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Phylogenetic Relationships in the Genus *Erimystax* (Actinopterygii: Cyprinidae) Based on the Cytochrome *b* Gene

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The phylogenetic and phylogeographic relationships of the North American cyprinid genus *Erimystax* were examined using sequences of the mitochondrial cytochrome *b* gene. Phylogenetic analyses were performed using parsimony and Bayesian approaches. Relationships within the genus were (((*Erimystax cahni* plus *Erimystax x-punctatus*)(*Erimystax dissimilis* plus *Erimystax harryi*))(*Erimystax insignis*))). Genetic distances among species were high and suggest that all speciation events occurred during the Miocene. Shallow divergence and wide geographic distribution in both *E. x-punctatus* and *E. dissimilis* suggest post-Pleistocene dispersal. *Erimystax x-punctatus* expanded its range northward into the upper Mississippi basin and eastward into the Ohio River basin from a putative Ozarkian refugium. *Erimystax dissimilis* expanded its range northward from a southern Appalachian refugium.

SEQUENCE variation of the mitochondrial cytochrome *b* gene was used to assess the phylogeny and biogeography of the cyprinid genus *Erimystax*. This genus is widely distributed in the Mississippi River basin; however, within each of the five species, individual populations are often localized because these fishes require stream habitat with gravel or rocky substrate and little or no sedimentation. Anthropogenic disturbances have resulted in fragmentation and extirpation of some populations (Trautman, 1981; Etnier and Starnes, 1993); this is dramatically illustrated by *Erimystax cahni*, now restricted to a total of 112-river km in the Clinch and Powell Rivers of Tennessee and Virginia (Jenkins and Burkhead, 1993). The disjunct nature of populations has also been attributed to vicariance or dispersal from southern refugia into areas impacted by Pleistocene glaciations (Wiley and Mayden, 1985; Strange and Burr, 1997).

Harris (1986) provided a complete synonymy and taxonomic history of *Erimystax* and described morphological variation supporting recognition of *Erimystax harryi* distinct from *Erimystax dissimilis*. Mayden (1989) presented the only explicitly phylogenetic treatment of relationships within *Erimystax*. Mayden's hypothesis of relationship was based on four morphological characters, two of which (stellate barbel, elongate basihyal) supported the monophyly of the genus. He proposed a monophyletic group, sister to *Erimystax x-punctatus*, consisting of *E. cahni*, *Erimystax insignis*, and *E. dissimilis* (*E. harryi* was not distinguished from *E. dissimilis*) supported by the presence of a narrowly developed maxillary process of the palatine. Presence of dark, distinct, lateral blotches supported monophyly of a group containing *E. insignis* and *E. dissimilis*. *Phenacobius* was the hypothesized sister group to *Erimystax*, an assessment subsequently supported by analysis of 12S and 16S mtDNA sequences (Simons and Mayden, 1999; Simons et al. 2003).

Wiley and Mayden (1985) considered the distribution of species within *Erimystax* to be consistent with a pre-Pleistocene vicariance hypothesis, that is, species observed today were extant at the beginning of the Pleistocene, and their current distributions reflect pre-Pleistocene drainage patterns. They suggested that isolated upper Ohio populations of *E. dissimilis* were preglacial relicts corresponding to unglaciated regions along the Illinoian Glacier. Other species have similar disjunct distributions, including *E. x-punctatus*, and three members of the genus *Etheostoma*: *Etheostoma camurum*, *Etheostoma maculatum*, and *Etheostoma tippecanoe* (Lee et al., 1980).

Strange and Burr (1997) examined mtDNA variation in the context of a Pleistocene vicariance hypothesis to explain the distribution of *E. dissimilis* and *E. harryi*. They interpreted interspecific patterns a result of either a pre-Pleistocene vicariant event or a Pleistocene dispersal of *E. dissimilis* from Appalachia to the Ozarks. They identified the Tennessee River drainage and the upper Green River as Pleistocene refugia and argued that Ohio River basin populations were the result of dispersal from the Tennessee River drainage. Upper Green River populations were presumed to have been isolated from reamining populations of *E. dissimilis* by Pleistocene Lake Green (Strange and Burr, 1997).

