Timing of clade divergence and discordant estimates of genetic and morphological diversity in the Slender Madtom, *Noturus exilis* (Ictaluridae)

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**ABSTRACT**

Phylogeographic relationships, the timing of clade diversification, and the potential for cryptic diversity in the Slender Madtom, *Noturus exilis*, was investigated using mitochondrial *Cyt b*, nuclear RAG2, shape analysis, and meristic and pigmentation data. Three well-supported and deeply divergent clades were recovered from analyses of genetic data: Little Red River (White River drainage) clade, Arkansas + Red River (Mississippi River) clade, and a large clade of populations from the rest of the range of the species. Recovered clades showed little to no diagnostic morphological differences, supporting previous hypotheses of morphological conservatism in catfishes, and indicating morphology may commonly underestimate diversity in this group of fishes. The Little Red River clade is the most distinct lineage of *N. exilis* with 11 POM pores (vs. 10 in other populations) and unique *Cyt b* haplotypes and RAG 2 alleles. However, treating it as a species separate from *N. exilis* would imply that the other major clades of *N. exilis* are more closely related to one another than they are to the Little Red River clade, which was not supported.

The UCLN age estimate for *Noturus* was 23.9 mya (95% HPD: 13.49, 35.43), indicating a late Oligocene to early Miocene origin. The age of *N. exilis* was estimated as late Miocene at 9.7 mya (95% HPD: 5.32, 14.93). Diversification within the species spanned the late Miocene to mid-Pleistocene. The largest clade of *N. exilis*, which dates to the late Miocene, includes populations from the unglaciated Eastern and Interior Highlands as well as the previously glaciated Central Lowlands. Diversification of this clade coincides with a drastic drop in sea-level and diversification of other groups of Central Highlands fishes (Centrarchidae and Cyprinidae). Sub-clades dating to the Pleistocene show that northern populations occurring in previously glaciated regions resulted from dispersal from populations in the Ozarks up the Mississippi River following retreat of the Pleistocene glaciers. Pre-Pleistocene vicariance, such as drainage pattern changes of the Mississippi River, also played a prominent role in the history of the species. The incorporation of a temporal estimate of clade diversification revealed that in some instances, phylogeographic breaks shared with other aquatic species were best explained by different or persistent vicariant events through time, rather than a single shared event.

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1. Introduction

For freshwater fishes of the Central Highlands region of North America (including the Eastern and Interior Highlands), vicariance and dispersal facilitated by landscape and climate alterations of Pleistocene glacial cycles, have been major factors shaping present-day distribution and diversity of many species (*Wiley and Mayden, 1985; Hocutt et al., 1986; Strange and Burr, 1997; Soltis et al., 2006*). Recent studies also have shown that pre-Pleistocene lineage diversification occurred both among and within these highland regions (*Kinzie and Keck, 2001; Berendzen et al., 2008; Hollingsworth and Near, 2009*), a result consistent with the Central Highlands Vicariance Hypothesis (CHVH; *Mayden, 1988*), which predicts the presence of a diverse highlands fish fauna prior to the Pleistocene glacial cycles.

Despite numerous tests of the CHVH (*Strange and Burr, 1997; Near et al., 2001; Faber et al., 2009*) relatively few phylogeographic studies of highland fishes have incorporated fossil-calibrated approaches to estimate the timing of clade divergence among and within the highland regions. Studies have been limited primarily to darters (*Percidae; Near and Bernard, 2004; Near and Keck, 2005; Hollingsworth and Near, 2009; Keck and Near, 2010; Near et al., 2011*) minnows (*Nagle and Simons, 2012*), and basses and
sunfishes (Centrarchidae; Near et al., 2003, 2005). Others have relied on clock-like rates of sequence evolution to estimate the timing of clade divergence for Highland fishes and to link phylogeographic patterns to geological events consistent with the estimated clade ages (e.g., Hardy et al., 2002; Eggé and Simons, 2006; Berendzen et al., 2008). Shared distributional patterns of species or clades also have been commonly used as support for the occurrence of common geological events or histories shared among taxa, ignoring the potential for pseudocongruent patterns among the studied groups (Avise, 2000). In the absence of temporal data, whether shared patterns observed across lineages are due to a single shared historical event or to different events at different points in time cannot be distinguished (Donoghue and Moore, 2003). Furthermore, incorporation of temporal data in phylogenetic studies allows for direct evaluation of the degree of diversity originating within and among these highland regions prior to versus during the Pleistocene and allows for a more precise interpretation of the geological events responsible for contemporary diversity patterns.

The Slender Madtom, Noturus exilis (Ictaluridae), is one of the most wide-ranging species of the North American genus Noturus with geographically isolated populations in the Eastern and Interior Highlands and the previously glaciated Central Lowlands (Page and Burr, 2011), and thus, offers the opportunity to further test the timing and degree of diversification of highland fishes. The species shows both genetic (Hardy et al., 2002) and morphological (Hubbs and Raney, 1944; Taylor, 1969) variation across its range. For example, LeGrande (cited in Robison and Buchanan, 1988) found that the Little Red River, a tributary of the White River in Arkansas, was karyotypically distinct from other populations. Hardy et al. (2002) found high levels of genetic structure and reduced levels of gene flow among populations and hypothesized that species divergence occurred prior to the Pleistocene using a molecular clock rate of evolution applied to mtDNA sequences. Estimates of fossil-calibrated divergence times have not been provided for the species and only one study of North American catfishes (Hardman and Hardman 2008) has used fossil-calibrated estimates for the diverse genus Noturus. These findings suggest long-term barriers to gene flow across much of the range of the species and the possible occurrence of several species lineages.

Although Taylor (1969) documented meristic, fin pigmentation, and body shape differences among several populations from the Interior Highlands, he concluded that the observed variation did not warrant taxonomic recognition. He noted that in general the morphology of madtom catfishes (Noturus) was conserved, especially among closely related species, a result supported by several recent examinations of madtom catfishes (e.g., Eggé and Simons, 2006, 2009). These observations, imply that morphology alone may commonly underestimate genetic and species diversity in this group of fishes.

The goals of this study were to examine the relative genetic and morphological variation in N. exilis to explore the possibility of currently unrecognized species diversity and to use this multi-faceted dataset to comparatively study morphological diversification relative to the occurrence of gene-based clade diversification to further evaluate whether morphology commonly underestimates genetic or species diversity in madtom catfishes. Also, catfish fossils were used to calibrate rates of sequence evolution to test the hypothesis of pre-Pleistocene divergence within Noturus exilis and commonly invoked for Central Highlands fishes. Lastly, inferred clade ages were compared to those of other similarly distributed species to identify whether shared phylogeographic patterns resulted from common or independent historical events.

