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ScienceDirect
Molecular Phylogenetics and Evolution 46 (2008) 974–985
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Phylogeography of the greenside darter complex, *Etheostoma blennioides* (Teleostomi: Percidae): A wide-ranging polytypic taxon

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Received 31 May 2007; revised 28 November 2007; accepted 28 November 2007
Available online 11 February 2008

Abstract

The greenside darter, *Etheostoma blennioides* (Teleostomi: Percidae), is a wide-ranging polytypic taxon that occurs throughout eastern North America. A previous morphological study recognized four subspecies (*blennioides*, *newmanii*, *gutselli*, and *pholidotum*), several morphological races, and three zones of morphological intergradation. We generated complete cytochrome *b* (1140 bp) sequence data for 51 individuals from across the range of the greenside darter inclusive of all of the currently recognized taxa to assess genetic variation and taxonomic boundaries. Both maximum parsimony and mixed model Bayesian analyses resulted in two strongly supported deeply divergent clades including (1) a Tennessee River drainage clade, and (2) an Ohio River and Great Lakes basins, Interior Highlands, and Atlantic slope clade. *Etheostoma blennius*, a closely related congener, nested within the Tennessee River clade of *E. blennioides*, rendering the complex paraphyletic. Test of alternative topologies failed to support the current taxonomic designations. The inclusion of nuclear sequence data from intron 1 of the S7 ribosomal protein (523 bp) from a subset of the populations was included to independently test whether the currently recognized taxa conform to distinct evolutionary lineages and also to clarify potential issues associated with ancestral hybridization. Although the nuclear data was less variable than the mitochondrial data, the monophyly of several of the subspecies could not be rejected.

Keywords: Greenside darter; *Etheostoma blennioides*; Cytochrome *b*; Systematics; Phylogeography

1. Introduction

An accurate understanding of species-level diversity is important for understanding general patterns of diversification. However, the criteria used to recognize populations as being specifically or subspecifically distinctive have not been universally accepted (Wilson and Brown, 1953; McKitrick and Zink, 1988; Frost et al., 1992; Mayden, 1997; Mayden, 1999; Wheeler and Meier, 2000). Recognition of taxa below the species level has been controversial and has lead to confusion with respect to the species boundaries within particular clades of organisms (Frost et al., 1992; Burbrink et al., 2000). By definition, the subspecies category is used for populations that display slight morphological differentiation in allopatry and evidence of intergradation (morphological intermediacy) in zones of contact with other conspecific populations (Wilson and Brown, 1953). However, evidence of intergradation is seldom rigorously tested in practice. Thus, for many groups, the subspecies rank is a category of taxonomic convenience rather than a meaningful biological entity (Wilson and Brown, 1953; Zink et al., 2000; Zink, 2004). In many instances, the recognition of subspecies masks the true evolutionary history of a group (Ball and Avise, 1992; Burbrink et al., 2000; Zink, 2004).

Mitochondrial DNA sequence divergences have been widely used to test and delineate taxonomic boundaries within morphologically variable taxa (Avise, 2000;

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1055-7903/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2007.11.023

Burbrink et al., 2000). Mitochondrial DNA divergence is routinely used to identify distinct lineages of organisms (Funk and Omland, 2003). However, authors have noted the problems associated with this type of distance-based approach to erecting taxa, due to problems of introgression, and maternal inheritance, common to mitochondrial DNA sequence data (Ballard and Whitlock, 2004; Sites and Marshall, 2004; Knowles and Carstens, 2007). A number of studies have noted discrepancies between morphologically defined subspecies and phylogenetic clades derived from mitochondrial DNA sequence data. The problems have lead many to question the validity of subspecies as units of evolution (Ball and Avise, 1992; Burbrink et al., 2000; Zink, 2004).

The darter genus *Etheostoma* (Teleostomi: Percidae) is one of the most speciose groups of North American freshwater fishes with over 150 species (Mayden et al., 1992; Page, 2000). The high level of diversity in the group has been attributed to the existence of: (1) strong sexual selection: nuptial males of most species exhibit exaggerated secondary sexual development including bright breeding coloration and/or accessory structures such as egg mimics (Page, 1983; Page and Bart, 1989; Porter et al., 2002; Mendelson, 2003); (2) low dispersal due to their benthic life style and poor swimming ability (Page, 1983); and (3) the fragmentation effects of Pleistocene glaciation (Page, 1983; Mayden, 1987; Mayden, 1988; Strange and Burr, 1997). Traditionally, darter species boundaries have been defined on the basis of breeding male pigmentation patterns and meristic differences (Page, 1983).

