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# Assessing phylogenetic resolution among mitochondrial, nuclear, and morphological datasets in *Nothonotus* darters (Teleostei: Percidae)

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## Abstract

External morphological characters are the basis of our understanding of diversity and species relationships in many darter clades. The past decade has seen the publication of many studies utilizing mtDNA sequence data to investigate darter phylogenetics, but only recently have nuclear genes been used to investigate darter relationships. Despite a long tradition of use in darter systematics few studies have examined the phylogenetic utility of external morphological characters in estimating relationships among species in darter clades. We present DNA sequence data from the mitochondrial cytochrome *b* (*cytb*) gene, the nuclear encoded *S7* intron 1, and discretely coded external morphological characters for all 20 species in the darter clade *Nothonotus*. Bayesian phylogenetic analyses result in phylogenies that are in broad agreement with previous studies. The *cytb* gene tree is well resolved, while the nuclear *S7* gene tree lacks phylogenetic resolution, node support, and is characterized by a lack of reciprocal monophyly for many of the *Nothonotus* species. The phylogenies resulting from analysis of the morphological dataset lack resolution, but nodes present are found in the *cytb* and *S7* gene trees. The highest resolution and node support is found in the Bayesian combined data phylogeny. Based on our results we propose continued exploration of the phylogenetic utility of external morphological characters in other darter clades. Given the extensive lack of reciprocal monophyly of species observed in the *S7* gene tree we predict that nuclear gene sequences may have limited utility in intraspecific phylogeographic studies of *Nothonotus* darters.

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## 1. Introduction

Molecular systematic studies have provided species-level phylogenetic hypotheses for several darter clades (Near, 2002; Near et al., 2000; Porter et al., 2002; Porterfield et al., 1999; Turner, 1997). These molecular phylogenies have provided a basis for comparative studies investigating the role of body size in the genetic structuring of darter populations (Turner and Trexler, 1998), the evolution of water column habitat utilization and dichromatism (Near, 2002), and the evolution of reproductive isolation (Mendelson, 2003). Substantial differences in species-level molecu-

lar phylogenies and traditional morphology-based relationships have been discovered in several darter clades (Near, 2002; Near et al., 2000; Porter et al., 2002). External morphology and patterns of male nuptial coloration were the basis for hypotheses of species-level relationships previous to these molecular studies, and morphological characters were often not presented as discretely coded character states and hypothesized relationships were not typically presented as trees estimated using phylogenetic methods (Page, 1974; Williams, 1975; Zorach, 1972; Zorach and Raney, 1967). Determination of the relative agreement among morphological and molecular phylogenetic analyses for most darter clades is constrained by the lack of datasets containing discretely coded morphological character states.

*Nothonotus* is a clade of 20 described darter species (Perciformes: Percidae) with a most recent common

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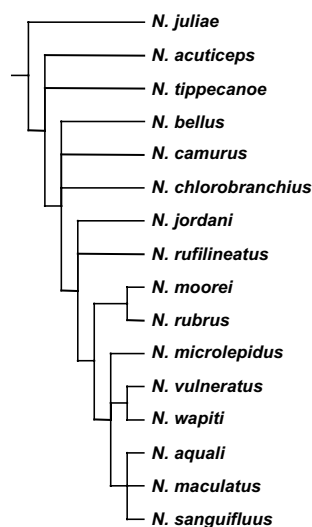
ancestor (MRCA) that has been dated close to 18 million years ago using time-calibrated mtDNA gene trees (Near and Keck, 2005). Unlike most other darter clades the diversity of external morphological and male nuptial coloration characters among *Nothonotus* species has been coded as discrete character states and used in maximum parsimony analyses (Etnier and Williams, 1989; Wood, 1996). The availability of molecular and morphological phylogenies that include specimens of all recognized *Nothonotus* species provides an opportunity to investigate the agreement of phylogenetic hypotheses inferred from morphological characters traditionally used in darter systematics with those resulting from analysis of aligned DNA sequence data.

Fig. 1 shows the previous phylogenetic hypotheses of *Nothonotus* species based on external morphology and male nuptial color patterns (Etnier and Williams, 1989), a combination of allozyme, external morphological, and behavioral characters (Wood, 1996), and mitochondrial cytochrome *b* gene sequences (Near and Keck, 2005). All of these phylogenetic hypotheses include a resolved clade of egg-guarding species referred to as the *N. maculatus* species clade and includes *N. aquali*, *N. maculatus*, *N. microlepidus*, *N. sanguifluus*, *N. vulneratus*, and *N. wapiti* (Etnier and Williams, 1989; Page, 1985; Zorach and Raney, 1967). In addition, these phylogenetic hypotheses depict the Ozark endemic *N. juliae* as the sister species to all other *Nothonotus* species and phylogenies based on allozyme and mtDNA sequence data result in a sister relationship between *N. acuticeps* and the Mobile Basin endemic *N. jordani* species clade (*N. chuckwachatte*, *N. douglasi*, *N. etowahae*, and *N. jordani*; Fig. 1). Despite appreciable agreement among these phylogenetic hypotheses some areas of disagreement and regions of poor phylogenetic resolution remain. All previous hypotheses of *Nothonotus* phylogeny

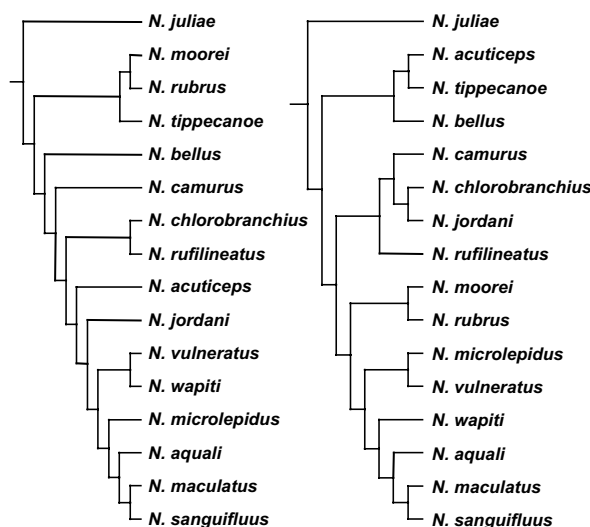
are based on phylogenetic datasets that are less than ideal. For instance, the most recent phylogenetic analyses have been based entirely on mtDNA gene sequences and carry all of the caveats of a single gene phylogeny, in particular the biological reality for potential conflict between the true species phylogeny and a single gene tree (Hudson, 1992; Maddison, 1997).