2. Methods

2.1. Specimen and tissue collection

Specimens of Noturus exilis were collected from across the range of the species using standard seineing techniques and a backpack electrofisher. A small piece of fin was removed from each specimen and placed in separate vials of 95% ethanol for DNA analysis. The remaining specimen was anesthetized in MS-222 and preserved in a 10% formalin solution and later transferred to 70% ethanol for permanent storage in the fish collection at the Florida Museum of Natural History. The 95% ethanol-preserved tissues not consumed by this study were cataloged and stored in a cryofreezer in the Genetic Research Repository at the Florida Museum of Natural History. Additional specimens and tissues of N. exilis were borrowed from various institutions. Institutional abbreviations provided in the materials examined follow Leviton et al. (1985).

2.2. DNA sequencing

Genomic DNA was extracted from fin or muscle tissue using a 5.0% Chelex solution with 3 µl of Proteinase K and digestion overnight or by using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc.) and following the manufacturer’s instructions. The extracted DNA was used as template for amplification of two markers by polymerase chain reaction (PCR). The mitochondrial Cytochrome b gene (Cyt b) was amplified and sequenced for 90 individuals from 41 localities from across the range of the species (Fig. 1 and Table 1). In addition, 58 individuals from the same 41 localities were amplified and sequenced for the nuclear recombination-activating gene (RAG2). In most cases, Cyt b sequence data were generated for multiple individuals per site to evaluate within- and among population variation and to assess phylogeographic patterns. Because RAG2 provided much lower levels of variation it was less informative for population-level comparisons. Therefore, only a subset of the individuals sequenced for Cyt b was sequenced for RAG2. Individuals sequenced for RAG2 were chosen to capture the overall clade diversity recovered by the Cyt b data set.

All PCR reactions were conducted using a PTC-200 or PTC-100 Peltier Thermocycler (MJ Research, Inc.). The complete coding region of the mitochondrial Cyt b gene was PCR amplified using previously published primers (Gluc2 and Pro-R1; Hardman and Page 2003). PCR reactions were 25 µl total volume and consisted of 1.5 mM MgCl2 (Bioline), 2.5 µl of 10 × NH4 PCR buffer (Bioline), 0.2 mM dNTP mix, 1 µl each of 10 pmol primers, and 0.5 U Biolase Tag DNA polymerase (Bioline). The thermal cycling protocol consisted of an initial denaturation at 94 °C for 30 s, followed by 25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s, and a final extension at 72 °C for 5 min.

RAG2 was PCR amplified using previously published primers (IcRAG2-F1 and IcRAG2-R1; Hardman and Page, 2003; Hardman, 2004) and the same reagents and concentrations as described for Cyt b. PCR cycling conditions followed those of Eggé and Simons (2006).

PCR purification and cycle sequencing of all products were performed by the Sanger Sequencing Facility at the University of Florida. The Pro-R1 PCR primer and the previously published IcYrb-R1 primer (Hardman and Page, 2003) were used in all Cyt b sequencing reactions. The primers used to amplify RAG2 also were used to sequence the marker for all individuals. Resulting sequences were edited and initially aligned using CodonCode Aligner (v2.0.6) with final alignments adjusted by eye.
2.2.1. Genetic variation

Pairwise sequence divergences and average pairwise divergences within and between recovered clades were generated using Mega v. 4 (Tamura et al., 2007) for both markers to examine patterns of sequence variation. Base compositional analysis was conducted to determine the average frequency of bases in both data sets using PAUP* 4.0b10 (Swofford, 2003).

2.2.2. Gene tree reconstruction

Independent phylogenetic hypotheses were generated for each dataset using the maximum parsimony criterion implemented in PAUP* 4.0b10 (Swofford, 2003). The most parsimonious trees were found using the heuristic search option and 100 random addition sequence replicates with the tree-bisection-reconnection option of branch-swapping and save multiple trees, branches of length zero collapsed, and retention of minimal length trees options. Node support was assessed using non-parametric bootstrap re-sampling procedure with 1000 pseudoreplicates.

Bayesian maximum likelihood analyses (BML) were used to generate phylogenetic hypotheses for each dataset independently and for a concatenated data set that included both the Cyt b and RAG2 genes. In all BML analyses, each gene was partitioned by codon position. The optimal model of sequence evolution for each data partition was determined from 56 progressively complex models tested in Modeltest v.3.7 (Posada and Crandall, 1998). The selected model under the Akaike Information Criterion (AIC) and its parameters were implemented in the respective BML analyses in Mr. Bayes 3.1.1 (Ronquist and Huelsenbeck, 2003) using the LSET and APPLYTO commands. Model parameter values were estimated for each partition independently using the ULINK command. Four Markov chains with default priors were used in all analyses and random trees were used to start each chain. Runs consisted of 5 million generations of Markov Chain Monte Carlo (MCMC) simulations. Four replicate runs were conducted to ensure the MCMC went through a sufficient number of iterations to allow convergence in the estimations of tree topology with the best maximum likelihood posterior probability. The burn-in of the MCMC analysis was determined by graphically examining the ML scores at each of the sampled generations to find where values converged. All trees recorded prior to the burn-in were discarded. The posterior probability or frequency at which a clade occurred in the remaining trees was used as an indication of node support. In all analyses, N. gyrinus (Cyt b GenBank No.: AY327295; RAG2 GenBank No.: AY327090), N. flavus (Cyt b: AY327287; RAG2: AY327086), and N. insignis (Cyt b: AY327301; RAG2: AYDQ492400) were used as outgroups. In the Cyt b dataset, N. albater (AY327268), N. baileyi (AY327273), N. eleutherus (AY327278), N. hildebrandi (AY327298), N. miurus (AY327307), and N. stigmosus (AY327319) also were used as outgroups; and N. lachneri (AY327090) and N. leptocanthus (AY327094) also were used as outgroups in the RAG2 analysis.

