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Phylogenetic Relationships of *Noturus stanauli* and *N. crypticus* (Siluriformes: Ictaluridae), Two Imperiled Freshwater Fish Species from the Southeastern United States

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***Noturus stanauli* and *N. crypticus* are two madtom catfish species of conservation concern with very restricted distributions in the Upper Tennessee River Drainage and Duck River. A recent phylogenetic analysis of *Noturus* using mtDNA sequences did not include these species. A new phylogenetic analysis of cytochrome *b* sequences including *N. stanauli* and *N. crypticus* is presented. Both maximum parsimony and Bayesian analyses indicate that *N. stanauli* and *N. crypticus* are closely related and nest within a genetically divergent clade comprising *N. fasciatus*, *N. baileyi*, *N. elegans*, and a paraphyletic *N. hildebrandi*. Limited intraspecific sampling of *N. stanauli* and *N. crypticus* revealed few genetic differences within these species. We discuss our results in the context of conservation status of these species and the use of phylogenetic trees and sequence data in the delimitation of cryptic species diversity in *Noturus*.**

THERE are 28 described species in the ictalurid catfish clade *Noturus*. Morphological variation and molecular data indicate that as many as nine additional *Noturus* species await description (Burr and Stoeckel, 1999; Hardman, 2004; Burr et al., 2005). Considering that over one quarter of the recognized species diversity in *Noturus* is undescribed is cause for concern, especially since 15 of the described species are considered by biologists as endangered, threatened, or vulnerable (Warren et al., 2000; Thomas and Burr, 2004).

Phylogenetic analyses of *Noturus* have aided in the identification of cryptic lineages and have served as a basis for studying geographic patterns of speciation in the clade (Grady and LeGrande, 1992; Hardman, 2004). However, due to the lack of available specimens, both of the potentially most imperiled species, *N. stanauli* and *N. crypticus*, were not included in the most recent of these phylogenetic analyses (Hardman, 2004). Previously, *N. stanauli* had been included in phylogenetic analyses of allozyme and morphological characters and was resolved as the sister species of either *N. hildebrandi* or *N. baileyi* (Grady and LeGrande, 1992). *Noturus stanauli* was described in 1980 and is only known from two localities in the Duck and Clinch rivers that are separated by over 1,000 river kilometers (Etnier and Jenkins, 1980; Etnier and Starnes, 1993). *Noturus crypticus* was recently described and is also known from two disjunct locations: Dunn Creek, Sevier County, Tennessee (Little Pigeon River) and Little Chucky Creek, Greene County, Tennessee (Nolichucky River; Burr et al., 2005). *Noturus crypticus* may be the most endangered freshwater fish species in the southeastern United States with only one specimen collected from Dunn Creek in 1940, and 14 specimens collected from Little Chucky Creek between 1991 and 2004 (Burr et al., 2005; Lang et al., 2005). In addition, historical collections of extirpated populations tentatively assigned to *N. elegans* from the middle Tennessee River Drainage in northern Alabama might represent an additional undescribed species closely related to *N. crypticus*, *N. elegans*, or *N. fasciatus* (Taylor, 1969; Boschung and Mayden, 2004; Burr et al., 2005).

We undertook this study to add *N. stanauli* and *N. crypticus* to the existing mtDNA phylogenetic database for ictalurids (Hardman and Page, 2003; Hardman, 2004; Wilcox et al., 2004). The complete cytochrome *b* (cyt*b*) gene was sequenced from a single population of three specimens each of *N. stanauli* and *N. crypticus*. The resulting phylogenetic trees indicate a close relationship between these two species in a clade of less-imperiled species. Closely related species that are not of conservation concern may serve as surrogate species for *N. stanauli* and *N. crypticus* in determining the basic biology and patterns of life history variation (Burr and Stoeckel, 1999), as well as aid in the development of captive propagation protocols (Rakes et al., 1999; Shute et al., 2005).