The greenside darter, *Etheostoma blennioides*, is one of several polytypic species in the genus *Etheostoma* (Miller, 1968; Mayden et al., 1992). It is a locally common species found in mid-sized streams, rivers, and occasionally lakes throughout the upland portions of the Mississippi River Basin, the Potomac River drainage on the middle Atlantic slope, and tributaries of the Great Lakes (Schwartz, 1965; Miller, 1968; Page and Burr, 1991). The most recent review of morphological diversity within the complex (Miller, 1968) recognized four subspecies: *E. b. newmanii* (Agassiz, 1854), *E. b. gutselli* (Hildebrand, 1932), *E. b. blennioides* (Rafinesque, 1819), and *E. b. pholidotum* (Miller, 1968),

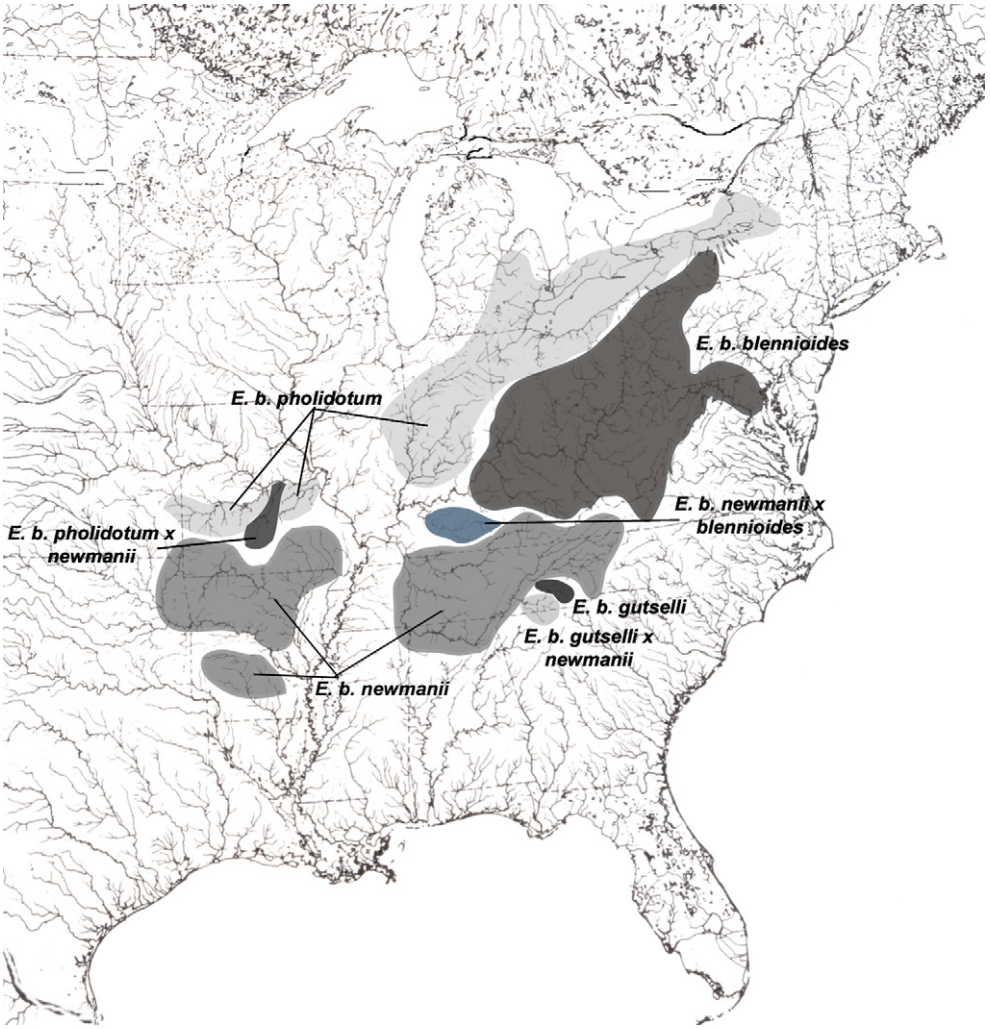

Fig. 1. Distribution of the currently recognized subspecies of *Etheostoma blennioides* following the taxonomic designations of Miller (1968).

several morphological races, and three zones of morphological intergradation (Gasconade, Green-Barren, and Hiwassee River systems) (Fig. 1).