Fig. 1. Localities examined for all gene-based analyses of Noturus exilis. Localities examined for the morphological data comparisons are provided in Section 5.
2.3. Estimation of divergence times

Compared to other groups of benthic North American fishes, such as darters (Percidae), catfishes have a relatively rich and well-documented set of fossils (e.g., Lundberg, 1975), which are valuable tools essential to estimating reliable node ages. A test for rate heterogeneity among lineages using the X² statistic implemented in PAUP* 4.0b10 revealed significant rate heterogeneity in *N. exilis* (*P* < 0.001). Thus, molecular divergence time estimates for *N. exilis* clades were generated using a relaxed clock method. The 95% highest posterior density (HPD) of divergence times was estimated using the uncorrelated lognormal model (UCLN; Drummond et al., 2006), which allows for uncertainty in fossil calibration ages, implemented in BEAST v. 1.6.1. (Drummond et al., 2006), which allows for uncertainty in fossil calibration ages, implemented in BEAST v. 1.6.1. (Drummond et al., 2006).
and Rambaut 2007). Three fossil-determined minimum bound log-

normal age estimates that represented prior distributions, and the

best-fit models of sequence evolution for the two-gene data set

were used to estimate the posterior probability density of diver-
gence times.

A separate alignment of the Cyt b + RAG2 sequences that in-
cluded the same N. exilis, N. gyrinus, N. flavus, and N. insignis se-
quences as in the combined phylogenetic analysis described
previously, plus two species of Ameiurus (A. brunnienis; A. catus),
two species of Ictalurus (I. furcatus; I. punctatus), and Cranoglan-
dis boulderus (Cyt b: AF16879; RAG2: DQ492401), was generated. Cra-

noglanis is member of the family Cranoglanidiidae, which is sister
to catfishes of the family Ictaluridae (Sullivan et al., 2006; Lundberg
et al., 2007), to which Noturus, Ameiurus, and Ictalurus belong. This

alignment was subjected to an independent BML analysis using the

same parameters as described previously for the two-gene analysis

of the more limited data set to estimate a tree that included taxa

encompassing selected fossils for estimating divergence times of

Noturus exilis clades and to serve as a target tree in the BEAST v.

1.6.1 analyses.

Node age constraints were set using lognormal priors with mini-

mum age bounds determined from a subset of dated catfish fossils

previously used by Lundberg (2007) to estimate lacantuniid catfish
divergence and by Hardman and Hardman (2008) to estimate Ameiurus
(Bullhead Catfish) diversification rates, and two Noturus fossils
(Lundberg, 1975) not used in previous studies. The mini-
mum age (Ma) estimates for fossils used as calibration points were:

1) Aspehus sp., 65 mya (lognormal prior mean and standard devi-
ation of 1.1 and root prior offset of 65); (2) Ictalurus, 19 mya (log-
normal prior mean and standard deviation of 1.0 and offset value
of 19) and (3) Noturus sp./N. furiosus, 2.6 mya (lognormal prior
mean and standard deviation of 1.0 and offset 2.6). Aspehus sp. is
the oldest known ictalurid fossil (63–65 Ma; Lundberg, 1975) and
was applied to the node representing the divergence of Ictaluridae
following Hardman and Hardman (2008; Fig. 5, node 1). The Ictalu-
rus fossil date (19 Ma; Lundberg 1975, 1992) was applied to the
node representing the Ictalurus clade, also following Hardman
and Hardman (2008; Fig. 5 node 2). The application of these fossils
and their corresponding dates as minimum age estimates for the
described nodes have been shown to be good predictors of catfish
divergence times (Lundberg et al., 2007; Hardman and Hardman,
2008). The third calibration point is based on two fossils of Noturus
described from the Pliocene (Fig. 5, node 3; Lundberg et al., 2007).
The use of 2.6 my as a minimum age estimate for the genus Noturus
was supported by estimates of Pliocene divergence times for other
species of Noturus (Egge and Simons, 2006). Other fossils were
available, but could not be placed phylogenetically given the esti-
mated phylogeny.

The birth–death process speciation prior was used to estimate
branching rates and a total of four independent runs of 60 million
generations each were conducted in BEAST v. 1.6.1. Resulting log
files were viewed in Tracer v. 1.4 (Drummond and Rambaut,
2007) to examine marginal probabilities of each run to ensure con-
vergence of parameter values and estimated node ages and
to ensure effective sample sizes (>1000). Samples recorded prior
to stationarity were discarded as burn-in. LogCombiner v. 1.4.6
was used to combine the resulting tree and log files of each run.
TreeAnnotater v. 1.4.6 (Drummond and Rambaut, 2007) was used to
summarize the posterior probability density of the combined
tree and log files. FigTree v. 1.1 (Drummond and Rambaut,
2007) was used to visualize the mean and 95% highest posterior
probability density estimates of divergence times for Noturus exilis
clades. A separate BEAST analysis was conducted without align-
ment data, using the same parameters as previously described,
to determine the effect of the calibration priors on divergence
time estimates.

2.4. Morphological variation

Examination of morphological variation focused on the same
geographic regions and river systems as that examined for genetic
variation; however, several populations for which tissues for gen-
etic data were unavailable were included in the morphological
comparisons. These included extirpated populations from the
Green River (Ohio River) system and Kentucky River system (Ohio
River) and a possibly extirpated population from the Licking River
(Ohio R.) of Kentucky. A list of the populations examined for mor-
phology is provided in Section 5.

2.4.1. Morphometrics

Measurements were taken on 281 specimens from 58 localities
distributed across the range of the species. Variation in body shape
was assessed using a truss network (Bookstein et al. 1985) of 16
interlandmark distances and an additional 16 standard measure-
ments including: (1) dorsal-fin base length, (2) dorsal-fin insertion
to adipose-fin insertion, (3) adipose-fin insertion to anal-fin inser-
tion, (4) anal-fin base length, (5) anal-fin origin to pelvic-fin origin,
(6) pelvic-fin origin to pectoral-fin origin, (7) dorsal-fin insertion
to pectoral-fin origin, (8) dorsal-fin insertion to pelvic-fin origin,
(9) dorsal-fin insertion to anal-fin origin, (10) body depth at the
posterior eye margin, (11) body width at the posterior eye margin(12)
body depth at dorsal-fin origin, (13) body width at dorsal-fin ori-

gin, (14) body width at pectoral-fin origin, (15) body width at
anal-fin insertion, (16) snout to posterior eye margin, (17) snout
to dorsal-fin insertion, (18) snout to adipose-fin insertion, (19)
snout to anal-fin origin, (20) snout to pelvic-fin origin, (21) snout
to pectoral-fin origin, (22) inter-orbital distance, (23) head length,
(24) dorsal-fin spine length, (25) pectoral-fin spine length, (26)
predorsal length, (27) adipose-fin base length, (28) dorsal-fin height,
measured from dorsal-fin origin to end of longest ray, (29)
anal-fin height, measured from anal-fin origin to end of long-

gest ray (30) pectoral-fin height, measured from pectoral-fin origin
to end of longest ray (31) caudal-fin height, measured along medial
ray, and (32) standard length (SL). Measurements were made on
the left side of the body on specimens >35 mm SL. Unless noted
otherwise, methods followed those described by Hubbs and Lagler

Measurements were analyzed using a multivariate Principal
Components Analysis (SYSTAT v8.0) of log-transformed data. The
first principal component recovered was considered to be a size
component and was not used in population comparisons. Variables
with high component loadings were considered potentially infor-
mative taxonomically and to have contributed most to any separa-
tion among taxa or populations in the PCA scatterplots.