MATERIALS AND METHODS

Three *Noturus stanauli* and a single *N. crypticus* specimen for this study were provided by P. L. Rakes and J. R. Shute of Conservation Fisheries Inc (CFI) of Knoxville, Tennessee. All four of these specimens were being used in a captive propagation program and were salvaged following their natural deaths in aquaria. Fishes were monitored daily at CFI and when dead specimens

were discovered, they were removed and stored at −20 C. The *N. stanauli* specimens originated from the Clinch River at Frost Ford, 11.8 air kilometers from Kyles Ford, Hancock County, Tennessee, and are cataloged in the University of Tennessee Research Collection of Fishes and the University of Tennessee Tissue Collection (UT 48.1238; tissues: UTTC 4647, UTTC 4802, and UTTC 4803). The single *N. crypticus* specimen originated from Little Chucky Creek at mouth of Jackson Branch, Greene County, Tennessee (UT 48.1237; UTTC 4801). Tissue biopsies from two additional *N. crypticus* specimens that were collected at the same locality as the CFI specimens (UTTC 4804, UTTC 4805) were provided by J. M. Grady.

We isolated DNA from ethanol preserved tissues using proteinase K digestion followed by protein precipitation with 4 M guanidine thiocyanate. Nucleic acids were precipitated using 100% ethanol at −20 C. DNA sequences of the complete cyt*b* gene were collected by PCR amplification using primers GLU-2 and PRO-R1 (Hardman and Page, 2003) with an annealing temperature of 50 C. Amplified PCR products were prepared for DNA sequencing with enzymatic degradation using shrimp alkaline phosphatase and exonuclease I, incubated at 37 C for 15 minutes and 80 C for 15 minutes. DNA sequences were generated with cycle sequencing using PCR and internal sequencing primers (Hardman, 2004) at the Molecular Systematics and Conservation Genetics Laboratory, Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT. Complete cyt*b* gene sequences were constructed from individual chromatogram files using the computer program Sequencher version 4.0 (Gene Codes, Ann Arbor, MI). Sequences were submitted to GenBank (DQ383657–DQ383662).

Seventy-two ictalurid cyt*b* sequences were downloaded from Genbank (see Hardman, 2004), including all but two of the described and extant *Noturus* species (*N. stanauli* and *N. crypticus* added in this study), all seven of the extant species of *Ameiurus*, and representative species from all other ictalurid genera except *Satan* and *Troglopluranis* (Hardman and Page, 2003; Hardman, 2004; Wilcox et al., 2004). Of the 30 *Noturus* species included in our analyses, two are undescribed (*N. aff. albater* and *N.* sp. ''Broadtail Madtom''), and 17 were sampled with multiple cyt*b* sequences. Because cyt*b* is a protein-coding gene with no deletions or insertions among ictalurids, alignment of the sequences was trivial and done by eye (available from the senior author upon request). All non-*Noturus* ictalurid taxa were used as outgroups in all phylogenetic analyses.

Both maximum parsimony and Bayesian methods were used to generate phylogenetic trees from the ictalurid cyt*b* alignment. Maximum parsimony analyses were executed using the computer program PAUP* 4.0 and a heuristic search with 100 replications of random taxon addition sequence and TBR branch swapping. Node support for the maximum parsimony phylogeny was determined through bootstrap analysis comprising 2,000 pseudoreplicates and using the TBR branch swapping algorithm on a starting tree to which taxa were added randomly. MrBayes 3.0 was used for a partitioned mixed-model Bayesian analysis with posterior probabilities estimated using metropolis-coupled Markov chain Monte Carlo (Larget and Simon, 1999; Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003). Modeltest 3.0 was used to perform maximum likelihood ratio tests to determine the optimal molecular evolutionary model for each of the three cyt*b* codon positions (Posada and Crandall, 1998). The appropriate molecular evolutionary models were assigned to data partitions in MrBayes 3.0 using the APPLYTO command, and model parameter values were estimated for each data partition using the UNLINK command. MrBayes 3.0 was run for 5×10^6 generations to ensure complete convergence of the Markov chain Monte Carlo algorithm sampling every 100th generation. We determined the burn-in period of the Bayesian analysis by plotting the maximum likelihood scores of the sampled generations. All tree samples with maximum likelihood scores beneath the asymptote were discarded as burn-in. Taxonomic congruence among remaining trees was summarized through a 50% majority-rule consensus. The frequency with which a particular clade occurred in the population of remaining trees was interpreted as its posterior probability. We interpret the posterior probability as a measure of how likely the clade is resolved in the optimal topology rather than any index of clade support.