The classification of *Nothonotus* has recently been questioned as a result of phylogenetic studies of darters using mtDNA gene sequences (Near and Keck, 2005). From the mid-1950's to the present day all darter species are classified into four genera, *Ammocrypta*, *Crystallaria*, *Etheostoma*, and *Percina* (Bailey and Etnier, 1988; Bailey and Gosline, 1955; Bailey et al., 1954; Boschung and Mayden, 2004; Jenkins and Burkhead, 1994; Kuehne and Barbour, 1983; Page, 1981, 1983; Simons, 1991). In a discussion of the validity of *Ammocrypta* (including *Crystallaria*), *Etheostoma*, and *Percina* Bailey et al. (1954) provide clear morphological synapomorphies for *Ammocrypta* and *Percina*; however, no apomorphies for *Etheostoma* are identified and the group is defined as containing any darter species that is not *Ammocrypta*, *Crystallaria*, or *Percina* (Bailey et al., 1954; Page, 1981). *Nothonotus* species have historically fallen into this rubric and since Bailey et al. (1954) *Nothonotus* has been classified as a subgenus of *Etheostoma* (Bailey and Etnier, 1988; Boschung and Mayden, 2004; Etnier and Starnes, 1993; Kuehne and Barbour, 1983; Page, 1981). Phylogenetic studies using mtDNA gene sequences have consistently resulted in a non-monophyletic *Etheostoma*, because species of *Nothonotus* and *Allohistium* are more closely related to other darter clades (Sloss et al., 2004; Song et al., 1998). Based on these results, Near and Keck (2005) recommended the treatment of *Nothonotus* as a distinct clade and not a subgenus of *Etheostoma*. Lang and Mayden (2007) obtain a similar pattern of *Etheostoma*

#### A. External morphology (Etnier and Williams 1989)



#### B. Allozymes & external morphology (Wood 1996; Figs 1B, 2)



#### C. mtDNA (Near and Keck 2005)

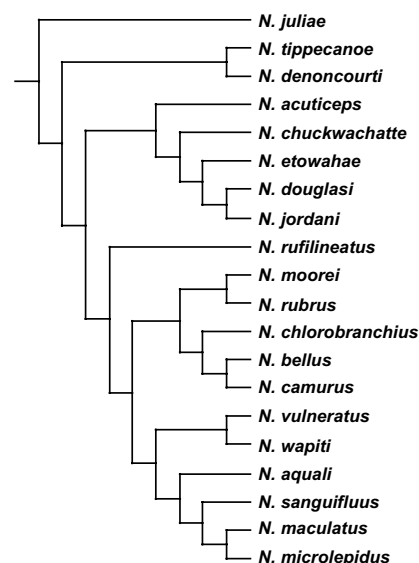


Fig. 1. Previous phylogenetic hypothesis for *Nothonotus*.

non-monophyly from a phylogeny inferred from mtDNA gene sequences, but their phylogenetic analysis of the nuclear encoded S7 intron 1 resulted in a monophyletic *Etheostoma* with weak node support. Lang and Mayden (2007) suggest this latter result should motivate the recognition of *Nothonotus* as a sub-clade of *Etheostoma*. However, our conclusions are based on the premise that the evolutionary history of a clade is described by any number of datasets and each can offer strengths and weaknesses for elucidating the phylogenetic relationships of the lineage. Given the distinct evolutionary history of *Nothonotus*, with strongly supported mtDNA inferred relationships closer to other darter lineages and weakly supported nuclear DNA inferred relationships with *Etheostoma*, and the distinct lack of morphological apomorphies for an inclusive *Etheostoma*, we suggest that *Nothonotus* be recognized as a distinct clade and not a subgenus of *Etheostoma*.

To investigate the agreement and degree of resolution between phylogenies inferred from different types of data and to determine the uniqueness of phylogenies inferred from combined data analyses, we collected mitochondrial and nuclear gene sequences from specimens of all *Nothonotus* species and utilized coded morphological character states used in previous phylogenetic studies. Combining morphological data sets with molecular data sets in maximum likelihood based analyses has become an option with the description of the Mk model by Lewis (2001). The results obtained using the Mk model in maximum likelihood phylogenetic analyses are typically very similar to those obtained using parsimony optimality criteria (Lewis, 2001; Nylander et al., 2004). A common assumption is that the nucleotide data will overwhelm any signal from morphological data in combined data phylogenetic analyses, but the combined analysis of morphological and molecular data generally results in phylogenies with higher resolution and support (Engstrom et al., 2004; Nylander et al., 2004; Wahlberg et al., 2005). Our analyses aim to provide insight as to the phylogenetic utility of external morphological and pigmentation characters traditionally used to investigate species level relationships in darter clades. Additionally, we discuss the prospect of using nuclear gene DNA sequence data to resolve phylogenetic relationships among closely related darter species and investigate phylogeographic patterns within species.

## 2. Materials and methods

All specimens were captured using seine nets or a backpack electrofishing unit (Table 1). Tissue biopsies were preserved in 100% ethanol and stored at 4 °C. Voucher specimens were fixed in 10% formalin for at least seven days, washed in tap water for two days, and transferred to 70% ethanol for long-term preservation. All specimens were deposited into either the University of Tennessee Research Collection of Fishes (UT) or the Yale Peabody Museum of Natural History fish collection (YPM). Darters

are classified in Percidae and we sampled two non-darter percids and nine other darter species from *Ammocrypta*, *Crystallaria*, *Etheostoma*, and *Percina* as outgroup species (Table 1).

Nucleic acids were extracted from tissue biopsies using the Qiagen DNAeasy DNA kits following the manufacturer's protocol. The mtDNA encoded cytochrome *b* (*cytb*) gene and the first intron of the nuclear encoded S7 ribosomal protein were amplified using PCR with primers and conditions outlined in Near et al. (2000) and Chow and Hazama (1998). Amplification products resulting from successful PCR were prepared for sequencing using Qiagen Qiaquick kits or by digesting with 1.0 unit of Exonuclease I and shrimp alkaline phosphatase and incubated for 15 min at 37 °C and 20 min at 80 °C. Treated PCR products were used as template for DNA sequencing that was performed by the DNA Sequencing Facility on Science Hill at Yale University or WM Keck Foundation Biotechnology Resource Laboratory at Yale University. Contiguous sequences were assembled from individual sequencing reactions using the computer program Sequencher version 4.5 (Gene Codes, Ann Arbor, MI, USA). Alignments of mtDNA *cytb* gene were performed by eye and the S7 intron was aligned using the computer program MUSCLE (Edgar, 2004). Four data partitions were identified: three codon positions in the protein coding *cytb* gene, and a single partition for the S7 intron. The optimal maximum likelihood model for each partition was determined with AIC as executed in the computer program Modeltest 3.0 (Posada and Crandall, 1998).

Discretely coded character states for external morphology and male nuptial coloration published in Etnier and Starnes (1993) and Wood (1996) were entered into MacClade 4.0 (Maddison and Maddison, 2000). Character state coding for outgroup species followed those presented in Etnier and Starnes (1993), examination of photographs of specimens from our field work, and examination of preserved museum specimens (Table 2).