To further identify potentially diagnostic characters, ranges of
measurements standardized by standard length were also
compared to identify populations with non-overlapping or margin-
ally-overlapping ranges. Measurements are presented as thou-
sandths of SL.

2.4.2. Pigmentation

Characteristics were described from 164 specimens and 34
localities from across the range of the species. The width or depth
of the distal black band was measured for caudal, anal and dorsal
fins; measurements were made at the middle of the fin, and values
were standardized for comparison by dividing the width or depth
of the band by the respective fin height or length. Notes were made
on the distribution of black coloration in the fin (e.g., covering all
rays, all rays except two, etc.). In the caudal fins of some individu-
als, the fin was black or dusky throughout and the exact width of
the distal black band could not be determined. In such cases, fins
were noted as ‘overall dusky’.
3. Results

3.1. Genetic variation

The 90 individuals of Noturus exilis sequenced for Cyt b had 49 different haplotypes (Table 1). Haplotypes were narrowly distributed geographically, with each restricted to a single river system or to a single tributary of a system (Table 1). Of the 1140 bp of the gene, 154 sites were variable, 91 of the variable sites were parsimony informative. Mean base composition for Cyt b was A = 0.27, C = 0.29, G = 0.14, and T = 0.30. Cytochrome b pairwise distances within N. exilis ranged from 0.0% to 10.0% with an overall average of 2.8% sequence divergence. Average pairwise divergences for the Cyt b gene within and between the major N. exilis clades recovered in Figs. 2 and 4–6 are reported in Table 2. In all cases, divergence between clades was more the twice that observed within the major recovered clades.

The 58 individuals sequenced for RAG2 had 11 alleles (Table 1). Alleles were more widely distributed than recovered haplotypes, with several shared across drainage divides, although the allele from the Little Red River system (Clade A) was not found at any other location (Table 1). Of the 814 bp sequenced for RAG2, 35 sites were variable, 15 of the variable sites were parsimony informative. Mean base composition for RAG2 was A = 0.25, C = 0.25, G = 0.25, T = 0.45. Pairwise-distances within N. exilis ranged from 0.0% to 0.4% with an overall average of 0.2% sequence divergence. Average pairwise distances for the RAG2 gene within and between the major N. exilis clades recovered in Figs. 2 and 4–6 are reported in Table 3. Within clade divergences were lower than between clade levels with the exception of Clade C, which had the same average divergence among members as that found between Clades B and C.

3.2. Models of sequence evolution

The best-fit model of sequence evolution found using Modeltest and AIC included TVMef+I+G for first positions of the Cyt b gene, HKY+I for second positions, and GTR+I+G for third positions. GTR+I, JC69 and HKY+I models were selected for first, second, and third codon positions, respectively, of the RAG2 gene.

3.3. Phylogeographic relationships

The BML and parsimony analyses of the genetic datasets resulted in identical topologies for Noturus exilis, differing only in support values for several clades. Therefore, only BML trees are shown and discussed, but both posterior probabilities and bootstrap support values are presented.

### Table 2

Average between clade and within clade cytochrome b sequence divergences. Clades correspond to those recovered in Figs. 2 and 4.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Average between clade divergences (%)</th>
<th>Average within clade divergence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade A</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Clade B</td>
<td>4.1</td>
<td>1.4</td>
</tr>
<tr>
<td>Clade C</td>
<td>5.2</td>
<td>4.6</td>
</tr>
</tbody>
</table>

3.3.1. Cytochrome b

Based on the mitochondrial data, N. exilis was recovered as a well-supported clade (1.0 pp/100% bs) with substantial geographic variation (Fig. 2). The species was comprised of three well-supported and deeply divergent clades: (A) a Little Red River (White R. drainage) clade, (B) an Arkansas + Red River (Mississippi River) clade, and (C) a large clade including populations from the rest of the range of the species. The larger clade (Clade C; Fig. 2) includes populations from the unglaciated Eastern and Interior Highlands as well as the previously glaciated Central Lowlands. Within the latter, well supported subclades were recovered for the upper White River system (including the North Fork White River), which was sister to all other clades, the Strawberry R (Black-White R. drainage), the upper Black and Current rivers, (Black-White R. drainage), and a Tennessee and Cumberland rivers clade that was sister to N. exilis from the Missouri River system, Bay Creek (lower Ohio R. drainage), upper Mississippi River systems, and other direct Mississippi River tributaries such as Clear Creek and the St. Francis River. Within Clade B, N. exilis from the Red River in Oklahoma was sister to N. exilis from the Arkansas + Neosho (Arkansas R. drainage) rivers.

3.3.2. Rag2

As expected, the nuclear RAG2 gene showed lower levels of resolution among populations of N. exilis compared to the mitochondrial marker (Fig. 3). Of the three deeply divergent clades of N. exilis recovered with the Cyt b data, RAG2 only supports the monophyly of the Little Red River clade (Clade A in Fig. 2). However, RAG2 also supported several of the subclades recovered with the Cyt b data, including the Tennessee and Cumberland rivers clade, and the Missouri River, upper, and middle Mississippi river systems, and Bay Creek of the lower Ohio River clade. There was no support for the monophyly of N. exilis from the Arkansas, Neosho, and Red rivers, and alleles were shared among the Strawberry and Current rivers of the Black River (White R. drainage), resulting in no resolution of relationships among these populations as seen with Cyt b. Noturus exilis from the upper White River was recovered as a clade but without the North Fork White River individuals.

3.3.3. Cyt b + RAG2

Analysis of the combined dataset recovered relationships identical to those seen in the Cyt b only analysis (Fig. 4). The trees only differed slightly in the posterior probability support values for several of the recovered clades and the total number of individuals included for the various populations examined.

