laterally along the body versus the orange of the northern form.

Estimation of Divergence Dates

Likelihood based estimates of divergence dates in the *Nocomis* phylogeny closely align with the Gulf Coast allopatric speciation model, which was proposed to account for the high degree of endemism in fishes of this region (Wiley and Mayden 1985, Swift et al. 1986, Near et al. 2003). This model invokes dramatic sea level fluctuations as having driven vicariant speciation events through the isolation and alteration of coastal river drainages. The major period of diversification in *Nocomis* occurred within the eastern clade at the close of the Pliocene and the onset of the Pleistocene, with ten of twelve nodes between thirteen extant lineages occurring between 3 and 2 mya. During this span

of one million years, at least two major sea level fluctuations occurred (Riggs 1984, Haq et al. 1987) The first and most dramatic of these occurred at the end of the Pliocene, ca. 2.5 mya, when mondial mean sea levels dropped from 50-80m above present day levels to an estimated 80-100m below (Swift et al. 1986, Riggs 1984, Haq et al. 1987). The second major fluctuation in this period occurred in the early Pleistocene, when the first in a series of at least three Pleistocene sea level oscillations brought sea levels from 80-100m below to 10-20m above present day levels. As sea levels fall, coastal drainages extend seaward and may form connections with other drainages that are isolated at periods of elevated sea levels. The phylogeny presented here is consistent with this model, with nearly all diversification in the eastern clade occurring during this time period (Figure 3). Estimated rates of diversification within this time period (r_{2-3} = 1.43 s/my) are extremely rapid, at over four times the global rate for this genus (r_G = 0.355 s/my) (Table 7). The diversification rate of 1.43 s/my within *Nocomis* lineages between 2 and 3 mya is among the highest Kendall-Moran estimates of diversification calculated from a molecular phylogeny (Klak et al 2004). Further, the phylogeny is characterized by short branch lengths during this time period followed by longer branches during the period from 1.5 mya to present (Figure 3). This pattern is consistent with a relatively rapid series of vicariant events, followed by long term isolation in coastal drainages.

Biogeography

The biogeographic implications of *Nocomis* phylogeny provide insight into the deep history of the southern Appalachian, Gulf and Atlantic Slope regions of North America and are consistent with several existing biogeographic hypotheses formulated on phylogenetic, distributional and geologic lines of evidence.

Northern Atlantic Slope

Nocomis raneyi is sister to all other populations in the eastern clade and diverged from these populations late in the Pliocene, 3.0 mya, 95% HPD: [2.6, 3.4]. This pattern of northern Atlantic slope drainages containing the sister taxon to the remaining species in the group is replicated in other clades of fishes. In the catostomid genus *Hypentelium*, the Roanoke River endemic *H. roanokense* is the sister taxon to all other populations of hogsuckers (Berendzen et al. 2003). Studfishes, Sub-genus *Xenisma* of *Fundulus,* also exhibit this pattern, with *Fundulus rathbuni*, occurring in the Roanoke, Pee Dee, Catawba and Cape Fear drainages, sister to all other studfish (Ghedotti et al. 2004). Logperch darters of the genus *Percina* also exhibit this pattern (Near and Benard 2004). *Percina rex*, which is distributed in the Roanoke and Chowan rivers, is sister to all other logperch. The divergence date of *N. raneyi* falls within the standard error of Near and Benard's (2004) estimate of the age of the node separating *P. rex* from other logperch. This phylogenetic and temporal congruence among drainages in four disparate lineages

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provides an argument for a common vicariant event that isolated drainages in the northern Atlantic Slope during the Pliocene.

Appalachian River

Geologists (Hayes and Campbell 1894, Hayes 1895, Simpson 1900, Eckel 1940, Conant 1964, Thornbury 1965) and later, biogeographers (Wiley and Mayden 1985; Mayden 1987a, 1987b, 1988; Swift et al. 1986) hypothesized that the present day conformation of the Tennessee River is a composite of ancient drainages; rivers in the present day upper Tennessee River drainage once flowed south through the Mobile Basin directly to the Gulf of Mexico. Thornbury (1965) employed analysis of sediment deposits and geomorphology of the region to argue that a gradient advantage of the lower Tennessee River brought about the capture of the upper Tennessee River from the Mobile Basin at the close of the Pliocene or early in the Pleistocene.