The existence of multiple morphologically distinctive forms and the proposed occurrence of zones of morphological intergradation within the *E. blennioides* complex provide an excellent opportunity to test the correlation of morphological subspecies with phylogenetic lineages inferred from DNA sequence data. The objectives of this study were (1) to determine phylogeographic relationships among specimens of *E. blenniodes* representing all of the currently recognized subspecies using complete cytochrome *b* (mtDNA) sequences, and (2) to test the efficacy of *E. blenniodies* subspecies and their current taxonomic boundaries (Miller, 1968) using two unlinked markers, cytochrome *b* (mtDNA) and S7 ribosomal intron-1 (nuclear DNA). The inclusion of nuclear gene sequences with mitochondrial sequences should provide a more accurate reconstruction of the phylogenetic history of the *E. blennioides* complex than that based on mitochondrial DNA alone (Ballard and Whitlock, 2004).

2. Methods

2.1. DNA extraction, amplification, and sequencing

Specimens of *E. blennioides* were collected using a backpack electrofisher and/or seines from streams inhabited by all of the currently recognized subspecies, morphological races, and from putative zones of morphological intergradation. Collected specimens were euthanized and either flash frozen or preserved in 95% ethanol. Fifty-one individuals representing 44 populations of *E. blennioides* were sampled from across the range (Table 1). Institutional abbreviations follow Leviton et al. (1985). Trees were rooted with *E. variatum*, *E. euzonum*, and *E. tetrazonum*. Several additional species of *Etheostoma* were used as functional ingroups to assess the monophyly of the *E. blennioides* group. We follow the hierarchical treatment of Jenkins et al. (1972) to designate bodies of water as basins, drainages, and systems.

Total genomic DNA was extracted from frozen or ethanol preserved tissues using the DNeasy Tissue Kit (Qiagen). The entire cytochrome *b* gene (1140 bp) and flanking tRNA regions (glutamine and threonine) were isolated by the polymerase chain reaction using the primers described in Schmidt and Gold (1993). Reactions were cycled according to the following temperature profile: initial denaturation of 94 °C for 2 min, followed by 25 cycles of 94 °C for 1 min, 47 °C for 1 min, and 72 °C for 75 s, and a final extension of 72 °C for 4 min. The first intron of the S7 ribosomal protein (523 bp) (Chow and Hazama, 1998) also was amplified for a subset of the ingroup specimens ($N = 24$) to investigate the relationships among the taxa using a nuclear marker and also to assess potential ancestral hybridization and/or ancestral polymorphism issues. The S7 intron was amplified using the primers identified in Chow and Hazama (1998) using a stepped thermal cycling profile with an initial denaturation step of 94 °C for 2 min, followed by 29 cycles of 94 °C for 1 min, and a stepped annealing protocol that consisted of 62 °C for 30 s (×2 cycles), followed by 61 °C for 30 s (×2 cycles), 59 °C for 30 s (×4 cycles), and 58 °C for 30 s (×21 cycles), a 72 °C for 1 min extension step for each cycle, and a single final extension of 72 °C for 4 min.

PCR products were electrophoresed on a 0.8% agarose gel and compared to a standard to assess the presence, size, and intensity of the amplified fragments. PCR products were purified using either QIAquick PCR purification kits (Qiagen) or ExoSAP (USB Corp.) and used in sequencing reactions (Applied Biosystems) according to the manufacturer's recommendations. Excess dye terminators, primers, and nucleotides were removed by gel filtration (Edge Biosystems) prior to sequencing. Cytochrome *b* and S7 sequences were determined on an ABI 373 or 3100 DNA sequencer. Chromatographs were initially checked for ambiguities by eye and then both strands were sequenced and aligned using Gene Works 2.5 (Oxford Biomolecular) or Sequencher (v.4.2) (GeneCodes).