Phylogenetic hypotheses were generated with partitioned mixed-model Bayesian analyses (Ronquist and Huelsenbeck, 2003) with posterior probabilities estimated using metropolis-coupled Markov chain Monte Carlo (Huelsenbeck et al., 2001; Larget and Simon, 1999). We performed separate analyses for the *cytb*, S7 intron, and morphological datasets. The optimal maximum likelihood models identified for each of the four data partitions in the molecular data were used in the computer program MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Morphological characters were treated as "standard" in MrBayes and used the Mk model (Lewis, 2001). This model allows for there to be *k* number of discrete character states and the rate of character change to be equal or to vary. We utilized a gamma distribution of among character rate variation that allowed a different rate of change for the individual morphological characters (Muller and Reisz, 2006; Nylander et al., 2004). Four sets of Bayesian

Table 1  
Specimens sampled, geographic localities, and catalog numbers

Species	Code	Locality	Lat (N)	Long (W)	Catalog number
<i>Perca flavescens</i>	PflaA	Lake Andrusia, Beltrami Co., MN	47 27 23	94 39 13	YFTC 261
<i>Sander vitreus</i>	SvitA	Mississippi River, Rock Island Co., IL	41 30 46	90 35 22	YFTC 312
<i>Crystallaria asprella</i>	CaspB	Cahaba River, Bibb Co., AL	32 56 52	87 08 26	YFTC 686
<i>Ammocrypta pellucida</i>	ApelA	Embarrass River, Cumberland Co., IL	39 15 01	88 10 27	YFTC 104
<i>Percina roanoka</i>	ProaA	Blackwater River, Franklin Co., VA	37 03 14	79 52 56	YFTC 76
<i>Percina evides</i>	PeviL	Black River, Jackson Co., WI	44 15 45	90 52 14	YFTC 1134
<i>Percina caprodes</i>	PcapD	Big Piney Fork, Sharp Co., AR	36 04 50	91 36 39	YFTC 396
<i>Etheostoma cinereum</i>	EcinA	Rockcastle River, Rockcastle Co., KY	37 17 33	84 13 14	YFTC 689
<i>Etheostoma blennioides</i>	EbleA	West Fork Pond River, Christian Co., KY	37 01 52	87 24 21	YFTC 756
<i>Etheostoma flabellare</i>	EflaB	Middle Fork of the Vermillion River, Vermillion Co., IL	40 14 06	87 46 18	YFTC 1438
<i>Etheostoma virgatum</i>	EvirF	Clear Creek, Rockcastle Co., KY	37 28 10	84 17 07	YFTC 781
<i>Nothonotus acuticeps</i>	NacuA	Nolichucky River, Greene Co., TN	36 06 00	83 02 51	YFTC 2205
<i>Nothonotus acuticeps</i>	NacuB	Nolichucky River, Greene Co., TN	36 06 00	83 02 51	YFTC 5659
<i>Nothonotus acuticeps</i>	NacuC	Nolichucky River, Greene Co., TN	36 06 00	83 02 51	YFTC 5660
<i>Nothonotus acuticeps</i>	NacuI	Nolichucky River, Greene Co., TN	36 06 00	83 02 51	YFTC 5666
<i>Nothonotus aquali</i>	NaquA	Buffalo River, Lewis Co., TN	35 27 44	87 32 10	YFTC 68
<i>Nothonotus aquali</i>	NaquB	Duck River, Bedford Co., TN	35 33 12	86 34 57	YFTC 2562
<i>Nothonotus aquali</i>	NaquD	Buffalo River, Lewis Co., TN	35 27 44	87 32 10	YFTC 69
<i>Nothonotus bellus</i>	NblmA	Goose Creek, Russell Co., KY	37 05 56	85 03 05	YFTC 754
<i>Nothonotus bellus</i>	NblmB	Long Creek, Macon Co., TN	36 35 01	85 55 47	YFTC 4865
<i>Nothonotus bellus</i>	NblmD	Petty's Fork of Russell Creek, Adair Co., KY	37 05 57	85 20 16	YFTC 6451
<i>Nothonotus bellus</i>	NblmF	Petty's Fork of Russell Creek, Adair Co., KY	37 05 57	85 20 16	YFTC 6453
<i>Nothonotus bellus</i>	NblmK	Middle Fork Drakes Creek, Allen Co., KY	36 41 31	86 20 53	YFTC 6306
<i>Nothonotus camurus</i>	NcamA	Middle Fork of the Vermillion River, Vermillion Co., IL	40 14 06	87 46 18	YFTC 1203
<i>Nothonotus camurus</i>	NcamC	Clinch River, Claiborne Co., TN	36 25 48	83 23 49	YFTC 2582
<i>Nothonotus camurus</i>	NcamI	Brimstone Creek, Scott Co., TN	36 18 17	84 30 18	YFTC 5249
<i>Nothonotus camurus</i>	NcamM	Little River, Blount Co., TN	35 45 56	83 51 23	YFTC 3309
<i>Nothonotus camurus</i>	NcamP	Nolichucky River, Greene Co., TN	36 06 00	83 02 51	YFTC 5669
<i>Nothonotus camurus</i>	NcamZ	Buck Creek, Pulaski Co., KY	37 18 34	84 33 59	YFTC 6492
<i>Nothonotus camurus</i>	NcamAA	Holston River, Jefferson Co., TN	36 06 02	83 40 09	YFTC 5640
<i>Nothonotus camurus</i>	NcamAK	Big South Fork, Scott Co., TN	36 32 49	84 39 56	YFTC 7063
<i>Nothonotus camurus</i>	NcamAM	Rockcastle River, Laurel/Rockcastle Co. line, KY	37 16 46	84 12 35	YFTC 7017
<i>Nothonotus camurus</i>	NcamAO	Sinking Creek, Laurel Co., KY	37 05 23	84 12 12	YFTC 6519
<i>Nothonotus chlorobranchius</i>	NchbA	Burningtown Creek, Macon Co., NC	35 15 23	83 28 20	YFTC 2120
<i>Nothonotus chlorobranchius</i>	NchbC	Little Pigeon River, Sevier Co., TN	35 44 21	83 24 59	YFTC 3094
<i>Nothonotus chlorobranchius</i>	NchbD	North Fork Mills River, Henderson Co., NC	35 24 22	82 38 39	YFTC 5967
<i>Nothonotus chlorobranchius</i>	NchbE	North Fork Mills River, Henderson Co., NC	35 24 22	82 38 39	YFTC 5968
<i>Nothonotus chlorobranchius</i>	NchbI	North Indian Creek, Unicoi Co., TN	36 08 36	82 25 42	YFTC 6803
<i>Nothonotus chuckwachatte</i>	NckwA	Tallapoosa River, Haralson Co., GA	33 51 49	85 12 47	YFTC 2275
<i>Nothonotus chuckwachatte</i>	NckwB	Cane Creek, Cleburne Co., AL	33 43 31	85 29 27	YFTC 5609
<i>Nothonotus chuckwachatte</i>	NckwC	Cane Creek, Cleburne Co., AL	33 43 31	85 29 27	YFTC 5610
<i>Nothonotus chuckwachatte</i>	NckwD	Cane Creek, Cleburne Co., AL	33 43 31	85 29 27	YFTC 5611
<i>Nothonotus denoncourti</i>	NdenA	Clinch River, Claiborne Co., TN	36 25 48	83 23 49	YFTC 2585
<i>Nothonotus denoncourti</i>	NdenB	Sequatchie River, Sequatchie Co., TN	35 04 45	85 35 35	YFTC 4108
<i>Nothonotus douglasi</i>	NdouA	Sipsey Fork, Winston Co., AL	34 17 07	87 23 57	YFTC 2116
<i>Nothonotus douglasi</i>	NdouB	Borden Creek, Lawrence Co., AL	34 19 46	87 22 37	YFTC 5540
<i>Nothonotus douglasi</i>	NdouC	Sipsey Fork, Winston Co., AL	34 17 07	87 23 57	YFTC 5567
<i>Nothonotus douglasi</i>	NdouD	Sipsey Fork, Winston Co., AL	34 17 07	87 23 57	YFTC 5568
<i>Nothonotus douglasi</i>	NdouF	Borden Creek, Lawrence Co., AL	34 19 46	87 22 37	YFTC 5539
<i>Nothonotus etowahae</i>	NetoA	Etowah River, Lumpkin Co., GA	34 32 06	84 03 48	YFTC 2272
<i>Nothonotus etowahae</i>	NetoC	Shoal Creek, Dawson Co., GA	34 25 57	84 07 45	YFTC 4089
<i>Nothonotus jordani</i>	NjorA	Conasauga River, Polk Co., TN.	35 00 35	84 43 55	YFTC 985
<i>Nothonotus jordani</i>	NjorB	Conasauga River, Polk Co., TN.	35 00 09	84 46 44	YFTC 2366
<i>Nothonotus jordani</i>	NjorD	Conasauga River, Polk Co., TN.	35 00 09	84 46 44	YFTC 2363
<i>Nothonotus jordani</i>	NjorE	Conasauga River, Polk Co., TN.	35 00 09	84 46 44	YFTC 2364
<i>Nothonotus jordani</i>	NjorF	Conasauga River, Polk Co., TN.	35 00 09	84 46 44	YFTC 2365
<i>Nothonotus juliae</i>	NjulA	Buffalo River, Searcy Co., AR	35 59 08	92 44 41	YFTC 897
<i>Nothonotus juliae</i>	NjulC	Buffalo River, Searcy Co., AR	35 59 08	92 44 41	YFTC 2957
<i>Nothonotus juliae</i>	NjulD	Buffalo River, Searcy Co., AR	35 59 08	92 44 41	YFTC 2958
<i>Nothonotus juliae</i>	NjulE	Buffalo River, Searcy Co., AR	35 59 08	92 44 41	YFTC 2959
<i>Nothonotus maculatus</i>	NmacA	Green River, Green Co., KY	37 15 13	85 30 10	YFTC 446
<i>Nothonotus microlepidus</i>	NmclA	Harpeth River, Cheatham Co., TN	36 07 23	87 05 57	YFTC 2249
<i>Nothonotus microlepidus</i>	NmclB	East Fork Stones River, Rutherford Co., TN	35 52 57	86 16 22	YFTC 5487
<i>Nothonotus microlepidus</i>	NmclC	East Fork Stones River, Rutherford Co., TN	35 52 57	86 16 22	YFTC 5488