3.4. Estimates of divergence times of recovered clades

The resulting chronogram for Noturus exilis (Figs. 5 and 6) was similar to the topology recovered in the Cyt b and Cyt b+RAG2 phylogenetic analyses (Figs. 2 and 4). Substantial among-branch nucleotide substitution rate heterogeneity was observed based on the coefficient of variation statistic (=0.50, 95% HPD: 0.23, 0.72). The UCLN age estimate for the Noturus clade was 23.9 mya (95% HPD: 13.49, 35.43), indicating an early Miocene to late Oligocene origin for the genus. The age of the MRCA of the N. exilis clade was estimated as late Miocene at 9.67 mya (95% HPD: 5.32, 14.93). The estimated ages for the MRCA of each of the three major clades (A–C) of N. exilis recovered ranged from mid-Pleistocene at 0.36 mya (95% HPD: 0.05, 1.00) for Clade A, which includes populations from the Little Red River system to the late Miocene at 5.47 mya (95% HPD: 3.16, 8.73) for the large Clade C (Table 4).

3.5. Morphological variation

Despite evidence for geographically defined, divergent genetic lineages of pre-Pleistocene origin, Noturus exilis shows little...
variation in the meristic characters examined. Only POM pores showed a modal difference between *N. exilis* from the Little Red River (White R. drainage) and *N. exilis* from elsewhere (Table 5). Fin pigmentation was highly variable, but substantial variation was observed within populations and was not definable geographically. Variation in body shape and general morphometric characters was observed. However, the range of variation observed among the three gene-based clades of *N. exilis* showed nearly complete overlap in the PCA and ranges of all variables were overlapping (Fig. 7, A.1). On a finer scale, substantial variation in body shape was noted among tributaries of the White River system (A.2, A), the Arkansas River system (A.2, B), the Ohio River system (A.2, C) and the Missouri River system (A.2, D). Variables with high component loadings that contributed most to observed separation among population in PCA scatterplots included: dorsal-fin insertion to pelvic-fin origin (0.638), dorsal-fin insertion to anal-fin origin (0.677), body depth at dorsal-fin origin (0.640), and body width at pelvic-fin origin (0.615) for PC2; and dorsal-fin insertion to pectoral-fin origin (0.732); head width at posterior eye margin (0.605), body width at dorsal-fin origin (0.621), snout to anterior eye margin (0.618), snout to dorsal-fin insertion (0.845), snout to pectoral-fin origin (0.688), head length (0.782) and snout to dorsal-fin origin (predorsal length, 0.769) for PC3.

4. Discussion

4.1. Genetic and morphological variation and implications for taxonomy

Clades recovered by the Cyt b gene were allopatrically distributed and divergence among clades was more than twice that observed within the major clades recovered, a pattern consistent with category I phylogeographic structure (Avise, 2000). This pattern is consistent with the previous hypothesis of long-term
barriers to gene flow among geographically definable populations of *Noturus exilis* (Hardy et al., 2002) and with criteria invoked under several species concepts. However, with the exception of Clade A (Little Red River clade), the pattern of divergence observed in the Cyt b tree is not replicated in the nuclear or morphological data sets.

Of the three divergent lineages of *Noturus exilis*, only Clade A can be distinguished by morphology and is recovered as a clade by both genetic markers examined. The Little Red River population, although regarded as a distinct genetic lineage with 11 POM pores (vs. 10 in populations in Clade B and C), unique Cyt b haplotypes (H14, H33, H11) and RAG 2 alleles, and a unique chromosome count (LeGrande cited in Robison and Buchanan (1988)), is not treated as a species separate from *N. exilis* given the current data.

Although we note that morphological similarity among clades does not necessarily imply that clades are conspecifics (Zink and McKitchick, 1995; Baric and Sturmbauer, 1999), elevation of the Little Red River clade to species implies that the other two major clades of *N. exilis* are more closely related to one another than they are to the Little Red River clade. Neither molecular dataset supports this conclusion. Alternatively, elevation of the Little Red River population would require that we also taxonomically recognize members of Clade B (Arkansas + Red rivers clade; the type locality for *N. exilis* falls within the range of Clade C); a decision not supported by either the nuclear or morphological data. Additional data are needed to resolve relationships among lineages of *N. exilis* and examination of additional morphological characters may support taxonomic recognition of the recovered clades.

The low levels or lack of geographically definable variation in morphology observed for the genetically divergent clades of *Noturus exilis* are consistent with previous observations of morphological conservatism among closely related madtom catfishes (Taylor, 1969; Egge and Simons, 2009). Genes and morphology are not expected to evolve at the same rate (Mayden, 2002) and disparity in the rate of evolution of morphology and genes has been previously demonstrated in *Noturus* catfishes (Thomas and Burr, 2004; Burr et al., 2005; Egge and Simons, 2006, 2009; Faber et al., 2009). In most studies, results have been similar to the findings herein – morphological characters were conserved across deeply divergent, gene-based clades. However, Thomas and Burr (2004) found that morphological divergence between the sister species *N. gladiator* and *N. stigmosus* was greater than genetic divergence inferred from
Cytochrome b. With this notable exception, the general observation of conserved morphology among closely related species of madtom catfishes and the low levels observed among genetically divergent clades of *N. exilis* (Taylor, 1969; Egge and Simons, 2006, 2009) indicate that morphology alone may commonly under-estimate diversity for this group of fishes.

Given the estimated early Miocene to late Oligocene age of the genus, the low-levels of morphological divergence in madtom catfishes is interesting considering the degree of morphological variation observed for other similarly-aged groups of small-bodied, benthic, Central Highlands stream fishes such as darters (Percidae). The relatively low levels of morphological divergence observed in madtom catfishes may be due, at least in part, to reduced levels of selection acting on morphological features. For example, darters rely on external morphological features such as color and fin shape for mate identification, selection, and antagonistic displays for nest sites. Thus, considerable variation in external morphology, particularly in color and pigmentation pattern, is observed among closely related species as a result of sexual selection (Page, 1983). Catfishes, however, rely less on visual cues, using other senses such as smell and taste to interact with the biotic and abiotic environment and are primarily nocturnal (Lundberg, 1970).