2.2. Data analyses

Phylogenetic hypotheses were generated using maximum parsimony (MP) and Bayesian inference (BI). Maximum parsimony analyses were performed using PAUP* 4.0b10* (Swofford, 2003) using equal weights and a heuristic search with tree-bisection reconnections (TBR) branch swapping with 10 random taxon addition replicates. Nodal support was assessed using a non-parametric bootstrap methodology (Felsenstein, 1985) with 10 random taxon addition replications per pseudoreplication.

A partitioned mixed-model Bayesian analysis (BI) was also conducted to assess relationships among the populations. ModelTest 3.06 (Posada and Crandall, 1998) was used to compare 56 nested models of DNA substitution in a hierarchical testing framework to infer the best model of DNA sequence evolution using Akaike Information Criterion (AIC) (Posada and Buckley, 2004). Each of the three codon positions of cytochrome *b* and the entire S7 intron was treated as a separate data partition and models of evolution separately were chosen for each partition using Modeltest.

Each data partition was assigned a distinct model of evolution using APPLYTO and UNLINK options in Mr.Bayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Posterior probabilities were estimated using the Metropolis-coupled Markov chain Monte Carlo (Huelsenbeck et al., 2001). Bayesian analyses were run for 5×10^6 generations using 4 chains, and trees were sampled every 100 generations resulting in 50,000 saved trees per analysis. Burn-in was determined by examining a plot of maximum likelihood scores against generations to determine the point at which likelihood values stabilized. Visual inspection suggested that stationarity was reached near 200,000

Table 1
Taxon, locality, drainage basin, museum voucher numbers, and Genbank Accession numbers for specimens of *Etheostoma blennioides* analyzed in this study