MATERIALS AND METHODS

Fishes were collected with seines and backpack electroshocker (for collection localities,

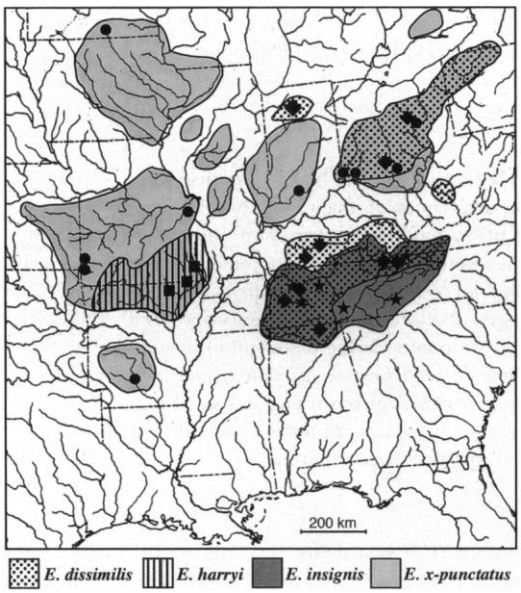

Fig. 1. Ranges and sampling localities of ♦*Erimystax dissimilis*, ★*Erimystax insignis*, ●*Erimystax x-punctatus*, ■*Erimystax harryi*. *Erimystax cahni* was excluded because of its extremely small range, which is overlapped by *E. dissimilis* and *E. insignis*.

see Materials Examined and Fig. 1) and frozen in liquid nitrogen and transported to the laboratory. DNA was extracted using DNeasy tissue kits (QIAGEN Inc.) following manufacturer's instructions. The mitochondrial cytochrome *b* gene was amplified using primers 14724 and 15915 (Schmidt and Gold, 1993). Amplification profile was denaturation at 94 C for 1 min; annealing at 48C for 1 min; and extension at 72 C for 2.5 min, repeated for 30 cycles. Reaction mixes contained: 2.5 μl (approximately 2 ng) genomic DNA; 0.6 μm of each dNTP (USB Corp.); 0.16 μm of each primer, 5 mM $MgCl_2$, 2.5 μl 10X buffer; 0.2 units of taq DNA polymerase (Promega Corp.); and distilled water to bring final reaction volume to 25 μl. Amplified products were purified using the QIAquick PCR cleanup kit (QIAGEN, Inc.) and eluted into 50 μl of elution buffer. Sequencing reactions were performed by the Advanced Genetic Analysis Center, University of Minnesota using amplification primers and 250 ng of purified PCR product. Sequences were edited with Sequencher 4.1 (Gene Codes Corp.) and aligned by eye. All sequences were deposited with GenBank. Sequences of *E. x-punctatus* from Big River, Missouri, were downloaded from GenBank (Raley and Wood, 2001).

Parsimony analyses were performed using PAUP* (vers. 4.0b10, D. L. Swofford, Sinauer Associates, Sunderland, MA, 1998, unpubl.). *Phenacobius catostomus*, *Phenacobius uranops*, and *Nocomis biguttatus* were included as outgroup taxa. Characters were equally weighted and unordered, and gaps were treated as missing data. All searches were heuristic; starting trees were obtained by stepwise addition with 1000 random addition sequence replicates and TBR branch swapping. Support for nodes was assessed by nonparametric bootstrapping (Felsenstein, 1985) with 1000 pseudoreplicates, simple taxon addition, TBR branch swapping, and maximum number of trees restricted to 1000. Analyses included (1) cytochrome *b* only and (2) cytochrome *b* plus Mayden's (1989) four morphological characters.

Bayesian analyses were performed using MrBayes 2.01 (Huelsenbeck and Ronquist, 2001). First, a hierarchy of evolutionary models was evaluated using two runs of ModelTest (Posada and Crandall, 1998), the first based on a neighbor joining tree and the second on one of 224 trees from the parsimony analysis. The parsimony tree was also used to evaluate a second hierarchy of 13 models incorporating site-specific rates. Akaike's (1974) information criterion (AIC) was used to identify the final model choice because the examined models were not nested.