4.2. Timing of clade diversification

The Central Highlands Vicariance Hypothesis (Mayden, 1988) predicts a widespread diverse pre-Pleistocene fish fauna and numerous studies have observed patterns of diversity and clade age estimates that are consistent with this hypothesis (e.g., Berendzen et al., 2008; Hollingsworth and Near, 2009; Keck and Near, 2010). Based on fossil evidence the genus *Noturus* arose prior to the Pleistocene (Lundberg, 1975), a result also supported herein by the late Oligocene age estimate for the MRCA of the genus. *Noturus exilis* was estimated to have originated in the Miocene, indicating that...
Fig. 5. Maximum credibility chronogram for catfish species estimated from the combined independent BEAST analyses using the mitochondrial cytochrome b and nuclear RAG2 genes. Bars show uncertainty in the estimated divergence with the length representing the 95% highest posterior density (HDP) of node ages. Numbers on nodes highlight the phylogenetic placement of fossil calibrations described in the methods: (1) *Astecephus*, 65 Ma; (2) *Ictalurus*, 19 Ma; and (3) *Noturus*, 2.6 Ma. Italicized numbers at nodes are mean node age estimates. Posterior probability and bootstrap support values for nodes are given in Figs. 2–4.
both the genus and species lineages were part of the diverse pre-Pleistocene fish fauna, lending further support to Mayden’s (1988) Central Highlands Vicariance Hypothesis. Diversification within *Noturus exilis* occurred more recently, spanning the pre-Pleistocene–Pleistocene boundary. Hardy et al. (2002) argued the degree of genetic structure and divergence within *Noturus exilis* arose before or very early in the Pleistocene. Using the poikilotherm mt DNA clock they estimated that *N. exilis* clades diverged 0.77–7.6 mya. Using multiple catfish fossils to calibrate rates of molecular divergence, we find on average younger age estimates for clades of *N. exilis*, which had mean ages of 0.36–5.47. The estimated age of Clade A, from the mid-Pleistocene is consistent with recent diversification within the Central Highlands associated with events of the Pleistocene glacial cycles (Hocutt et al., 1986). Contradictory to predictions of the CHVH, Hocutt et al. (1986) hypothesized that the Pleistocene glacial cycles were the primary generator of the diversity and distribution of fishes in the Central Highlands region and that this diversity was relatively young. Diversification of the two other clades (B and C) of *N. exilis*, however, occurred prior to the Pleistocene (Pliocene and Miocene), demonstrating that both ancient and recent events have played prominent roles in the evolutionary history of the species.

![Fig. 6. Maximum credibility chronogram for *Noturus exilis* estimated from the combined independent BEAST analyses using the mitochondrial cytochrome b and nuclear RAG2 genes. Italicized numbers at nodes are mean node ages. Bars show uncertainty in the estimated divergence with the length representing the 95% highest posterior density (HDP) of node ages. Age estimates and the 95% HPD for focal nodes (A–D) are provided in Table 4. In the Neogene geologic time scale shown ‘Ps’ = Pleistocene, ‘Pl’ = Pliocene, and ‘Mi’ = Miocene (in part). Posterior probability and bootstrap support values for nodes are given in Figs. 2–4.](image)

Table 3

<table>
<thead>
<tr>
<th>Clade</th>
<th>Average between clade divergences (%)</th>
<th>Average within clade divergence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.17</td>
<td>0.09</td>
</tr>
<tr>
<td>C</td>
<td>0.23</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 4

<table>
<thead>
<tr>
<th>Node</th>
<th>Posterior mean age (mya)</th>
<th>95% HPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.36</td>
<td>0.05, 1.00</td>
</tr>
<tr>
<td>B</td>
<td>3.68</td>
<td>1.78, 6.13</td>
</tr>
<tr>
<td>C</td>
<td>5.47</td>
<td>3.16, 8.73</td>
</tr>
<tr>
<td>D</td>
<td>9.67</td>
<td>5.32, 14.93</td>
</tr>
</tbody>
</table>

Diversification within *Noturus exilis* occurred more recently, spanning the pre-Pleistocene–Pleistocene boundary. Hardy et al. (2002) argued the degree of genetic structure and divergence within *Noturus exilis* arose before or very early in the Pleistocene. Using the poikilotherm mt DNA clock they estimated that *N. exilis* clades diverged 0.77–7.6 mya. Using multiple catfish fossils to calibrate rates of molecular divergence, we find on average younger age estimates for clades of *N. exilis*, which had mean ages of 0.36–5.47. The estimated age of Clade A, from the mid-Pleistocene is consistent with recent diversification within the Central Highlands associated with events of the Pleistocene glacial cycles (Hocutt et al., 1986). Contradictory to predictions of the CHVH, Hocutt et al. (1986) hypothesized that the Pleistocene glacial cycles were the primary generator of the diversity and distribution of fishes in the Central Highlands region and that this diversity was relatively young. Diversification of the two other clades (B and C) of *N. exilis*, however, occurred prior to the Pleistocene (Pliocene and Miocene), demonstrating that both ancient and recent events have played prominent roles in the evolutionary history of the species.

Although the estimated diversification time of genus *Noturus* (Miocene to late Oligocene) is younger than the estimate recovered by Hardman and Hardman (2008; late Eocene–early Oligocene), it supports their speculation of climate change of the Oligocene and Miocene potentially playing a more prominent role in North American fish species diversification than the Eocene–Oligocene climate-shift. Near et al. (2005) emphasized the impact of the dramatic cooling event of the latter on North American freshwater fishes in general, and particularly for the Centrarchidae (basses and sunfishes) for which the age of the root node was estimated at the Eocene–Oligocene boundary. However, Hardman and Hardman (2008) noted that most of the extant genera of Centrarchidae and much of the diversification within Ictaluridae was Oligocene to Pliocene in age. The latter was also observed based on the clade ages estimated herein for diversification within each of the catfish genera (*Ictalurus*, *Ameiurus*, and *Noturus*) examined. Diversification...
Table 5

Frequency distribution of preopercular mandibular pores (POM) for *Noturus exilis* from clades A–C of Figs. 2 and 4. Numbers in bold highlight modal POM counts for each clade.

<table>
<thead>
<tr>
<th>Species/clade</th>
<th>POM pores</th>
<th># Ind.</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Noturus exilis</em> (Clade A)</td>
<td>7</td>
<td>50</td>
<td>10.9</td>
</tr>
<tr>
<td><em>Noturus exilis</em> (Clades B + C)</td>
<td>1</td>
<td>104</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Fig. 7. Scatterplot of factor scores generated from the Principle Components Analysis of all morphometric characters and individuals of *Noturus exilis* examined. Individuals/populations are grouped by clades as recovered in Figs. 2 and 4. Component loadings for variables measured are provided in the text.

among genera and for the family predated the Eocene–Oligocene cooling event, indicating that, as suggested by Hardman and Hardman (2008), this event had minimal impact on North American catfish divergence and that climate shifts or geological events, such as the warming event of the Oligocene or sea level fluctuations of the Miocene–Pliocene, likely played a more influential role in their diversification.