(continued on next page)

Table 1 (continued)

Species	Code	Locality	Lat (N)	Long (W)	Catalog number
<i>Nothonotus microlepidus</i>	NmclD	East Fork Stones River, Rutherford Co., TN	35 52 57	86 16 22	YFTC 5489
<i>Nothonotus moorei</i>	NmorA	Middle Fork Little Red River, Van Buren Co., AR	35 39 11	92 18 56	YFTC 2896
<i>Nothonotus moorei</i>	NmorC	Middle Fork Little Red River, Van Buren Co., AR	35 39 11	92 18 56	YFTC 2898
<i>Nothonotus moorei</i>	NmorL	Middle Fork Little Red River, Van Buren Co., AR	35 31 20	92 26 26	YFTC 2884
<i>Nothonotus rubrus</i>	NrubB	Bayou Pierre, Copiah Co., MS	31 52 12	90 29 52	YFTC 4912
<i>Nothonotus rubrus</i>	NrubC	Bayou Pierre, Copiah Co., MS	32 00 10	90 41 22	YFTC 4913
<i>Nothonotus rubrus</i>	NrubD	Foster's Creek, Copiah Co., MS	31 56 18	90 37 17	YFTC 4914
<i>Nothonotus rufilineatus</i>	NrufH	Horse Creek, Hardin Co., TN	35 10 50	88 12 35	YFTC 2493
<i>Nothonotus rufilineatus</i>	NrufI	Horse Creek, Hardin Co., TN	35 10 50	88 12 35	YFTC 2494
<i>Nothonotus rufilineatus</i>	NrufAM	Elk River, Moore/Franklin Co., TN	35 09 49	86 19 06	YFTC 5747
<i>Nothonotus rufilineatus</i>	NrufAV	Grinders Creek, Lewis Co. TN	35 27 44	87 32 10	YFTC 770
<i>Nothonotus rufilineatus</i>	NrufBB	Duck River, Coffee Co., TN	35 29 07	86 07 18	YFTC 6595
<i>Nothonotus rufilineatus</i>	NrufBK	Grinders Creek, Lewis Co. TN	35 27 44	87 32 10	YFTC 771
<i>Nothonotus rufilineatus</i>	NrufCQ	Duck River, Coffee Co., TN	35 29 07	86 07 18	YFTC 6594
<i>Nothonotus sanguifluus</i>	NsgfA	Rockcastle River, Rockcastle Co., KY	37 17 33	84 13 14	YFTC 940
<i>Nothonotus sanguifluus</i>	NsgfB	Scott Creek, Warren Co., TN	35 34 17	85 42 43	YFTC 2444
<i>Nothonotus sanguifluus</i>	NsgfG	Collins River, Warren Co., TN	35 44 46	85 41 54	YFTC 2472
<i>Nothonotus sanguifluus</i>	NsgfH	Cane Creek, VanBuren Co., TN	35 46 57	85 24 16	YFTC 3128
<i>Nothonotus sanguifluus</i>	NsgfI	Cane Creek, VanBuren Co., TN	35 46 57	85 24 16	YFTC 3129
<i>Nothonotus sanguifluus</i>	NsgfO	Buck Creek, Pulaski Co., KY	37 09 05	84 26 18	YFTC 6504
<i>Nothonotus sanguifluus</i>	NsgfQ	Buck Creek, Pulaski Co., KY	37 09 05	84 26 18	YFTC 6506
<i>Nothonotus sanguifluus</i>	NsgfT	Big South Fork, Scott Co., TN	36 32 49	84 39 56	YFTC 7070
<i>Nothonotus sanguifluus</i>	NsgfV	Big South Fork, Scott Co., TN	36 32 49	84 39 56	YFTC 7068
<i>Nothonotus sanguifluus</i>	NsgfZ	Rockcastle River, Laurel/Rockcastle Co. line, KY	37 16 46	84 12 35	YFTC 7022
<i>Nothonotus tippecanoe</i>	NtipA	Green River, Green Co., KY	37 15 59	85 34 52	YFTC 6424
<i>Nothonotus tippecanoe</i>	NtipB	Big South Fork, Scott Co., TN	36 32 49	84 39 56	YFTC 7084
<i>Nothonotus tippecanoe</i>	NtipF	Licking River, Pendleton Co., KY	38 47 22	84 22 03	YFTC813
<i>Nothonotus tippecanoe</i>	NtipG	South Fork Kentucky River, Clay Co., KY	37 16 24	83 38 47	YFTC 459
<i>Nothonotus vulneratus</i>	NvulA	Taccoa River, Fannin Co., GA	34 45 51	84 14 56	YFTC 2203
<i>Nothonotus vulneratus</i>	NvulB	Oconoluftee River, Jackson Co., NC	35 28 07	83 19 28	YFTC 2204
<i>Nothonotus vulneratus</i>	NvulE	Clinch River, Claiborne Co., TN	36 25 48	83 23 49	YFTC 2591
<i>Nothonotus vulneratus</i>	NvulG	Daddys Creek, Cumberland Co., TN	36 03 32	84 47 32	YFTC 460
<i>Nothonotus vulneratus</i>	NvulH	Nolichucky River, Greene Co., TN	36 06 00	83 02 51	YFTC 5677
<i>Nothonotus vulneratus</i>	NvulI	Little River, Blount Co., TN	35 47 16	83 52 55	YFTC 5776
<i>Nothonotus wapiti</i>	NwapB	Elk River, Giles/Lincoln Co., TN	35 04 56	86 49 60	YFTC 4097
<i>Nothonotus wapiti</i>	NwapE	Elk River, Giles/Lincoln Co., TN	35 04 56	86 49 60	YFTC 4100
<i>Nothonotus wapiti</i>	NwapF	Elk River, Giles/Lincoln Co., TN	35 04 56	86 49 60	YFTC 4101