RESULTS

The patterns of nucleotide variation in cyt*b* within Ictaluridae and among *Noturus* species were very similar to those reported by Hardman and Page (2003), Hardman (2004), and Wilcox et al. (2004). Specifically, third codon positions were the most variable and cyt*b* changes did not appear to be saturated for contrasts within *Noturus*. Maximum parsimony analysis resulted in 96 most parsimonious trees with a length of 2708 steps, a consistency index (excluding uninformative characters) equal to 0.286, and a retention index equal to 0.758 (Fig. 1). *Noturus*

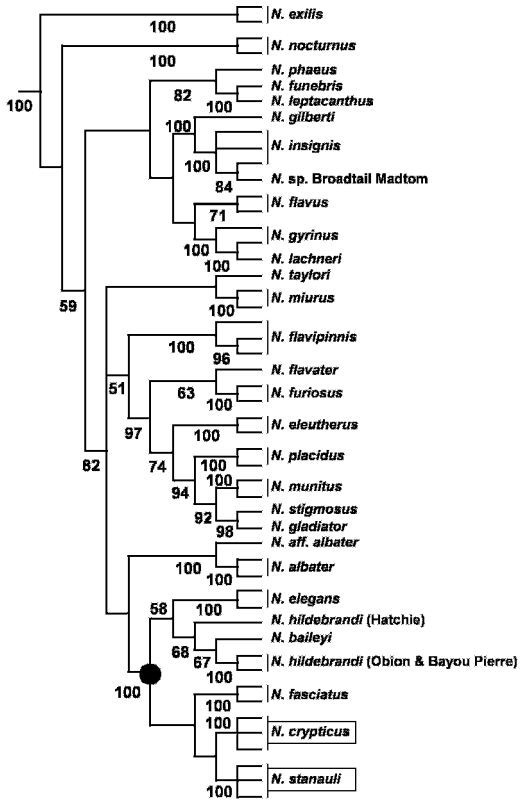

Fig. 1. Strict consensus of 96 trees resulting from maximum parsimony analysis of the ictalurid cytochrome *b* alignment. *Noturus stanauli* and *N. crypticus* are highlighted with a box. The most recent common ancestor of the *Noturus hildebrandi* species clade is marked with a black dot. Only relationships among *Noturus* species are shown. Numbers at nodes report percent recovery in bootstrap pseudoreplicate analysis.

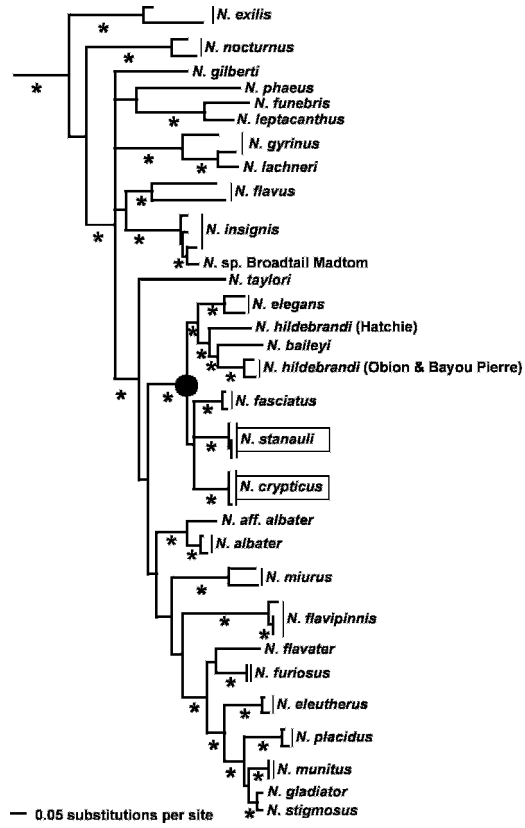

Fig. 2. Fifty percent majority rule consensus phylogram resulting from Bayesian analysis of the ictalurid cytochrome *b* alignment. Only relationships among *Noturus* species are shown. *Noturus stanauli* and *N. crypticus* are highlighted with a box. The most recent common ancestor of the *Noturus hildebrandi* species clade is marked with a black dot. Asterisks at nodes indicate a 95% or greater Bayesian posterior probability.