	Taxon[a]	Locality	Drainage/system	Museum voucher	GeneBank (cyt *b*)	GeneBank (S7)
1	*E. b. newmanii*	French Broad River, Transylvania Co., NC	Tennessee River	TU 194282	EU296645	
2	*E. b. newmanii*	Nolichucky River, Green Co., TN	Tennessee River	No voucher	EU296678	
3	*E. b. newmanii*	Little River, Blount Co., TN	Tennessee River	TU 190439	EU296651	
4	*E. b. newmanii*	Citico Creek, Monroe Co, TN	Little Tennessee River	TU 192693	EU296663	EU296721
5	*E. b. newmanii*	Pigeon River, Cocke Co., TN	Pigeon River	TU 191501	EU296658	
6	*E. b. newmanii*	West Fork Sugar Creek, Lawrence Co., TN	Tennessee River	TU 187563	EU296649	
7	*E. b. newmanii*	Duck River, Bedford Co., TN	Duck River	TU 191470	EU296652	EU296722
8	*E. b. gutselli*	Cowee Creek, Macon Co., NC	Little Tennessee River	—	EU296650	EU296713
9	*E. b. gutselli*	Tuckasegee River, Swain Co., NC	Little Tennessee River	TU 188865	EU296642	EU296712
10	*E. b. gutselli*	Tuckasegee River, Swain Co., NC	Little Tennessee River	TU 188865	EU296643	EU296711
11	*E. b. gutselli*	Deep Creek, Swain Co., NC	Little Tennessee River	—	EU296669	EU296701
12	*E. b. gutselli*	Pigeon River, Haywood Co., NC	Pigeon River	TU 188875	EU296640	EU296700
13	*E. b. gutselli*	Tuckasegee River, Swain Co., NC	Little Tennessee River	—	EU296644	EU296710
14	*E. b. newmanii x E. b. gutselli*	Hiwasse River, Polk Co., TN	Hiwassee River	TU 191899	EU296637	
15	*E. b. gutselli*	Jonathon Creek, Haywood Co., NC	Pigeon River	—	EU296641	EU296708
16	*E. b. gutselli*	Jonathon Creek, Haywood Co., NC	Pigeon River	—	EU296639	EU296699
17	*E. b. gutselli*	Little Tennessee River, Macon Co., NC	Little Tennessee River	—	EU296698	
18	*E. b. gutselli*	Little Tennessee River, Macon Co., NC	Little Tennessee River	—	EU296681	EU296705
19	*E. b. newmanii* × *E. b. gutselli*	Brasstown Creek, Towns Co., GA	Hiwassee River	—	EU296695	
20	*E. b. newmanii* × *E. b. gutselli*	Brasstown Creek, Towns Co., GA	Hiwassee River	—	EU296684	EU296704
21	*E. b. newmanii* × *E. b. gutselli*	Toccoa River, Fannin Co., GA	Hiwassee River	—	EU296679	EU296702
22	*E. b. newmanii* × *E. b. gutselli*	Toccoa River, Fannin Co., GA	Hiwassee River	—	EU296680	EU296703
23	*E. b. newmanii* × *E. b. gutselli*	Coopers Creek, Fannin Co., GA	Hiwassee River	—	EU296636	EU296709
24	*E. b. newmanii* × *E. b. gutselli*	Valley River, Cherokee Co., NC	Hiwassee River	—	EU296696	
25	*E. b. newmanii* × *E. b. gutselli*	Valley River, Cherokee Co., NC	Hiwassee River	—	EU296697	EU296706
26	*E. b. newmanii* × *E. b. gutselli*	Big Lost Creek, Polk Co., TN	Hiwassee River	TU 191899	EU296638	
27	*E. b. blennioides*	Monocacy River, Fredrick Co., MD	Potomac River	—	EU296656	
28	*E. b. blennioides*	Seeley Creek, Chemung Co., NY	Susquehanna River	—	EU296659	
29	*E. b. pholidotum*	Ganarga Creek, Ontario Co., NY	Great Lakes	—	EU296661	EU296720
30	*E. b. blennioides*	Loramie Creek, Miami CO., OH	Ohio River	TU 192694	EU296667	
31	*E. b. blennioides*	Stillwater River, Darke Co., OH	Ohio River	TU 192696	EU296664	
32	*E. b. blennioides*	Stillwater River, Darke Co., OH	Ohio River	TU 192696	EU296665	
33	*E. b. newmanii*	West Fork Stones River, Rutherford Co., TN	Cumberland River	TU 187552	EU296646	EU296714
34	*E. b. pholidotum*	Big Blue River, Johnson Co., IN	Wabash River	TU 191382	EU296647	
35	*E. b. pholidotum*	Middle Fork Vermilion River, Vermilion Co., IL	Wabash River	TU 188918	EU296648	EU296707
36	*E. b. pholidotum*	Laurel Creek, Ontario, Canada	Great Lakes	—	EU296670	
37	*E. b. pholidotum*	Carroll Creek, Ontario, Canada	Great Lakes	—	EU296671	EU296719
38	*E. b. pholidotum*	Auglaize River, Auglaize Co., OH	Great lakes	TU 192691	EU296660	
39	*E. b. newmanii* × *E. b. blennioides*	Trammel Fork, Allen Co., KY	Green-Barren River	TU 190384	EU296662	
40	*E. b. newmanii* × *E. b. blennioides*	East Fork Little Barren River, Metcalfe Co., KY	Green-Barren River	TU 192692	EU296672	
41	*E. b. newmanii* × *E. b. blennioides*	South Fork Barren River, Metcalfe Co., KY	Green-Barren River	TU 191397	EU296673	
42	*E. b. pholidotum*	Niangua River, Laclede Co., MO	Osage River	TU 191135	EU296653	
43	*E. b. pholidotum*	Tavern Creek, Miller Co., MO	Osage River	TU 188905	EU296654	EU296718
44	*E. b. pholidotum*	Little Sac River, Greene Co., MO	Osage River	TU 191337	EU296668	
45	*E. b. pholidotum*	Cole Camp Creek, Benton Co., MO	Osage River	—	EU296676	EU296717

(*continued on next page*)

Table 1 (*continued*)