analyses were run, one for each molecular dataset and morphology, and an analysis where all three datasets were combined. Only a single operational taxonomic unit (OTU) was scored for each species in the phylogenetic analyses using the morphological data; however, multiple OTUs were scored for each species (except *N. maculatus*) in the combined data analyses. In each analysis MrBayes 3.1 was run for  $6.0 \times 10^6$  generations to ensure convergence of the MC3 algorithm and the number of generations discarded as the burn-in was determined by plotting the maximum likelihood score versus the number of generations. The posterior probabilities of nodes in the phylogeny were calculated from the set of post burn-in trees. All resulting phylogenetic hypothesis were rooted with the eleven outgroup species (Table 1).

In addition to the Bayesian analysis we used maximum parsimony to analyze the morphological data. The most parsimonious trees were found using PAUP\* 4.0 (Swofford, 2003) with heuristic tree searches and 100 addition sequence replicates. All characters were treated as unordered and node support was assessed with a bootstrap analysis using 2000 pseudoreplicates.

### 3. Results

Taxon sampling for this phylogenetic analysis included 90 individuals sampled from all 20 *Nothonotus* species. The only species sampled with a single specimen was *N. maculatus* (Table 1). The aligned *cytb* sequences consisted of 1140 nucleotide sites and did not include any insertions or deletions. The size of the nuclear S7 ribosomal protein intron 1 ranged from 516 base pairs (bp) to 530 bp among *Nothonotus* species and the length of the S7 ribosomal protein intron 1 alignment was 549 bp including gaps. All new sequences were submitted to GenBank with accession numbers EU094658–EU094820.

Intraspecific uncorrected pairwise nucleotide distances were less than 1.0% for most species, but five species showed a maximum intraspecific genetic distance higher than 1.0% for the *cytb* gene and seven species exhibited maximum intraspecific genetic divergences exceeding 1.0% at the nuclear S7 intron (Table 3). Maximum intraspecific genetic distances at the S7 locus were much higher than the maximum genetic distances observed for mitochondrial *cytb* in *Nothonotus aquali*, *N. douglasi*, and *N. microlepidus* (Table 3).

Table 2  
Coding of discrete morphological characters for *Nothonotus* species

Species	1	2	3	4	5	6	7	8	9	10	11	12
<i>Nothonotus juliae</i>	1	0	0	0	0	0	0	0	0	0	1	0
<i>Nothonotus denoncourti</i>	0	1	1	0	0	0	0	1	0	1	1	0
<i>Nothonotus tippecanoe</i>	0	1	1	0	0	0	0	1	0	1	1	0
<i>Nothonotus rufilineatus</i>	1	1	1	0	1	1	0	1	0	0	0	0
<i>Nothonotus acuticeps</i>	1	1	1	1	0	0	0	0	0	0	0	0
<i>Nothonotus chuckwachatte</i>	0	1	1	1	1	1	0	1	0	0	0	0
<i>Nothonotus etowahae</i>	0	1	1	1	1	0	0	1	0	0	0	0
<i>Nothonotus douglasi</i>	0	1	1	0	1	0	0	1	0	0	0	0
<i>Nothonotus jordani</i>	0	1	1	1	1	1	0	1	0	0	0	0
<i>Nothonotus moorei</i>	1	1	1	0	1	0	0	1	1	1	1	0
<i>Nothonotus rubrus</i>	1	1	1	0	1	1	0	1	1	1	1	0
<i>Nothonotus chlorobranchius</i>	1	1	1	0	1	1	0	0	0	0	0	0
<i>Nothonotus camurus</i>	1	1	1	0	1	1	0	0	0	0	0	0
<i>Nothonotus bellus</i>	1	1	1	0	1	1	0	0	0	0	0	0
<i>Nothonotus vulneratus</i>	1	1	1	0	1	1	1	1	1	0	0	1
<i>Nothonotus aquali</i>	1	1	1	0	0	1	1	1	1	0	0	0
<i>Nothonotus sanguifluus</i>	1	1	1	0	0	1	1	1	1	0	0	0
<i>Nothonotus maculatus</i>	1	1	1	0	0	1	1	1	1	0	0	0
<i>Nothonotus microlepidus</i>	1	1	1	0	1	1	1	1	1	0	0	0
<i>Nothonotus wapiti</i>	1	1	1	0	1	0	0	1	1	0	0	1
Outgroup	0	0	0	0	0	0	0	0	0	0	0	0

Character 1: Dark lines on side of body, (0) absent, (1) present; character 2: color on breast, (0) absent, (1) present; character 3: scales on nape, (0) absent, (1) present; character 4: scales on opercle, (0) absent, (1) present; character 5: dark bands on fin margins, (0) absent, (1) present; character 6: red spots on side of body, (0) absent, (1) present; character 7: round halos on side of body, (0) absent, (1) present; character 8: sexual dimorphism in median fin coloration, (0) absent, (1) present; character 9: scales on upper cheek, (0) absent, (1) present; character 10: scales on belly, (0) absent, (1) present; character 11: modal number of vertebrae, (0)  $\geq 37$ , (1)  $\leq 36$ ; character 12: bright color on anal fin of males, (0) absent, (1) present.

Table 3  
Uncorrected pairwise DNA sequence divergence observed within *Nothonotus* species sampled with three or more specimens

Species (number sampled)	Min <i>cytb</i>	Max <i>cytb</i>	Mean <i>cytb</i>	Min S7	Max S7	Mean S7
<i>Nothonotus acuticeps</i> (4)	0.00088	0.00439	0.00263	0	0	0
<i>Nothonotus aquali</i> (3)	0.00088	0.00526	0.00351	0.01149	0.03643	0.02366
<i>Nothonotus bellus</i> (5)	0.00175	0.00965	0.00614	0	0.00766	0.00249
<i>Nothonotus camurus</i> (10)	0	0.00965	0.00590	0	0.01156	0.00236
<i>Nothonotus chlorobranchius</i> (5)	0.00263	0.03070	0.01771	0	0.01924	0.00862
<i>Nothonotus chuckwachatte</i> (4)	0	0.00439	0.00219	0	0.00383	0.00191
<i>Nothonotus douglasi</i> (5)	0.00088	0.00351	0.00207	0	0.02323	0.01291
<i>Nothonotus jordani</i> (5)	0	0.01670	0.00773	0	0.00382	0.00193
<i>Nothonotus juliae</i> (4)	0	0.00175	0.00088	0	0.00386	0.00193
<i>Nothonotus microlepidus</i> (4)	0	0.00439	0.00307	0	0.01530	0.00510
<i>Nothonotus moorei</i> (3)	0	0.00176	0.00117	0	0	0
<i>Nothonotus rubrus</i> (3)	0	0	0	0	0	0
<i>Nothonotus rufilineatus</i> (7)	0.00088	0.02018	0.01295	0	0.02536	0.01195
<i>Nothonotus sanguifluus</i> (10)	0	0.02281	0.01542	0	0.00958	0.00414
<i>Nothonotus tippecanoe</i> (4)	0	0.00351	0.00175	0	0	0
<i>Nothonotus vulneratus</i> (6)	0.00351	0.02807	0.01749	0.00191	0.01153	0.00500
<i>Nothonotus wapiti</i> (3)	0	0.00088	0.00058	0	0.00574	0.00319

The minimum (Min) and maximum (Max) intraspecific divergences observed at the mitochondrial cytochrome *b* gene (*cytb*) and the nuclear encoded S7 intron 1 is reported for each species.