A recent study by Kozak et al. (2006) provides compelling genetic evidence for this ancient connection based on deep divergence between populations of dusky-sided salamanders in the upper and lower Tennessee River. Kozak et al. noted this pattern in multiple lineages of the *Eurycea bisilineata* complex and argued that divergence at this level (5%-9%) was a decisive indicator that the upper and lower reaches of the modern day Tennessee River basin had separate outlets to the Gulf of Mexico until the middle Miocene/ middle Pliocene.

While phylogeographic patterns among *Nocomis* populations do not directly support the Appalachian River hypothesis, they are consistent with and may provide a time scale to the paleodrainage model offered by Kozak et al. (2006). The Tennessee and

Ohio River distributed *N. micropogon* diverged from Gulf and Atlantic Slope populations 2.9 mya, 95% HPD: [2.5, 3.3], at the close of the Pliocene. This divergence may represent the severance of the upper Tennessee River and its outlet to the Gulf of Mexico through the Mobile Basin. While other scenarios are possible, genetic and geologic lines of evidence leave little doubt as to the existence of the ancient Appalachian system. The timing of this node falls directly on Thornbury's (1965) prediction, yet occurs more recently than Kozak et al.'s estimate of four to seven MYA.

Direct Gulf and Lake Pontchartrain Tributaries

Nocomis populations in Gulf and Mississippi River tributaries west of the Mobile basin are sister to remaining Atlantic/Gulf Slope populations and diverged from these other lineages 2.7 mya, 95% HPD:[2.3, 3.0],. These populations represent at least one undescribed species, with a deep 1.8 mya, 95% HPD:[1.4, 2.1], divergence between gulf tributaries and rivers draining into the Mississippi River. The meristic and morphological similarity between these populations and distantly-related populations in the adjacent Mobile Basin (Lachner and Wiley 1971) argue that these characters are perhaps more influenced by environmental factors than phylogeny.

Atlantic and Gulf Slopes

The rapid diversification among lineages of the Gulf and Atlantic slopes may represent the contemporaneous establishment of present day drainage configurations throughout the coastal plain. This clade is characterized by short branch lengths deep in the clade and a series of rapid divergences at the onset of the Pleistocene, between 2.5,

and 2.0 mya. The 95% highest posterior density distributions on these nodes overlap, and likely represent an abrupt series of vicariant events that isolated populations in coastal drainages. This isolation is evidenced by the markedly long branches from 2 mya to present.

Nocomis populations occupying northern and southern portions of this region diverged 2.5 mya, 95% HPD: [2.2, 2.8]. The northern portion, containing New and Roanoke river populations, represents at least one undescribed species of *Nocomis*. Divergence between New and Roanoke populations is deep, dating to 2.0 mya, 95% HPD: [1.6, 2.4]. The southern portion of this clade contains *N. leptocephalus* (Pee Dee, Catawba, Edisto, and Santee Rivers) sister to *N. bellicus* (Mobile Basin) plus *N. interocularis* (Savannah, Altamaha, and Chattahoochee rivers). Divergences within and among these groups are deep and successive, with reciprocal monophyly observed among populations in all of the major Gulf and Atlantic Slope drainages mentioned above.