Four independent Bayesian runs were performed using the model determined with ModelTest, a random starting tree, uniform interval priors except base composition, which assumed a dirichlet prior. In each run, the Markov Chain Monte Carlo (MCMC) was run for 2,000,000 generations with four chains; chains were heated (T = 0.5) to better explore parameter space of the model. The MCMC chain was sampled every 1000 generations. Preliminary analysis indicated that the MCMC chain reached stationarity, where likelihood and model parameters plateau, well before 250,000 generations; 250,000 generations were discarded as burn-in.

Genetic divergence within and among clades was calculated using both uncorrected "p" and likelihood corrected distances. Model parameters were estimated using PAUP* on the consensus topology obtained from the Bayesian analyses. Distances between sister taxa were corrected for inherited ancestral polymorphism using the formula $\Delta = \Delta xy - 0.5(\Delta x + \Delta y)$, where Δxy is the average of all pairwise comparisons between the two groups, and Δx and Δy are the average of all pairwise comparisons within each of the two groups (Nei and Li, 1979).

The hypothesis of constant rates among lineages, that is, clocklike substitution rates, was

tested using likelihood ratio tests (Huelsenbeck and Crandall, 1997). Two tests were conducted based on the consensus tree topology obtained in the Bayesian analyses. In each test, likelihood values were obtained with and without enforcing a molecular clock using PAUP*. The tests included (1) all taxa using parameter values from the Bayesian analyses and (2) all taxa using parameter values estimated with PAUP*. All tests used the substitution model determined with ModelTest. Significance was assessed using a χ^2-distribution, df = number of OTU − 2, α = 0.05. Divergence times were estimated using substitution rates in cytochrome *b* of North American cyprinids calibrated against the fossil record (Dowling et al., 2002). Dowling et al. (2002) used Tamura-Nei distances; the distances used in this paper (uncorrected "p" and likelihood corrected distance) bracket their calibration and thus provide a conservative estimate of divergence.

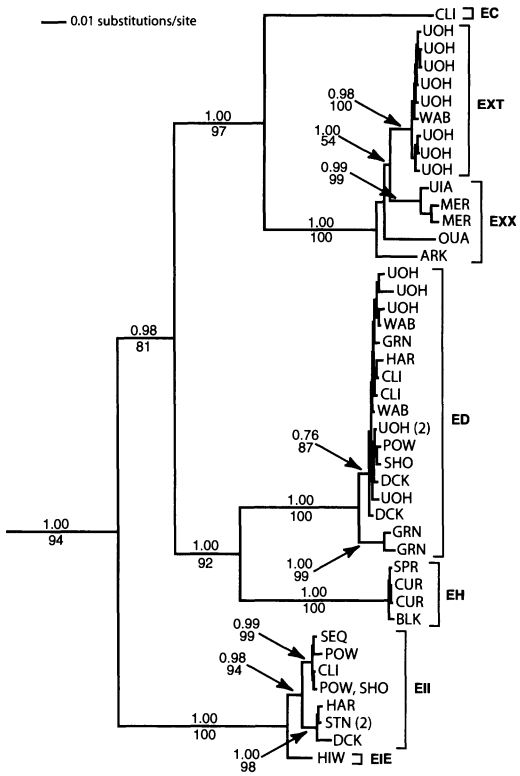

Fig. 2. Bayesian consensus topology for the genus *Erimystax*. Posterior probabilities marked above nodes. Parsimony bootstrap values are below nodes. Outgroups are not shown. Number of identical haplotypes are indicated in parentheses. Abbreviations for sampled river systems: ARK Arkansas River, BLK Black River, CLI Clinch River, CUR Current River, DCK Duck River, GRN Green River, HAR Harpeth River, HIW Hiwassee River, MER Meramec River, OUA Ouachita River, POW Powell River, SEQ Sequatchie River, SHO Shoal Creek, SPR Spring River, STN Stones River, UIA Upper Iowa River, UOH Upper Ohio River, WAB Wabash River. Abbreviations for taxon names: EC *Erimystax cahni*, EXT *Erimystax x-punctatus trautmani*, EXX, *Erimystax x. x-punctatus*, ED *Erimystax dissimilis*, EH *Erimystax harryi*, EII *Erimystax insignis insignis*, EIE *Erimystax insignis eristigma*.