Bayesian analysis of the *cytb* alignment resulted in a set of posterior trees that were fairly well resolved and similar to previous analyses of *Nothonotus* phylogeny (Fig. 2). The node representing the MRCA of *Nothonotus* and most internal nodes in the *cytb* phylogeny were supported with significant Bayesian posterior probabilities. A notable exception was the MRCA of the Mobile Basin endemic *N. jordani* clade (*N. jordani*, *N. etowahae*, *N. douglasi*, and *N. chuckwachatte*) that was supported with a non-

significant Bayesian posterior probability, but the MRCA node of *N. acuticeps* and the *N. jordani* species group was supported with a significant posterior probability. Five of the 19 *Nothonotus* species sampled with multiple specimens were not reciprocally monophyletic in the *cytb* Bayesian phylogeny. In two cases haplotypes from sister species pairs (*N. bellus*–*N. camurus* and *N. sanguifluus*–*N. microlepidus*) did not sort to exclusive lineages (Fig. 2). The three specimens sampled from *N. wapiti* were reciprocally

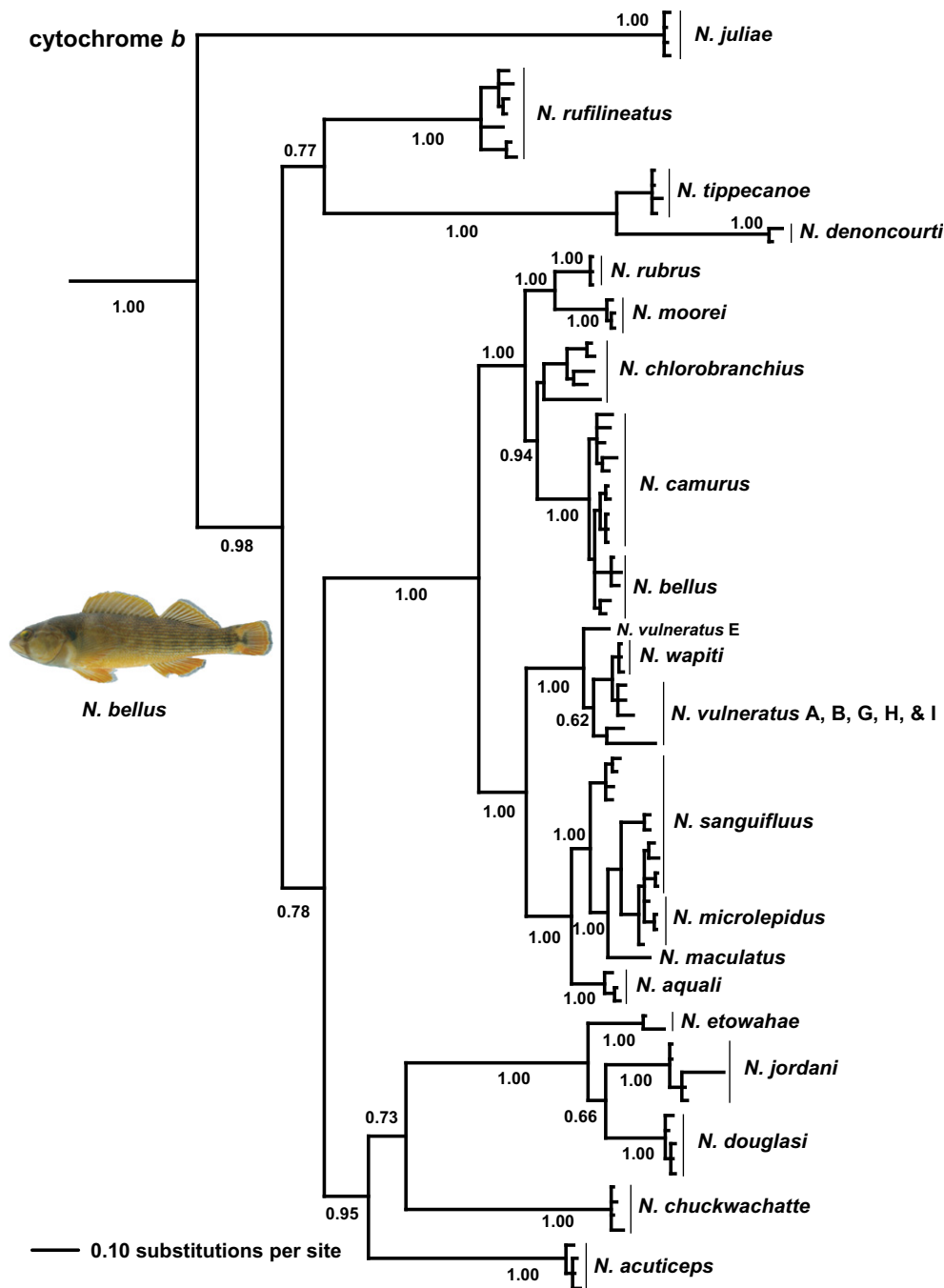


Fig. 2. Phylogeny of *Nothonotus* resulting from Bayesian analysis of mitochondrial cytochrome *b* gene sequences. Numbers at nodes represent Bayesian posterior probabilities.

monophyletic and nested in a paraphyletic group of *N. vulneratus* haplotypes.

The Bayesian phylogeny inferred from the nuclear encoded S7 ribosomal protein intron 1 was much less resolved than the *cytb* phylogeny with only three internal nodes supported with significant posterior probabilities (Fig. 3). The alleles sampled from individual *Nothonotus* species were reciprocally monophyletic in only five of the 19 species sampled with two or more individuals. This was not limited to paraphyly between sister species as seen in the *cytb* phylogeny, but alleles sampled from *N. douglasi*,

*N. aquali*, *N. rufilineatus*, and *N. camurus* were widely distributed throughout the S7 Bayesian phylogeny (Fig. 3).