The timing and geographic pattern of diversification within this clade of *Nocomis* is consistent with the "White's River" hypothesis of Wood and Mayden (1992). Wood and Mayden invoked the existence of a river proposed by White (1953) to explain the biogeography of *Notropis lutipinnis* and *N. chlorocephalus*, a clade of minnows exhibiting a similar distribution and phylogeny to the Atlantic/Gulf Slope clade of *Nocomis*. White (1953) observed asymmetry among tributaries of Atlantic Slope river drainages, noting that northeast flowing tributaries are long and sub-parallel, while opposing tributaries northeast of the main channels are short and irregular in direction. White interpreted this asymmetry as evidence for the former existence of a large river flowing from areas adjacent to the headwaters of the Chattahoochee River northeast

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along the Piedmont of North Carolina and Virginia to the Potomac. White proposed that this river was dismembered by successive stream captures from steeper gradient, southeast flowing rivers along the Atlantic Slope that flowed perpendicular to the coastline. The timing, relationships and branch lengths within the Atlantic/Gulf Slope clade are consistent with the rapid dismemberment of such a river at the onset of the Pleistocene.

Interior Highlands and Central Lowlands

The divergence between the western clade (*N. biguttatus* species group) and the eastern clade is dated to the Miocene-Pliocene boundary 5.3 mya, 95% HPD: [4.5, 6.1]. The timing of this divergence coincides with a sharp drop in sea levels (Figure 3) and a period of rapid diversification in *Micropterus* (Near et al. 2003). This temporal congruence may represent the effect of a shared geologic/climatologic event that influenced diversification in these fishes.

Divergences within the western clade are recent relative to the eastern clade. It is possible that populations in this region were not as dramatically impacted by sea level fluctuations as were coastally distributed populations, or perhaps, diversity in this clade was reduced through local extinctions during glacial episodes. *Nocomis asper* diverged from the common ancestor of *N. biguttatus* and *N. effusus* 1.0 mya, 95% HPD: [2.6, 3.4]. Divergence between *N. biguttatus* and *N. effusus* is recent, 0.4 mya, 95% HPD: [0.3, 0.6].

CONCLUSION

This investigation demonstrates that sea-level fluctuation can provide a mechanism for dramatic bursts of cladogenesis in coastally-distributed, riverine fishes. Herein, close temporal concordance between significantly elevated rates of diversification in *Nocomis* and changes in mean global sea levels are demonstrated, indicating that large-scale sea level fluctuations can significantly alter gene flow within and among coastal drainage basins. Further, this study demonstrates the utility of geologic histories and Bayesian estimates of divergence dates in the absence of fossil calibration points for dated phylogenies. The complex phylogeographic history of *Nocomis* retains the genetic signatures of ancient events and the spatial arrangements of ancient river drainages. Molecular results generated in this study reinforce faunistic and geologic arguments for the hypothesized existence of extinct rivers, such as White's River and the Appalachian River. Phylogeographic hypotheses presented in this study provide ample area for future investigation of codistributed riverine taxa. Methods and analyses similar to those used in this study could be employed to test the generality of these results.

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Table 1.1. List of PCR and sequencing primers used in this study.

Primer name	Primer sequence (5' to 3')	Source
Cytochrome *b*		
HA	CAA CHA TCT CCG GTTTAC AAG AC	Schmidt et al. 1998
LA	GTG ACT TGA AAA ACC ACC GTT G	Schmidt et al. 1998
HE	ATA TAT GGG TGT TCT ACA GGT ATG CC	Schmidt et al. 1998
LD	CCA TTC GTC ATC GCC GGT GC	Schmidt et al. 1998
HC	CCA TAG TTG ACG TCA CGG CA	Schmidt et al. 1998
LC	ATA CAT GCC AAC GGA GCA TC	Schmidt et al. 1998
IRBP		
Cyp109F	AAC TAC TGC TCR CCA GAA AAR	Chen pers comm. 2006
Cyp1001R	GGA AAT GCA TA TTG TCT GCA	Chen pers comm. 2006
Rhodopsin		
Rh193	CNT ATG AAT AYC AYC CTC AGT ACT ACC	Wood pers comm. 2006
Rh1073r	CCR CAG CAC ARC GTG GTG ATC ATG	Wood pers comm. 2006

Table 1.2. Guidelines for interpretation of Bayes factors presented by Jeffreys (1961). Modified from Kass and Raftery (1995).