Results

The data matrix contained 50 OTUs with 1116 characters. Positions 1–10 and 1127–1140 of the cytochrome *b* gene were trimmed from the data matrix as these were missing in most sequences. Of the included characters, 307 were parsimony informative. Within *Erimystax*, there were 44 unique haplotypes among 47 specimens. Shared haplotypes were present in two specimens of *E. dissimilis*, one from Walhonding River and one from Big Darby Creek, Ohio. In *E. insignis*, one specimen from Shoal Creek, Alabama, and one specimen from Powell River, Tennessee, shared a haplotype, as did both specimens from the Stones River, Tennessee.

Parsimony analysis found 256 equally parsimonious trees, TL = 889, CI = 0.60, excluding uninformative characters. The genus *Erimystax* was recovered as monophyletic in all analyses with bootstrap support of 94%. *Erimystax insignis* was sister to a clade containing all remaining taxa. *Erimystax harryi* plus *E. dissimilis* was sister to *E. cahni* plus *E. x-punctatus*. Inclusion of the morphological characters with the cytochrome *b* dataset do not alter these results.

Model selection identified GTR+SSR as the best base substitution model based on the NJ and parsimony trees (AIC = 10715.80). This model was used in pairwise comparisons of genetic distance and in the Bayesian analyses. The Bayesian analyses produced a consensus topology consistent with the parsimony analysis, differing only in resolution among OTUs (Fig. 2). The genus *Erimystax* was monophyletic with a posterior probability of 1.00. Posterior probabilities of species groups and species were also high, ranging from 0.98 to 1.00.

The molecular clock hypothesis was not rejected in the two tests: (1) all taxa, Bayesian parameters, $-2 \log \Lambda = 42.99$, $\chi^2_{48} = 65.17$; and (2) all taxa, estimated parameters, $-2 \log \Lambda = 40.69$, $\chi^2_{48} = 66.17$. Genetic distances between clades indicated that all speciation events occurred more than 6 MYA. Uncorrected "p": *E. x-punctatus* versus *E. cahni*, $\delta = 0.088$, 6.7–8.3 MYA; *E. dissimilis* versus *E. harryi*, $\delta = 0.081$, 6.2–7.6 MYA; *E. dissimilis*, *E. harryi* versus *E. x-punctatus*, *E. cahni*, $\delta = 0.092$, 7.0–8.7 MYA; *E. insig-*

nis versus all *Erimystax*, δ = 0.082, 6.3–7.7 MYA. ML corrected distances: *E. x-punctatus* versus *E. cahni*, δ = 0.108, 8.2–10.2 MYA; *E. dissimilis* versus *E. harryi*, δ = 0.108, 8.2–10.2 MYA; *E. dissimilis*, *E. harryi* versus *E. x-punctatus*, *E. cahni*, δ = 0.135, 10.4–12.8 MYA; *E. insignis* versus all *Erimystax*, δ = 0.125, 9.5–11.8 MYA.

Discussion

Phylogenetic relationships of species in the genus *Erimystax*, based on cytochrome *b*, differ from those suggested by morphological data (Mayden, 1989). The characters from his analysis may be best interpreted as synapomorphies for the genus, with reversals to the plesiomorphic condition in *E. x-punctatus* (maxillary process) and *E. x-punctatus* plus *E. cahni* (lateral blotches). Alternatively, lateral blotches might be independently derived in *E. insignis* and *E. dissimilis* plus *E. harryi*. The corrected sequence difference between *E. dissimilis* and *E. harryi* (10.8%) supports recognition of these as separate species (Harris, 1986).

Sequence divergence among species is high. Molecular clock analyses suggest that all speciation events in *Erimystax* occurred in the late Miocene. The relationships (Fig. 2) and divergence values suggest that speciation events producing the species pairs *E. x-punctatus* plus *E. cahni* and *E. dissimilis* plus *E. harryi* occurred within a relatively short period of time. It is possible that the ranges of the ancestral species of each of these two clades were disrupted by a common event. *Erimystax dissimilis* and *E. harryi* occur on either side of the Mississippi River, whereas *E. x-punctatus* occurs on both sides of the Mississippi River (Fig. 1). The distribution of *E. x-punctatus* may reflect dispersal from west to east since the derived haplotypes in this species are east of the Mississippi River. Although speciation was not a function of Pleistocene effects, glaciation and subsequent events affected distribution of populations and haplotype diversity. This is particularly evident in *E. x-punctatus* and *E. dissimilis*, both of which inhabit previously glaciated areas.