Phylogenetic analysis of the 12 discretely coded morphological characters resulted in very little resolution among *Nothonotus* species (Fig. 4). Despite the lack of resolution, both analyses resulted in phylogenies that placed *N. juliae* as the sister species to all other *Nothonotus*, a relationship consistent with the phylogenies inferred from *cytb* and S7 (Figs. 2 and 3). Maximum parsimony analysis found 611 most parsimonious trees with 24 inferred changes. The strict consensus of these trees is presented in Fig. 4. The

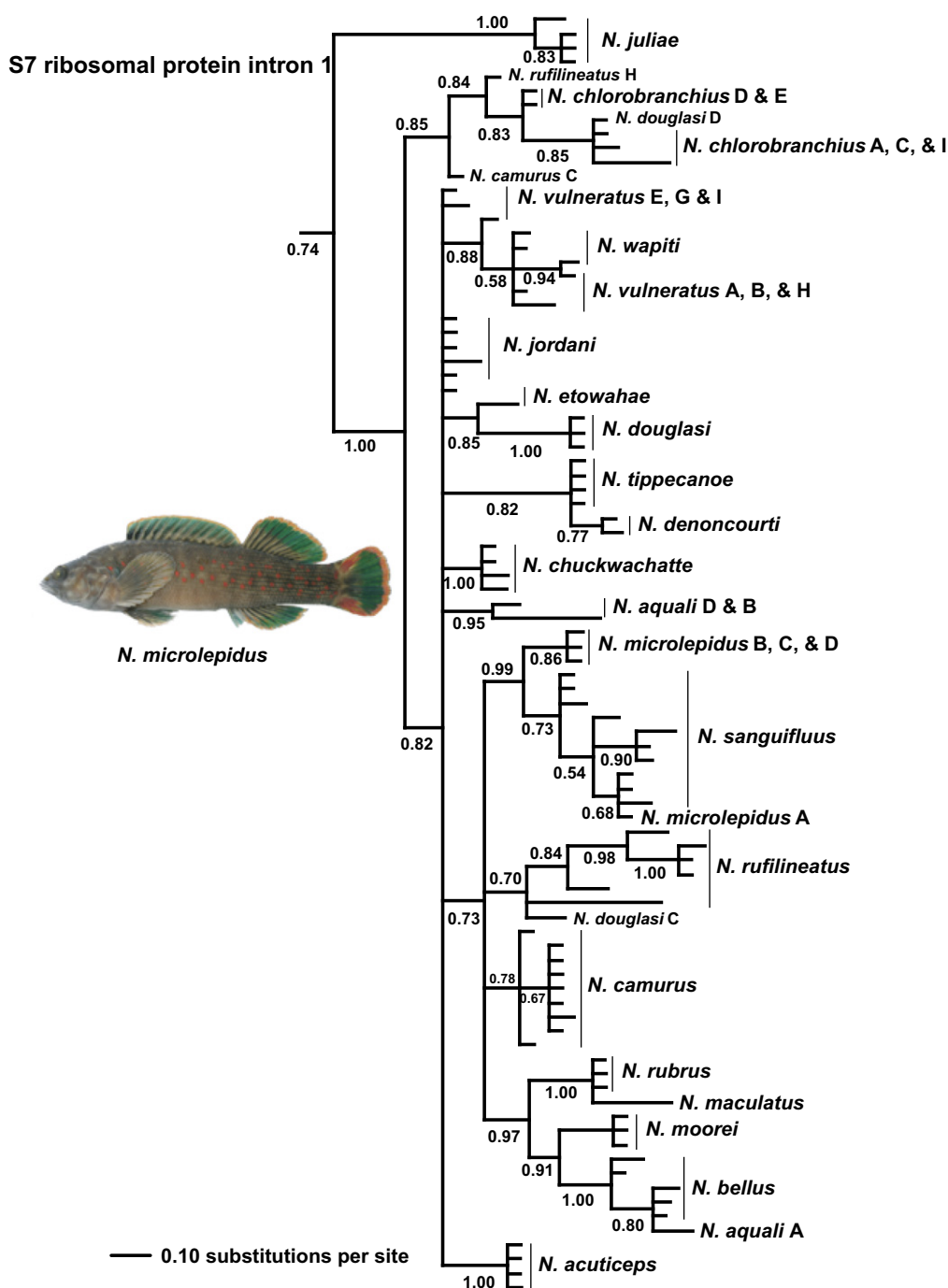


Fig. 3. Phylogeny of *Nothonotus* resulting from Bayesian analysis of nuclear encoded S7 ribosomal protein intron 1 gene sequences. Numbers at nodes represent Bayesian posterior probabilities.

only node supported with a bootstrap score  $\geq 75$  in the maximum parsimony tree was the node depicting *N. tippecanoe* and *N. denoncourti* as sister species (Fig. 4). No nodes were supported with significant posterior probability score in the Bayesian analysis.

The Bayesian analyses of the combined molecular and morphological data resulted in a phylogeny similar to the *cytb* Bayesian phylogeny with regard to specific relationships and degree of resolution (Fig. 5). Similar to the *cytb* phylogeny the combined data phylogenetic analyses

resulted in *N. juliae* as the sister species to all other *Nothonotus* species, *N. tippecanoe* and *N. denoncourti* as sister species, and monophyly of the six species in the *N. maculatus* clade that exhibit male parental care (Fig. 5). The monophyly of the Mobile Basin endemic *N. jordani* species clade was not supported in greater than 50% of the post burn in Bayesian phylogenies; however, the monophyly of clade containing the species in the *N. jordani* clade and the upper Tennessee River endemic *N. acuticeps* was supported with a significant posterior probability. The MRCA of the clade

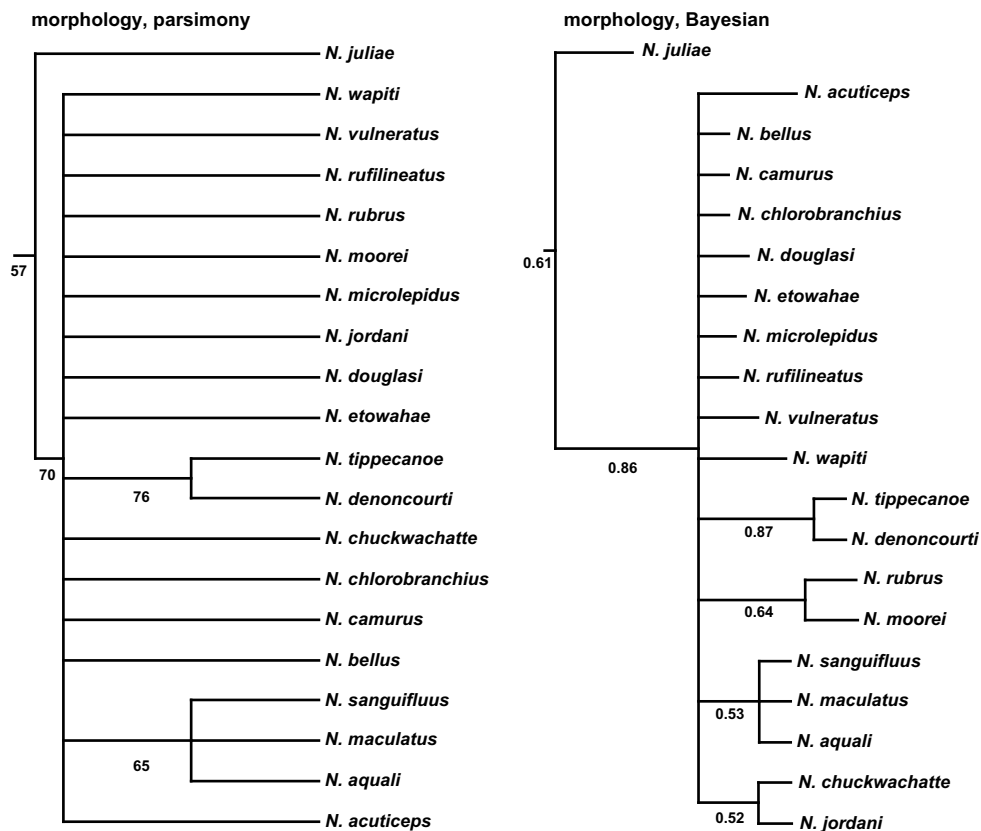


Fig. 4. Phylogenies for *Nothonotus* species resulting from maximum parsimony and Bayesian analyses of external morphological characters. Numbers at nodes in the parsimony phylogeny represent percent recovery in bootstrap analysis, and those in the Bayesian phylogeny represent posterior probabilities.