$2 \log_e (B_{10})$	B_{10}	Evidence against M_0
0 to 2	1 to 3	not worth more than a mention
2 to 6	3 to 20	positive
6 to 10	20 to 150	strong
>10	>150	very strong

Table 1.3. List of sequences obtained for each of the three selected markers for each taxon.

Taxon	Cyt b (1140 bp)	IRBP (849 bp)	Rhodopsin (872 bp)
N. asper	5	2	2
N. bigutattus	7	2	2
N. effusus	6	2	2
N. l. bellicus	16	5	5
N. l. interocularis	9	5	5
N. l. leptocephalus	19	8	8
N. platyrhynchus	4	1	1
N. micropogon	6	3	3
N. raneyi	6	2	2
Total	78	30	30

Table 1.4. Partition schemes P1-P5 and the associated best-fitting model for each partition as determined by the AIC in the software package Mr. Modeltest (Nylander 2004).

Partition	Model
P1: Single Partition	GTR + I + Γ
P2: Partition By Genome	
mtDNA	GTR + I + Γ
Nuclear DNA	TVM + I + Γ
P3: Partition by Codon Position	
1st	GTR + I + Γ
2nd	F81 + Γ
3rd	GTR + Γ
P4: Partition by Gene	
Cyt *b*	GTR + I + Γ
IRBP	TVMef + Γ
Rhodopsin	GTR + I + Γ
P5: Partition by Gene by Codon Position	
Cyt *b*	
1st	SYM + I + Γ
2nd	F81 + Γ
3rd	SYM + I + Γ
IRBP	
1st	SYM + I + Γ
2nd	SYM + I + Γ
3rd	SYM + I + Γ
Rhodopsin	
1st	GTR + I
2nd	F81 + I
3rd	GTR + Γ

Table 1.5. Mean Harmonic likelihood scores and Bayes factor comparisons for partition schemes P1-P5 after 2 million generations of MCMC.

Partition Scheme	Mean Harmonic Likelihood	P1	P2	P3	P4	P5
Single Partition (P1)	-14732.24	*				
Partition By Genome (P2)	-14532.67	399.14	*			
Partition by Codon Position (P3)	-14528.93	205.31	7.48	*		
Partition by Gene (P4)	-14552.76	358.96	-40.18	-47.66	*	
Partition by Gene by Codon Postion (P5)	-14530.17	404.14	5	**-2.48**	45.18	*

Table 1.6. Mean harmonic likelihood scores and Bayes factor comparison of partition schemes P3 and P5 after 10 million generations of MCMC.

Partition Scheme	Mean Harmonic likelihood	P3	P5
Partition by Codon Position (P3)	-14531.44	*	
Partition by Gene by Codon Position(P5)	-14568.07	**-73.26**	*

Table 1.7. Kendal Moran estimates of diversification rate (r) for one million year intervals. Period from 2-3 mya differs significantly from the global rate (0.35 s/my). Null distribution generated from 20,000 birth-death trees under the global estimate of *Nocomis* diversification.