	Taxon[a]	Locality	Drainage/system	Museum voucher	GeneBank (cyt *b*)	GeneBank (S7)
46	*E. b. newmanii* × *E. b. pholidotum*	Gasconade River, Pulaski Co., MO	Gasconade River	TU 191329	EU296655	
47	*E. b. newmanii*	Middle Fork Little Red River, Searcy Co., AR	White River	TU 192690	EU296657	
48	*E. b. newmanii*	Kings River, Carrol Co., AR	White River	TU 191360	EU296674	EU296715
49	*E. b. newmanii*	South Fork Spring River, Fulton Co., AR	White River	TU 188813	EU296675	
50	*E. b. newmanii*	Cadron Creek, Van Buren Co., AR	Arkansas River	TU 188834	EU296677	
51	*E. b. newmanii*	Saline River, Saline Co., AR	Ouachita River	TU 188843	EU296666	EU296716
52	*E. blennius*	Duck River, Bedford Co., TN	Duck River	—	EU296682	EU296723
53	*E. blennius*	Duck River, Bedford Co., TN	Duck River	—	EU296683	
54	*E. blennius*	Horse Creek, Hardin Co., TN	Duck River	UT 91.6471	EU296690	EU296724
55	*E. swannanoa*	Little Pigeon River, Sevier Co., TN	Pigeon River	UT 91.6578	EU296685	
56	*E. zonale*	Middle Fork Little Red R., Van Buren Co., AR	White River	UT 91.6631	EU296686	
57	*E. rupestre*	Conasauga R., Bradley Co., TN	Conasauga River	UT 91.7506,	EU296687	
58	*E. lynceum*	Clarks Creek, Chester Co., TN	Hatchie River	UT 91.6680	EU296688	
59	*E. rafinesquei*	Little Barren R., Metcalfe Co., KY	Green-Barren River	UT 91.7337	EU296689	
60	*E. euzonum*	Middle Fork Little Red R., Van Buren Co., AR	White River	UT 91.6629	EU296691	EU296725
61	*E. thalassinum*	Middle Little R., Caldwell Co., NC	Catawba River	INHS 64072	EU296692	
62	*E. variatum*	French Cr. and small trib, Crawford Co., PN	Ohio River	INHS 39195	EU296693	EU296727
63	*E. tetrazonum*	Maries R., Osage Co., MO	Osage River	—	EU296694	EU296726

[a] Taxonomic designations according to Miller (1968).

generations. However, the first 1,000,000 generations were discarded to assure stationarity. The remaining (non-discarded) trees were used to calculate posterior probabilities on a 50% majority rule consensus tree. Results from four separate analyses were compared to provide additional confirmation of convergence among likelihood values, tree topologies, and posterior distributions. Branch support was tested using Bayesian posterior probabilities (*BPP*) (Holder and Lewis, 2003).

Particular aspects of the evolutionary history of the *E. blennioides* complex were examined by quantitatively assessing alternative phylogenetic hypotheses, using cytochrome *b* and S7 intron separately using three tests. First, using maximum parsimony criteria, tests were conducted using the *a priori* hypotheses of subspecies groups according to Miller (1968). These hypotheses included (1) monophyly of *E. b. newmanii*, (2) monophyly of *E. b. pholidotum*, (3) monophyly of *E. b. gutselli*, (4) monophyly of *E. b. blennioides*, and (5). monophyly of *E. blennioides* (*sensu stricto*). Trees were constrained according to the above criteria and length differences relative to the unconstrained maximum parsimony tree were compared. Second, specific hypotheses were statistically assessed using the Shimodaira–Hasegawa test (SH test) (Shimodaira and Hasegawa, 1999) as implemented in PAUP. Results from the parsimony analyses, including constrained and unconstrained trees, were compared to the optimal tree derived from the Bayesian analysis to determine whether any of the hypotheses could be statistically rejected. The SH test was performed using 1000 bootstrap replicates. Based on overall likelihood values, trees that were significantly different from the best tree were rejected ($P < 0.05$). Finally, an analysis using Bayesian posterior probabilities was used to statistically test alternative phylogenetic hypotheses. Post-stationary trees from the BI were loaded into PAUP* and filtered according to the constraints listed above. The resultant trees from the constraint filtering were divided by the total number of post-stationary trees to arrive at a probability of monophyly for a particular test. If 5% or less of the post-stationary trees recovered a particular relationship, the hypothesis was statistically rejected under Bayesian criteria (Weisrock et al., 2006).