containing *N. camurus*, *N. bellus*, and *N. chlorobranichius*, and the MRCA of *N. wapiti* and *N. vulneratus* (exclusive of *N. vulneratus* E) were supported with significant posterior probabilities, but these nodes were not supported in the *cytb* phylogeny (Figs. 2 and 5). In the combined data phylogeny three of the 19 *Nothonotus* species with more than one specimen sampled were not reciprocally monophyletic. Both *N. camurus* and *N. bellus* were reciprocally monophyletic in the combined data phylogenetic analysis; however, haplotypes sampled from these two species were not reciprocally monophyletic in the *cytb* phylogeny.

#### 4. Discussion

The phylogenetic analyses for *Nothonotus* presented in this study used one of the most comprehensive datasets ever compiled to investigate relationships among species in a darter clade. Despite the large amount of data compiled for this investigation the results of our phylogenetic analyses are strikingly similar to previous efforts using external morphology, behavioral characters, and allozyme variation shown in Fig. 1 (Etnier and Williams, 1989; Wood, 1996). In particular, all phylogenetic analyses of *Nothonotus* have resulted in *N. juliae* as the sister species of all other *Nothonotus* species, a sister species relationship between *N. moorei* and *N. rubrus*, and a clade containing the species that exhibit parental care through male egg

guarding. The exceptions are the phylogenies resulting from analysis of the S7 intron and external morphology datasets that lacked resolution, including the monophyly of species that exhibit male parental care (Figs. 3 and 4).

Does the substantially different phylogenetic resolution obtained with the mitochondrial *cytb* gene sequences and either the nuclear gene sequences or external morphology provide any direction for studying species-level relationships in other darter clades? Few published studies have examined the phylogenetic utility of external morphological characters in resolving relationships among darter species. Braasch and Mayden (1985) used 46 discrete external morphological characters to examine relationships among species in the darter clade *Catnotus*. The phylogenies were completely resolved and subsequent phylogenetic analyses of *Catnotus* using *cytb* DNA sequences found broad agreement with Braasch and Mayden's (1985) morphological phylogeny (Porterfield et al., 1999). Page (1974,1981) combined discrete and continuous characters from larger numbers of darter species for phenetic analyses to determine if predefined genera and subgenera clustered in the resulting phenograms. The results of these analyses were inconclusive, as genera and subgenera failed to cluster. However, it was not clear if such relationships resulted from differences at discrete or continuous characters. Unfortunately, the most extensive analysis of darter phylogeny using discrete morphological characters is a Ph.D.



taxonomy and phylogeny (Etnier and Williams, 1989; Kinziger et al., 2001; Raney and Suttkus, 1966; Stauffer and Van Snik, 1997; Wood, 1996). Despite the lack of phylogenetic resolution the morphological characters do provide evidence for at least three nodes in the *Nothonotus* phylogeny that is independent of the *cytb* and *S7* gene trees.

When compared to the nuclear gene *S7* phylogeny (Fig. 3) the higher phylogenetic resolution and greater frequency of reciprocally monophyletic species in the *cytb* phylogeny is expected, because ancestral genetic polymorphisms will sort faster for mitochondrial haplotypes than nuclear gene alleles (Hudson and Coyne, 2002). This is due to the differences in the effective population sizes across genomes, where the mitochondrial genome has an effective population size that is one quarter that of a given set of linked nuclear autosomal loci (Avice et al., 1988; Birky et al., 1983; Moore, 1995). Despite the expected difference in resolution between mitochondrial and nuclear gene trees, mitochondrial gene trees can be incongruent with the species tree due to introgression facilitated by interspecific hybridization (Avice, 1994; Maddison, 1997) and comparing mitochondrial and nuclear gene trees provides a way to identify instances of introgression (Bachtrog et al., 2006; Shaw, 2002). Hybridization and introgression between *N. chlorobranchius* and *N. camurus* has been documented using allozyme data in an area of secondary contact between these two species in the Nolichucky River in Tennessee (Eisenhour, 1995); however, we did not detect any patterns consistent with introgression in our limited sampling of these two species.

We propose that the lack of reciprocal monophyly observed in both the nuclear *S7* and mitochondrial *cytb* phylogenies is due to internodes in the *Nothonotus* phylogeny that are short when scaled by the ancestral effective population sizes along specific branches. This lack of sorting is extensive in the *S7* phylogeny (Fig. 3), but limited to sister species contrasts in the *cytb* tree (Fig. 2). A previous study using external fossil calibrations to estimate molecular divergence times with a *Nothonotus cytb* phylogeny resulted in ages that were all less than 1.5 million years for each MRCA node containing species that are not reciprocally monophyletic in the *cytb* phylogeny (Near and Keck, 2005), indicating that short branches in evolutionary time coupled with relatively large effective population sizes may explain the lack of mtDNA haplotype sorting for these *Nothonotus* species. However, the sister species *N. microlepidus* and *N. sanguifluus*, while non-monophyletic, exhibit similar patterns in both the *cytb* and *S7* phylogenies (Figs. 2 and 3). The MRCA node for this species pair, and *N. maculatus*, also dates to approximately 1.4 million years suggesting that these species may have distinctly different population sizes or dynamics than the other sister species pairs with MRCA nodes of the same age.

The results from our analyses have provided a valuable phylogenetic hypothesis of *Nothonotus* that includes data from a mitochondrial gene, a nuclear gene, and external morphology (Fig. 5). We predict that phyloge-

gies of other darter clades using limited external morphological characters will provide much less resolution than mtDNA sequence data. Despite lower resolution these morphological phylogenies can provide independent verification for key nodes in the phylogeny. Nuclear gene data has only recently been applied to darter phylogenetics (Lang and Mayden, 2007; Morrison et al., 2006; Page et al., 2003), but our study is the first to find extensive lack of reciprocal monophyly among species (Fig. 3). The utilization of DNA sequences from multiple nuclear genes is clearly becoming the state of the art in molecular systematics and we envision that such data will drive future investigations of darter phylogenetics. Despite the lack of resolution in the nuclear *S7* gene tree, combination with the *cytb* and morphological datasets resulted in a tree that was more resolved with slightly better node support than the *cytb* phylogeny. This indicates that in addition to identifying instances when the mitochondrial gene tree can be misleading the addition of the nuclear gene dataset to mtDNA datasets can improve the resolution of species level relationships. On the other hand, our study indicates that the prospect of resolving intraspecific relationships or phylogeographic patterns among most *Nothonotus* species using nuclear gene DNA sequences is not very promising. This conclusion is supported by the finding that a very long divergence time between sister species is needed to observe reciprocal monophyly for large numbers of autosomal loci (Hudson and Coyne, 2002). In cases such as *Nothonotus* where introgression does not appear to obscure the mitochondrial gene tree, perhaps the best prospect for examining patterns of intraspecific phylogeography will come from mitochondrial gene data.

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