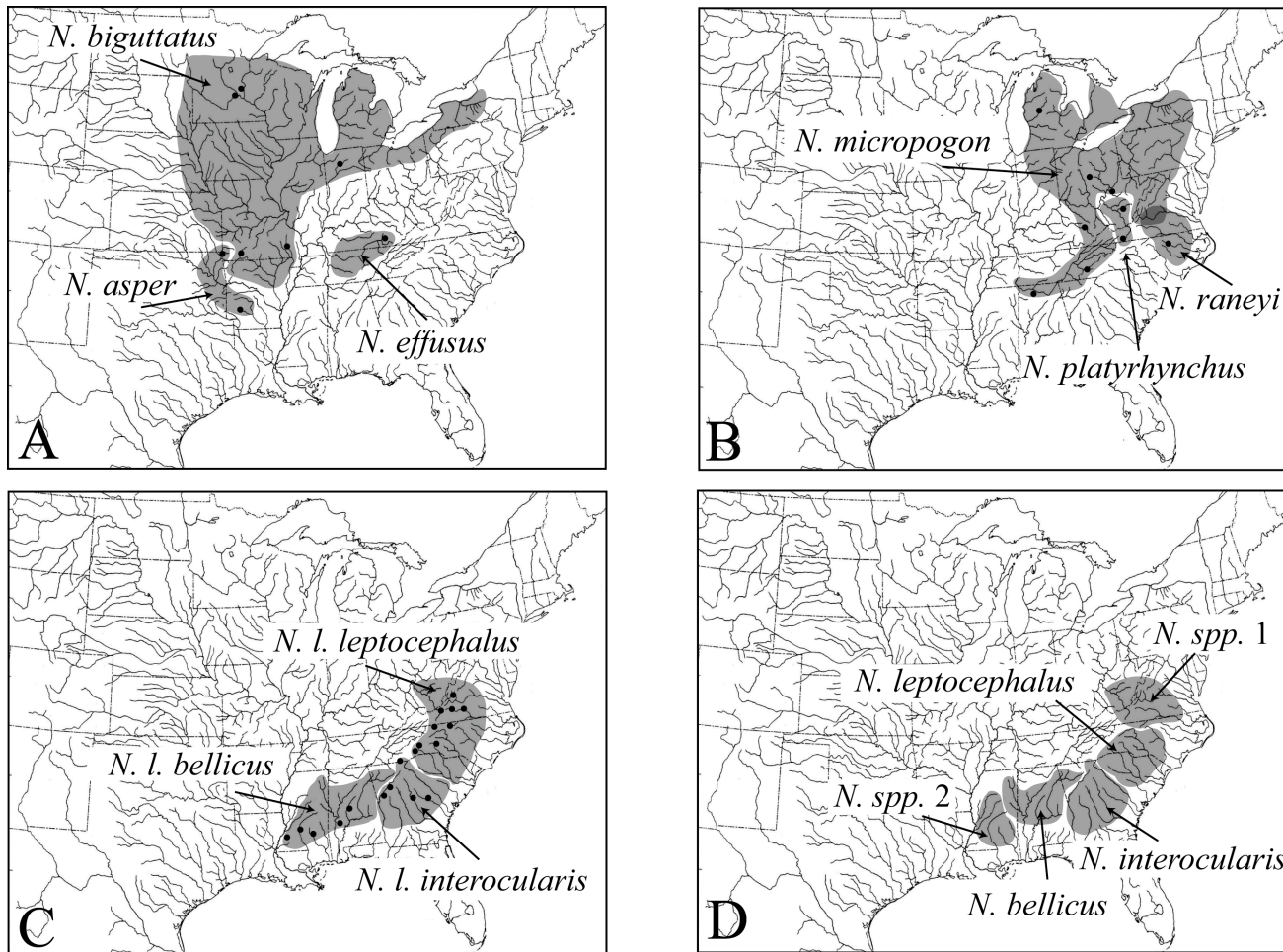| Time interval	KM estimate of r	P -value
0-1 mya	0.65	0.990
1-2 mya	0.23	0.714
2-3 mya	1.43	0.002
3-4 mya	0.50	0.241
4-5 mya	Null	-