Erimystax x-punctatus exhibits more haplotype variation than other species in the genus. Four groups can tentatively be recognized distributed in the following basins, Arkansas River, Ouachita River, Ozark/Upper Mississippi Rivers, and Ohio River. Corrected genetic distances range from 2.4% between the Ozark/Upper Mississippi clade and the Ohio River clade to 3.0% between the Arkansas River clade and the Ohio, Ozark/Upper Mississippi River clade. The Ohio River group is monophyletic and exhibits low levels of divergence among populations in the East Fork of the White River (Wabash drainage) and the Miami, Little Miami, Scioto, and Muskingum rivers of the Upper Ohio River Basin. This group corresponds to the subspecies *E. x. trautmani* Hubbs and Crowe (1956). Harris (1986) included the White River populations of the Arkansas River Basin in this subspecies, an area not represented in my samples of *E. x-punctatus*. The remaining haplotypes, which represent the nominate subspecies *E. x. x-punctatus*, do not constitute a monophyletic group in these analyses. Samples from the Meramec and Upper Iowa Rivers are a monophyletic group, sister to the Ohio clade. The relationship between the Meramec and Upper Mississippi River basin is consistent with those for other highland fishes and is likely caused by post-Pleistocene dispersal (Near et al. 2001; Berendzen et al., 2003).

The remaining two "groups" within *E. x. x-punctatus* are each represented only by a single specimen. The Ouachita River specimen is sister to the Ohio plus Ozark/Upper Mississippi clade and the Upper Arkansas River specimen is sister to all haplotypes detected in this species. The apparent paraphyly of *E. x. x-punctatus* might be an artifact of sampling, incomplete lineage sorting, or might indicate that this subspecies is not monophyletic. Paraphyly of *E. x. x-punctatus* would not be particularly surprising given that these subspecies were not defined in a phylogenetic context. Clearly, additional samples from the Ouachita, Arkansas, and Missouri River tributaries and the Upper Mississippi River are needed to resolve the status of these regional variants.

Erimystax dissimilis is widespread in the Ohio River system. Populations are disjunct, and some are separated by great distances. Some disjunctions are likely the result of anthropogenic habitat alterations, but others are more puzzling. *Erimystax dissimilis* occurs in the Clinch, Powell, and Upper Holston Rivers of Tennessee and Virginia but is absent from the Blue Ridge province of the Upper Tennessee River even though suitable habitat seems available. It is present in the lower Tennessee system downstream of the Sequatchie River. This distribution is similar to that of the *Ethestoma tippecanoe* species group (Stauffer and van Snik, 1997; Kinzinger et al. 2001).

My results for *E. dissimilis* differ from those reported by Strange and Burr (1997). Their results, from a Dollo parsimony analysis of restriction-site data, indicated a monophyletic Tennessee River clade sister to an upper Ohio River clade, whereas I found remarkably little geo-

graphic structure to sequence variation in cytochrome *b*. Two divergent clades (1.4%) were identified in both parsimony and Bayesian analyses. The larger of these contained haplotypes from across the range of *E. dissimilis* and represented all sampled localities (including Green River). Haplotypes of the second clade are restricted to the Green River system of Kentucky. Paraphyly of Green River haplotypes was also observed by Strange and Burr (1997). Possible explanations for this pattern include (1) isolation of Green River with subsequent dispersal from other populations into the Green River; (2) range expansion from a Green River refugium where dispersers were sampled from one of the two Green River clades; (3) isolation of Green River but with incomplete lineage sorting in this drainage. Distinguishing among these hypotheses requires additional sampling.

Erimystax insignis is the sister taxon to a clade containing remaining *Erimystax* species. The species contains two recognized subspecies: *Erimystax insignis insignis* in the Cumberland River and upstream in the Tennessee River to the Sequatchie River, and *Erimystax insignis eristigma* in the upper Tennessee River. Specimens in the Clinch and Powell Rivers are considered intergrades between the two forms (Hubbs and Crowe 1956; Harris, 1986). The single specimen of *E. i. eristigma* included in this study (Hiwassee River sample) was sister to a clade containing *E. i. insignis* and presumed intergrades from the Clinch and Powell Rivers. The latter clade contains two reciprocally monophyletic groups, one in the Tennessee River and one in the Duck and Cumberland Rivers. Increased sampling across the range of *E. insignis*, particularly in the Blue Ridge physiographic province of Tennessee and North Carolina is needed to determine the status of subspecific forms.