4.3. Phylogeographic relationships

Geographic variation in *N. exilis* displays a number of interesting biogeographic features. Data from both Cyt b and RAG2 indicate that populations in the Illinois and Skunk river systems in the glaciated regions of Illinois and Iowa are more closely related to those in the Ozarks, particularly the Missouri River and its tributaries and the St. Francis River, than they are to populations in the Eastern Highlands. This suggests that northern populations are the result of dispersal from populations in the Ozarks up the Mississippi River following retreat of the Pleistocene glaciers, which is further supported by the estimated mid-Pleistocene (mean node age = 0.57, 95% HDP: 0.24–1.05) diversification for this clade of *N. exilis* (Fig. 6). This finding is consistent with the Leading Edge Model of population expansion (Hewitt, 1996), which states that aquatic taxa in northern glaciated portions of North America are comprised of lineages persisting and/or differentiating in southern refugia during the Pleistocene. These lineages then expanded northward into newly available habitats as glaciers receded. Similar results have been found for other taxa in the upper Mississippi River system, including *Etheostoma caeruleum*, *Percina evides*, and *Noturus flavus* (Near et al., 2001; Ray et al., 2006; Faber et al., 2009).

Bay Creek, a tributary of the Ohio River in southern Illinois, also seems to have been populated by Ozarkian fishes, rather than by invasions from closer tributaries of the Ohio River, viz., the lower Tennessee and Cumberland rivers.

The phylogeographic position of Clear Creek, a small direct tributary of the Mississippi River in southern Illinois and adjacent to Bay Creek, was of particular interest. Hardy et al. (2002) found shared haplotypes between the Clear Creek system and the Little Red River (White River) of Arkansas. Although our sample sizes were smaller, we found no shared haplotypes between these regions and both the Cyt b and RAG2 data indicate that the Little Red River population is genetically distinct and divergent from all others examined. As with Bay Creek, Clear Creek is allied with Ozarkian fishes, but those from the Missouri and St. Francis rivers and not the White River system as previously proposed.

The Cyt b data suggest that populations of *Noturus exilis* in the Black River system (Current, Black, and Strawberry rivers) of the lower White River drainage are more closely related to populations in the Eastern Highlands, Missouri River drainage, and previously glaciated regions than they are to those in the upper White river system. The RAG2 data did not resolve these relationships, and we know of no other fish that shows this exact pattern. However, distributions of other upland Ozarkian fishes do support a phylogeographic break between the upper White River and the Black River (lower White River). For example, populations of the Rainbow Darter, *Etheostoma caeruleum*, from the Black River system are more closely related to those from the middle and lower Mississippi river systems (e.g., Meramac and St. Francis) than to the upper White River system (Ray et al., 2006). The relationship of the Black River clade to the clades containing Eastern Highlands and Missouri River populations was unresolved. Hellbenders, *Cryptobranchus alleganiensis*, from the upper White River system are more closely related to those from the Tennessee River of the Eastern Highlands and Meramac and Missouri Rivers than to populations from the Black River (e.g., Current and Eleven Point rivers; Routman et al., 1994). In *Noturus exilis*, the Black River system is allied with those from the Eastern Highlands and Missouri River systems. Other madtom catfishes (*N. albater* and *N. maydeni*; Egge and Simons, 2006), minnows (*Cyprinidae*: *Luxilus*: Mayden, 1988) and basses (*Centrarchidae*: *Ambloplites*: Cashner and Suttikus, 1977) also support a Black-White River vicariance.

The phylogeographic break observed in many taxa between the upper and lower White River system is often attributed to Mayden’s (1988) pre-Pleistocene drainage hypothesis that states that the Mississippi River flowed through the area that is now the Black River. Under this historical drainage pattern scenario, populations of fishes in each of the Black River system tributaries would have been isolated from each other and from the upper White River system and the other major lower White River tributary, the Little Red River system. Hardy et al. (2002) suggested that the observed phylogenetic divergence between the Black River and upper White River was likely due to pre-Pleistocene separation of these drainages, as noted by Mayden (1988). The pattern and timing of clade divergence observed herein for the clades of *N. exilis* from the White River system also are congruent with this aspect of Mayden’s (1988) pre-Pleistocene drainage hypothesis. *Noturus exilis* from the upper and lower White River (excluding the Little Red River of Clade A) were recovered as divergent sub-clades within the larger Clade C, which had a diversification age estimate of 5.47 mya (95% HDP 3.16, 8.73). This result supports the hypothesis of vicariance associated with pre-Pleistocene drainage pattern shifts leading to the observed genetic divergence among the upper and lower White River system populations of *N. exilis*.

Although, the same general phylogeographic break in the White River system is observed across multiple taxa, the timing of the break has only been estimated for the sister species *Noturus albater* from the upper White River and Little Red River system, and *N.
maydeni from the Black River system (lower White River). The estimated divergence time for these species is 2.7–3.4 mya (Egge and Simons, 2006). Although calculated under different methods (using the cyprinid molecular clock of Dowling et al. (2002)) this implies isolation leading to divergence of these species was younger than that inferred for Noturus exilis. Estimates for the timing of diversification for other species that show this geographic break have not been generated. Thus, it is unclear if other species are consistent with the timing of divergence estimated for the Slender Madtom. Given the two different estimates for catfish divergence, it is likely that the commonly observed pattern of divergence among populations from the upper White River and those from the Black River system, shared by many taxa, is not associated with a single vicariant event, but may be better explained by vicariance associated with multiple of persistent events through time. This finding supports previous studies that caution against reliance of shared patterns of clade divergence among taxa, in the absence of temporal data, to infer historical processes responsible for contemporary patterns of species or clade diversity (Donoghue and Moore, 2003; Keck and Near 2005).

At a larger, more general scale, disparity is seen in clade ages estimated for Noturus exilis and those estimated for other small upland, benthic fishes with similar geographic patterns of diversity in the Central Highlands. For example, populations of the Corrugated Darter, E. basilare, which is restricted to a small portion of the Cumberland River drainage in the Eastern Highlands, diverged between 2–8 mya (Hollingsworth and Near, 2009). Although the estimated divergence time for all Eastern Highland populations (all within Clade C) of N. exilis (at 5.47 mya), is comparable to that estimated for E. basilare, divergence among Cumberland River populations (middle versus lower Cumberland River) of N. exilis, which are geographically more widespread than populations of the Corrugated Darter, was estimated at only 2.5 mya. Such disparity in clade ages for fishes with similar habitat preferences and geographic ranges provides further support that multiple events or persistent historical processes, rather than a single shared vicariant event, may often best explain shared biogeographic patterns (Donoghue and Moore, 2003).

5. Materials examined

5.1. Morphology

Numbers in parentheses are numbers examined for measurements, meristics, and pigmentation, respectively.