Figure 1.1. Geographic ranges of *Nocomis* species. Black dots indicate sampling localities. A: *N. biguttatus* species group, *sensu* Lachner and Jenkins 1971b. B: *N. micropogon* species group *sensu* Lachner and Jenkins 1971a. C: *N. leptocephalus* species group *sensu* Lachner and Wiley 1971. D: Proposed taxonomic changes and areas containing undescribed diversity (this study).
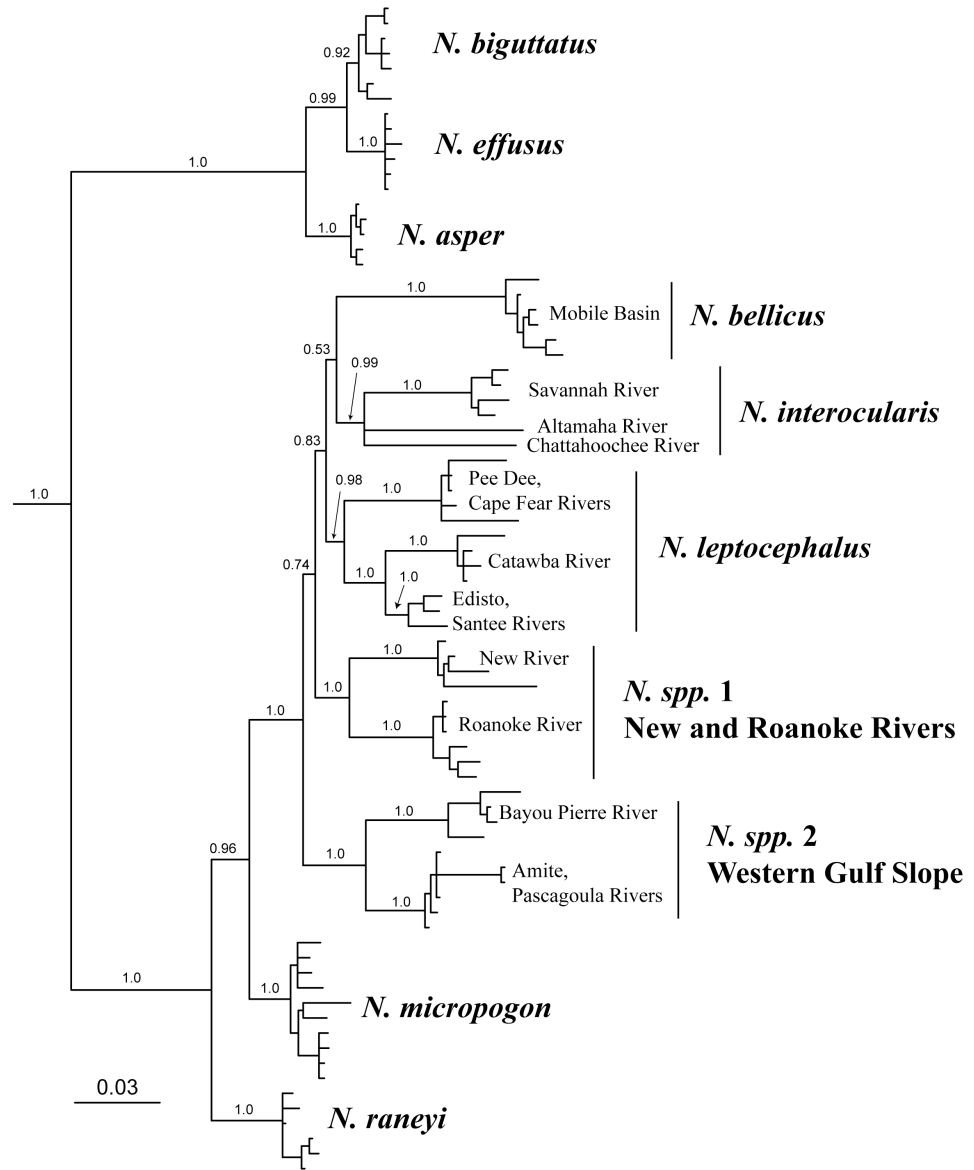
Figure 1.2. Phylogenetic hypothesis generated through mixed-model, partitioned Bayesian analysis of DNA sequence data, showing relationships among *Nocomis* populations. Numbers above nodes indicate Bayesian posterior probabilities. Outgroup taxa have been pruned for clarity. Species names follow this study.