was monophyletic in all of these trees, but basal nodes were not convincingly resolved as judged by the bootstrap analysis (Fig. 1). *Noturus stanauli* and *N. crypticus* were resolved as sister species though not supported in the bootstrap analysis. Both *N. stanauli* and *N. crypticus* were included in a clade containing *N. elegans*, *N. baileyi*, *N. fasciatus*, and a paraphyletic *N. hildebrandi*. We refer to this monophyletic group of species as the *N. hildebrandi* clade. This clade was strongly supported with a bootstrap score of 100% (Fig. 1).

Tree samples and parameter estimates from the first 1.0×10^6 generations of the Bayesian analysis were designated as burn-in and discarded. The phylogeny inferred from the remaining 4.0×10^6 generations is represented as a 50% majority-rule consensus tree, with branch lengths calculated as the mean value of the posterior probability estimate across all post burn-in generations (Fig. 2). The Bayesian tree topology is very similar to the maximum parsimony tree (Fig. 1), particularly in the presence of a strongly supported clade containing *N. stanauli*, *N. crypticus*, *N. fasciatus*, *N. baileyi*, *N. elegans*, and a paraphyletic *N. hildebrandi* (Fig. 2). Despite the resolute placement of *N. stanauli* and *N. crypticus* in this clade, resolution of the relationships between them and *N. fasciatus* is lacking.

Genetic divergence at cyt*b* between *N. stanauli* and *N. crypticus* averaged 5.2% in uncorrected pairwise contrasts. Greater genetic divergence at cyt*b* was observed when *N. crypticus* and *N. stanauli* were compared to other species in the *N. hildebrandi* species clade, with uncorrected distances ranging from 5.9% (*N. crypticus* vs. *N.*

fasciatus) to 7.8% (*N. crypticus* vs. *N. hildebrandi* from the Hatchie R.). Hardman (2004) observed a mean pairwise genetic divergence at cyt*b* of 10.9% among all *Noturus* species. When compared to all other *Noturus* species, the substantial genetic divergence of cyt*b* for both *N. crypticus* and *N. stanauli*, and the strict reciprocal monophyly of sampled individuals, could indicate a long history of reproductive isolation that may have resulted in their observed distinction. Phylogenetic patterns from the mtDNA analyses, the presence of unique allozyme profiles, and morphological differences all support the recognition of these two species (Etnier and Jenkins, 1980; Grady and LeGrande, 1992; Burr et al., 2005).

Two haplotypes were found among the three sampled *N. crypticus* specimens that differed by a single nucleotide substitution, and there were two haplotypes found among the three *N. stanauli* specimens that differed by two nucleotide substitutions. Despite the genetic uniqueness when compared to other *Noturus* species, these results indicate very little intraspecific divergence of cyt*b* in both *N. crypticus* and *N. stanauli*.

Discussion

The phylogenetic trees and levels of genetic divergence inferred from cyt*b* offer several clues to the discovery of additional species in *Noturus*. As pointed out by Burr et al. (2005), and illustrated in Taylor's (1969) monograph, morphological conservatism is a persistent characteristic of *Noturus* species. The lack of morphological distinction results in a paucity of characters typically used to identify and diagnose species in other clades of North American freshwater fishes (Taylor, 1969; Burr et al., 2005). As such, undescribed species potentially remain as cryptic lineages, hidden within the ranges of currently recognized widespread species (e.g., *N. gyrinus*, *N. flavus*, *N. miurus*, and *N. insignis*).