5.1.1. Mississippi River basin: Arkansas river drainage

AR: Pope Co.: McCoy Creek TU 182899 (10); Van Buren Co.: Cadron Creek TU 188831 (5, 5, 5); Washington Co.: Illinois River UAIC 12550.09 (5, 5, 5); Yell Co.: Fourche LaFave River TU 98015 (6). KS: Cherokee Co.: Spring River-Neosho River UAIC 10460.09 (14), USNM 172051 (1). OK: Adair Co.: Tyner Creek UAIC 2758.01 (5); Adair-Sequoyah Co. Line: Little Creek TU 2254 (4); Delaware Co.: Flint Creek TU188767 (10). Illinois River Drainage: IL: Bureau Co.: East Bureau Creek INHS 55800 (1, 1, 1); McClain Co.: Buck Creek-Mackinaw River INHS 85850 (4); LaSalle Co.: Buck Creek-Fox River INHS 88189 (3). Missouri River Drainage: KS: Wabaunsee Co.: Kansas River UAIC 7999.10 (4, 4, 4). MO: Dallas Co.: Greasy Creek-Osage River UAIC 10104.09 (4); Texas Co.: Piney River-Gasconade River TU 57612 (2), UF 172043 (2), USNM 201394 (1, 1, 1); Warren Co.: Massie Creek UAIC 7995.05 (3); Webster Co.: Niangua River USNM 244959 (2, 2, 2). Ohio River Drainage: Cumberland River: KY: Trigg Co.: Donaldson Creek UF 167337 (5, 5, 5); TN: Montgomery Co.: Piney Fork-Red River UF 167327 (5, 5, 5); Williamson Co.: Mayes Creek-Harpeth River UAIC 13633.02 (3, 3, 3). Green River: KY: Adair Co.: Green River SIUC 61238 (5, 5, 5); Grayson Co.: Nolin River SIUC (uncataloged SIUC lot, collected 7 July 1959, 4, 4, 4). Kentucky River: KY: Grant Co.: Eagle Creek SIUC 66647 (5, 5, 5). Licking River: KY: Pendleton Co.: South Fork Licking River SIUC 19120 (2, 2, 2). Tennessee River: AL: Limestone Co.: Piney Creek UAIC 13287.03 (5, 5, 5), Swan Creek UAIC 13289.03 (5, 5, 5); TN: Coffee Co.: Duck River TU 30304 (4, 4, 4); Lewis Co.: Grinders Creek-Buffalo River UF 172084 (5, 0, 5); Wayne Co.: Stokes Branch-Duck River UF 172062 (5, 0, 5); Wayne Co.: Hog Creek UF 172003 (3, 0, 3). Direct Ohio River Tributaries: IL: Pope Co.: Bay Creek UF 167309 (5, 5, 5). Red River Drainage: AR: Polk Co.: Two Mile Creek TU 48515 (8). White River Drainage: Black River: AR: Fulton Co.: South Fork Spring River TU 188810 (8); MO: Shannon Co.: Jacks Fork Current River UAIC 1077.05 (1); Texas Co.: South Prong Current River UF 170722 (7, 0, 5). Buffalo River: AR: Baxter Co.: Buffalo River TU 49703 (1). James River: MO: Christian Co.: Finley Creek USNM 263606 (2). Little Red River: AR: Stone Co.: Middle Fork Little Red River UAIC 11370.08 (2, 2, 2); Van Buren Co.: Archev Fork UAIC 11368.06 (2, 2), TU 187869 (5, 31, 5); Hartsugg Creek TU 182855 (22, 22). North Fork White River: MO: Douglas Co.: Indian Creek UF 171996 (1, 0, 1); Ozark Co.: North Fork White River UAIC 118680.1 (3, 3, 3). Upper White River: AR: Benton Co.: War Eagle Creek UAIC 12586.12 (2, 2, 2); White River TU 50018 (5). West Fork White River: AR: Washington Co.: Hutchins Creek USNM 201395 (6). Other Mississippi River Systems or Tributaries: Clear Creek: IL:Union Co.: Clear Creek UF 167389 (5, 5, 5); Hutchins Creek UF 172053 (5). Mera- mec River: MO: Franklin Co.: Bourbeuse River UAIC 103211.4 (4, 4, 4); Washington Co.: Big River SIUC 25996 (5, 5, 5). Rock River: IL: Ogle Co.: Leaf River INHS 54181 (9). Pecatonica River: IL: Winnebago Co.: Grove Creek Braasch & Smith 1962 (uncataloged, 5). Skunk River: IA: Story Co.: Bear Creek UF 170791 (3, 3, 3). Middle Fork Little Red River: AR: Fulton Co.: South Fork Spring River UF 172043 (7, 0, 5). Tennessee River: TN: Jackson Co.: South Fork Tennessee River UF 172035 (4).

5.2. Molecular

Materials examined arranged by gene and locality number used in Table 1 and Fig. 1. Following locality numbers are tissue voucher number, followed by specimen voucher number in parentheses (if available). Non-institutional abbreviations used are as follows: AMS are collections by A.M. Simons and JDE are collections by J.J.D. Egge, loaned through the Bell Museum (University of Minnesota); and NJL are collections by N.J. Lang loaned through the University of St. Louis.

5.2.1. Cytochrome b

Site 1: AMS02-01-2, AMS 02-01-3; Site 2: KU 630, 631 (KU 23543); Site 3: KU 7892, 7907 (KU 38695); Site 4: FLMNH 2008-0238, 2008-0239 (UF 172043); Site 5: FLMNH 2008-0228, 2008-0229, 2008-0230, 2008-0231, 2008-0232 (UF 172072); Site 6: NAFF 1241 (STL 834.01); Site 7: AMS 02-17-3, 02-17-4, 02-16-1, 02-16-2; Site 8: AMS02-04-1, 02-04-2; Site 9: FLMNH 2008-0234, 2008-0235, 2008-0236 (UF 171996); Site 10: AMS 02-05-1, 02-05-2; Site 11: JDE 03-13-3, 03-13-4; Site 12: FLMNH 2008-0223, 2008-0224, 2008-0225, 2008-0226 (UF 172035); Site 13: AMS 99-34-1; Site 14: NAFF 1234 (STL 272.04); Site 15: KU 8104, 8105 (KU 38748); Site 16: STL 271.02; Site 17: NAFF 1242, 1243, 1244 (STL 271.02); Site 18: NAFF 1249 (STL 400.2); Site 19: NAFF 1235 (STL 268.01); Site 20: AMS 01-06-1, 01-06-2, JDE03-11-1, 03-11-2; Site 21: JDE 03-10-1, 03-10-2; Site 22: NAFF 1240 (STL 458.01); Site 23: FLMNH 2007-0445, 2007-0448.
5.2.2. RAG2