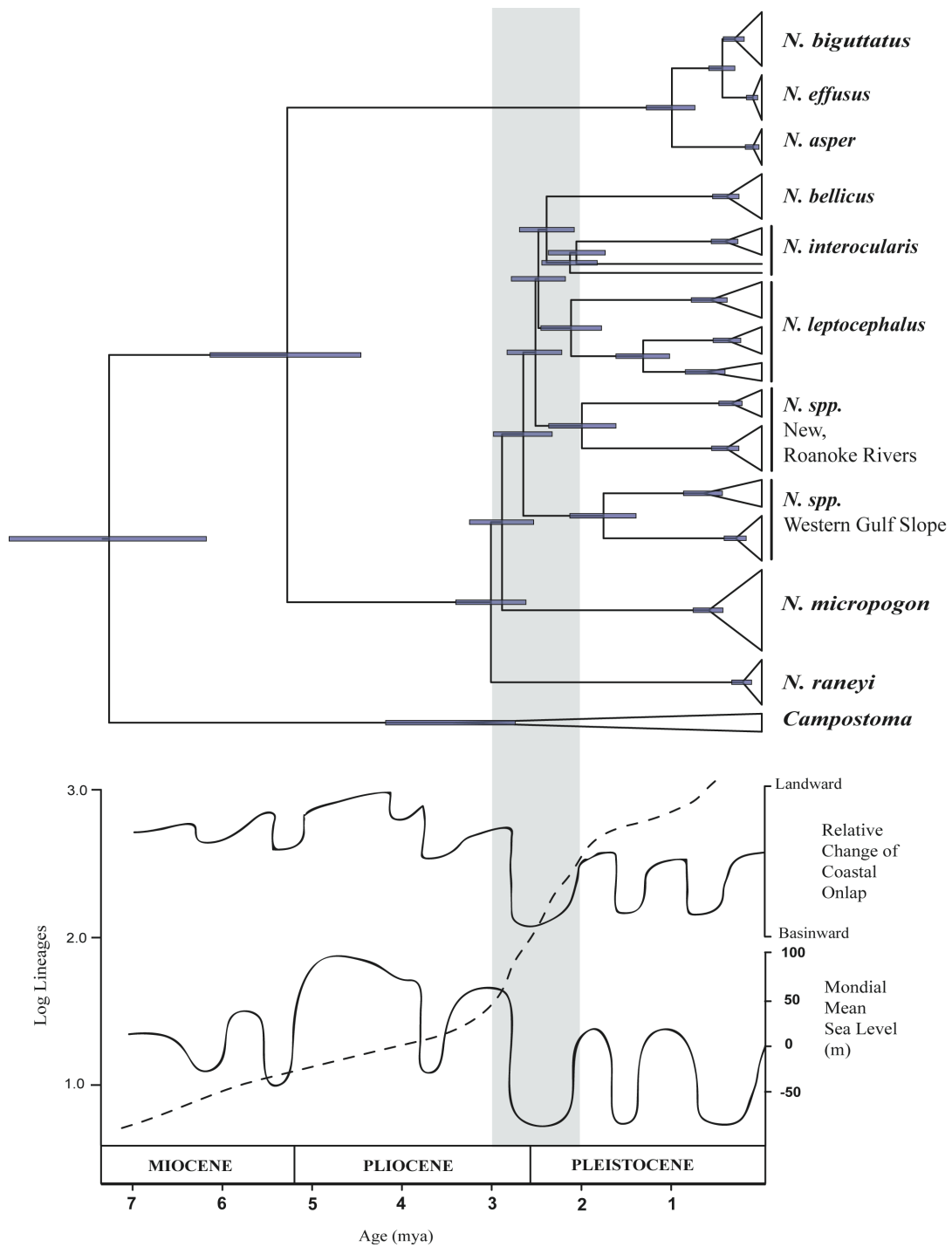
Figure 1.3. Chronogram of the partitioned Bayesian phylogeny of *Nocomis*. Divergence dates are plotted along the x-axis and grey bars on nodes indicate the 95% highest posterior density for each date. Vertical grey bar highlights a period of significantly elevated cladogenesis (r = 1.43 s/my, p = 0.002) from the global rate (r_G = 0.35 s/my). The dotted line plots the log of the number of *Nocomis* lineages through time (LTT), and black curves represent relative coastal onlap and global mean sea levels (redrawn from Haq et al. 1987).