The data we present in this paper support two conclusions. First, *N. stanauli* and *N. crypticus* are nested in a larger *N. hildebrandi* species clade (Figs. 1, 2). Burr et al. (2005) resolved *N. fasciatus* and *N. crypticus* as sister species, but did not include *N. stanauli* in their analyses. Both maximum parsimony and Bayesian analyses with our taxon sampling result in a close phylogenetic relationship among *N. crypticus*, *N. fasciatus*, and *N. stanauli*. Second, each of these species is genetically distinct and divergent from all other *Noturus* species. However, our results, and those of Hardman (2004), indicate that several more cryptic species of *Noturus* await description. For example, our new phylogenies (Figs. 1, 2) and Hardman (2004) demonstrate that three *Noturus* species are paraphyletic with strongly supported intervening nodes (*N. insignis*, *N. gyrinus*, and *N. hildebrandi*). While phenomena such as ancestral polymorphism can result in a similar pattern (Hudson and Coyne, 2002), the paraphyly observed among haplotypes of these species is compelling and warrants more detailed examination. Another line of evidence comes from the observed genetic divergence at cyt*b* among intraspecific comparisons (Hardman, 2004). There is substantial intraspecific divergence at cyt*b* for at least three geographically widespread *Noturus* species (*N. exilis* 3.5%, *N. flavus* 5.6%, and *N. miurus* 3.2%; Hardman, 2004). Also, examination of mtDNA ND4 haplotype variation in *N. exilis* revealed intraspecific divergence as high as 4.8% (Hardy et al., 2002). Some of these intraspecific cyt*b* genetic divergences approach those observed when comparing *N. stanauli* and *N. crypticus* with other species in the *N. hildebrandi* clade, and all of these intraspecific cyt*b* divergences are much greater than the lowest interspecific divergence observed among any two *Noturus* species (*N. stigmosus* vs. *N. gladiator* 0.9%).

The implications of recognizing the extant biodiversity in *Noturus* through species descriptions and reconstruction of phylogenetic relationships extend well beyond the esoteric interests of ichthyologists, ecologists, and evolutionary biologists. The freshwater biodiversity hotspot located in the southeastern portion of the United States is nearing a critical conservation crisis (Lydeard and Mayden, 1995; Etnier, 1997; Warren et al., 2000). Since most governmental management strategies for imperiled taxa are aimed at the rank of species, the failure to adequately recognize alpha taxonomic diversity can have dire consequences, namely, the extinction of undescribed evolutionary lineages and species (Daugherty et al., 1990). This threat is particularly acute for *Noturus*, as the clade is characterized by morphological conservatism and cryptic species. However, the challenge of discovering and describing *Noturus* species is compounded by the fact that the most endangered and threatened species in the clade are characterized by small and fragmented geographic ranges that dramatically increase the risk of extinction (Etnier and Jenkins, 1980; Etnier and Starnes, 1993; Burr et al., 2005). This risk is made apparent by the extirpation of at least three populations in the middle Tennessee River Drainage of northern Alabama that are either conspecific with species in the *N. hildebrandi* clade or were distinct and undescribed species

(Taylor, 1969; Boschung and Mayden, 2004; Burr et al., 2005).

To counteract the disheartening extinction of potentially undescribed *Noturus* species, taxonomists and evolutionary biologists can continue to contribute most effectively to the conservation of our unique freshwater fish fauna through the description of cryptic species using innovative combinations of traditional characters and newer molecular data (Page et al., 2003; Burr et al., 2005). In addition, there is a battery of novel approaches that use phylogenetic information to assess conservation priorities that minimize the loss of evolutionary history (Faith, 1992; Mace et al., 2003), and methods that integrate phylogenetic hypotheses with geographic distributions to identify specific biodiversity hotspots that merit conservation (Moritz and Faith, 1998; Sechrest et al., 2002). As more phylogenies accumulate for North American freshwater fish clades, taxonomists and conservation biologists hopefully will embrace such new methods for the ultimate goal of preserving this exceptional fauna.

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