The reported distributional discontinuities of upper Ohio River fishes, including *E. dissimilis*, *E. x-punctatus* and the darters, *Etheostoma camurum*, *Etheostoma maculatum*, and *E. tippecanoe*, are likely an artifact of inadequate collection records prior to effects of European settlement on the Ohio River. Descriptions of the Ohio River in the early 1800s mention sandbars, and rock and gravel bars, and state that it was fordable in several places between Cincinnati and Pittsburgh (Trautman, 1981). Although the Ohio River now presents a formidable barrier to species intolerant of slow water and sedimentation, this probably was not true 200 years ago. The low sequence divergences reported here for upper Ohio River populations of *E. x-punctatus* and *E. dissimilis* and by Kinzinger et al. (2001) for *E. tippecanoe* indicate relatively recent isolation. Levels of divergence also indicate that these distributions reflect post-Pleistocene dispersal rather than relictual pre-Pleistocene populations as suggested by Wiley and Mayden (1985).

Material Examined

Sequences generated for this study were deposited with GenBank under the accession numbers AY486010–AY486057. *Erimystax cahni* UAIC 7945.02, Clinch River, Hancock County, TN. *Erimystax dissimilis*: JFBM 35783–1, JFBM 35783–2, Duck River, Bedford County, TN; JFBM 34951–2, JFBM 34951–3, Drakes Creek, Warren County, KY; JFBM 55125–3, Powell River, Claiborne County, TN; JFBM 34852–1, JFBM 34852–2, Tippecanoe River, Fulton County, IN; JFBM 41841–1, JFBM 41841–2, Clinch River, Hancock County, TN; JFBM 38232–2, Green River, Green County, KY; JFBM 38270–1, JFBM 38270–2, JFBM 38270–3, Big Darby Creek, Pickaway County, Ohio; JFBM 41838, JFBM 38295–1, JFBM 38126–1, Walhonding River, Coshocton County, OH; UAIC 9863.06–1, Harpeth River, Cheatam County, TN; UAIC 11822.01–1, Shoal Creek, Lauderdale County, Al. *Erimystax harryi*: JFBM, 41840–1, Spring River, Sharp County, AR; JFBM 30467–2, JFBM 30467–3, Current River, Clay County, AR; JFBM 41834–1, Black River, Reynolds County, MO. *Erimystax insignis*: JFBM 34908–1, JFBM 34908–2, East Fork Stones River, Rutherford County, TN; JFBM 35126–1, JFBM 35126–2, Powell River, Claiborne County, TN; JFBM 35547–3, Sequatchie River, Marion County, TN; JFBM 37259–1, Clinch River, Hancock County, TN; JFBM 41836–1, Duck River, Bedford County, TN; UAIC 9863.07, Harpeth River, Cheatam County, TN; UAIC 11822.02, Shoal Creek, Lauderdale County, AL; UAIC 13120.01, Valley River, Cherokee County, NC. *Erimystax x-punctatus*: JFBM 41835–3, Ouachita River, Hot Spring County, AR; JFBM 41839–1, East Fork White River, Jackson County, IN; JFBM 37865–2, Shoal Creek, Newton County, MO; JFBM 41837–1, Whitewater River, Hamilton County, OH; JFBM 38386–1, JFBM 38386–2, JFBM 38386–3, Little Miami River, Clermont County, OH; JFBM 38371–2, JFBM 38371–3, Paint Creek, Ross County, OH; JFBM 38127–1, JFBM 38127–3, Walhonding River, Coshocton County, OH; JFBM 31619, Iowa River, Fillmore County, MN; AF117172, AF117173, Big River, Jefferson County, MO. *Nocomis biguttatus* Elevenpoint River, Randolph County, MO. *Phenacobius catostomus*, Cahaba River, Bibb County, AL. *Phenacobius uranops* UAIC 7922.13, North Fork Holston River, Scott County, VA.

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