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APPENDIX 1.1

Materials Examined**:**

Nocomis asper:

Nasp8, AMS 99-02, South Fork Ouachita River, Montgomery County, AR; Nasp9, JJDE 03-17, Elk River, McDonald County, MO; Nasp201, Nasp202, AMS 01-07, Flint Creek, Benton County, AR; Nasp 203 AMS 01-05, Shoal Creek, Newton Co, MO.

Nocomis biguttatus:

Nbig1, 96SC065, Sunrise River, Chisago County, MN; Nbig2, AMS 99-33, Black River, Reynolds County, MO; Nbig6, Nbig46, Nbig47, AMS 99-35, Tippecanoe River, Fulton County, IN; Nbig7, PBB 00-20, Seely Brook, Anoka County, MN.

Nocomis effusus:

Neff10, Neff11, Neff50, Neff51, Neff52, AMS 01-25, Otter Creek, Wayne County, KY.

Nocomis leptocephalus bellicus:

Nbel14, Nbel44, Nbel45, JJDE 03-20, Bayou Pierre, Copiah County, MS; Nbel16, Nbel39, Nbel40, Nbel41, AMS 00-10, Hachemedega Creek, Coosa County, Al; Nbel88, Nbel89, Blue Girth Creek, Bibb County, AL; Nbel90, Nbel91, Bowie Creek, Covington County, MS; Nbel92, Brushy Creek, Amite County, MS; Nbel93, Nbel94, Nbel95, Nbel96, JJDE 03-24, Pearl River, Marion County, MS.

 Nocomis leptocephalus interocularis:

Nint70, Nint71, BCN 04-10, Horsepasture River, Transylvania County, NC; Nint74, Nint86, Nint87, Acorn Creek, Carroll County, GA; Nint76, Nint77, AMS 05-06, Hencoop Creek, Anderson County, SC; Nint78, Nint79, Chinquapin Creek, Aiken County, SC; Nint81, AMS 05-05, Mountain Creek, Greenville County, SC; Nint84, Candler Creek, Jackson County, GA.

 Nocomis leptocephalus leptocephalus:

Nlep12, Nlep48, Nlep49, AMS 01-54, County Line Creek, Caswell County, NC; Nlep13 Nlep53. Nlep54, Nlep55, AMS 01-50, Catawba River, McDowell County, NC; Nlep21, Nlep22, Nlep23, AMS 01-55, Stony Creek, Giles County, VA; Nlep24, Nlep26, PBB 03-05, Dan River, Patrick County, VA; Nlep27, PBB 03-09, North Fork New River, Watauga County, NC; Nlep30, Nlep31, Nlep32, PBB 03-03, Ararat River, Patrick

County, VA; Nlep65, BCN 04-07, Deep River, Randolph County, NC; Nlep66, South Fork New River, Watauga County, NC; Nlep72, Uwharrie River, NC.

Nocomis micropogon:

Nmic5, AMS 01-42, Walhonding River, Coshocton County, OH; Nmic17, AMS 01-30, South Fork Kentucky River, Owsely County, KY; Nmic18, AMS 02-30, Shoal Creek, Lauderdale County, AL; Nmic56, BCN 04-01, Elk River, Braxton County, WV; Nmic68, BCN 04-09, South Toe River, Yancey County, NC.

Nocomis platyrhynchus:

Npla29, PBB 03-03, North Fork New River, Watauga County, NC; Npla58, Npla59, BCN 04-02, East Fork Greenbriar River, Pocahontas County, WV;Npla67, BCN 04-08, South Fork New River, Watauga County, NC.

Nocomis raneyi:

Nran62, Nran63, Nran97, Nran98, Nran99, Nran200, BCN 04-04, Tar River, Vance/Franklin County, NC.