3. Results

3.1. Nucleotide variability and sequence divergence

Sequence data were obtained from 51 individuals of *E. blennioides* (Table 1) for cytochrome *b* (1140 bp) (Genbank Accession Nos. EU296636–EU296698). Average base frequencies for cytochrome *b* were as follows; ($A = 0.226$, $C = 0.303$, $G = 0.169$, $T = 0.302$, $x^2 = 19.24$, $P = 1.00$). Thirty-seven haplotypes were recovered from 51 individuals included in the analysis, and no haplotypes were shared between drainage basins. The average uncorrected pairwise sequence divergence excluding identical haplotypes among the ingroup was 4.95% (0.09% to 8.42%, Table 2).

Table 2
Uncorrected cytochrome *b* sequence divergence for select populations of *Etheostoma blennioides* (*sensu stricto*), *Etheostoma blennius*, and outgroups

	Population	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Toccoa River Dr.	—												
2	Hiwassee River Dr.	0.026	—											
3	Upper Pigeon River Dr.	0.028	0.033	—										
4	Upper Little Tennessee River Dr.	0.020	0.027	0.021	—									
5	Tennessee River Dr.	0.017	0.026	0.025	0.010	—								
6	Cumberland River Dr.	0.066	0.069	0.073	0.072	0.073	—							
7	Great Lakes–Wabash–Green–Barren Dr.	0.063	0.067	0.073	0.073	0.073	0.025	—						
8	Osage–Gasconade Dr.	0.073	0.079	0.083	0.081	0.082	0.030	0.026	—					
9	Upper Ohio River Dr.	0.065	0.071	0.073	0.073	0.075	0.033	0.037	0.043	—				
10	Arkanasas-White River Dr.	0.076	0.080	0.084	0.082	0.082	0.036	0.033	0.040	0.040	—			
11	Ouachita River Dr.	0.069	0.073	0.078	0.077	0.078	0.029	0.030	0.033	0.039	0.035	—		
12	*Etheostoma blennius*	0.041	0.044	0.041	0.045	0.043	0.075	0.075	0.082	0.076	0.085	0.081	—	
13	Outgroups	0.129	0.130	0.132	0.132	0.131	0.125	0.125	0.129	0.130	0.131	0.127	0.134	—

Variability in S7 sequences was significantly lower than that observed in cytochrome *b* sequences for a subset of the populations of *E. blennioides* (Genbank Accession Nos. EU296699–EU296727). Average base frequencies for S7 were as follows; ($A = 0.257$, $C = 0.183$, $G = 0.230$, $T = 0.330$, $x^2 = 3.48$, $P = 1.00$). The average uncorrected pairwise sequence divergence excluding identical haplotypes among the ingroup was 0.60% (0.20% to 1.6%).

3.2. Phylogenetic analysis

Both the MP (not shown) and BI (Fig. 2) of the mitochondrial data resulted in two deeply divergent clades of greenside darters: (1) a Tennessee River drainage clade, and (2) an Ohio River basin, Great Lakes, Interior Highland, Atlantic slope clade. Both analytical methods failed to recover the *E. blennioides* complex as a monophyletic group. Tennessee River basin populations of greenside darters were monophyletic and sister to *E. blennius*, a Tennessee River drainage endemic that co-occurs with *E. blennioides* in the Duck and Sequatchie Rivers in east central Tennessee. Uncorrected cytochrome *b* divergence between Tennessee River populations of *E. blennioides* and *E. blennius* averaged 4.25% (4.12–4.30%). Generally, deeper relationships were in agreement in both the MP and BI trees. However, overall, the BI tree was better resolved, particularly at the terminal nodes. Thus, the remainder of the results and discussion will focus on the BI analysis.

The Tennessee River drainage clade was strongly supported ($BPP = 100$) and includes greenside darters that are currently recognized as *E. b. newmanii*, *E. b. gutselli*, and *E. b. newmanii* × *E. b. gutselli*. Seven individuals of *E. b. gutselli* from the Upper Little Tennessee River system nested within a clade consisting of *E. b. newmanii* specimens from throughout the Tennessee River drainage. There is a relatively low degree of cytochrome *b* sequence divergence ($x = 0.97\%$, range = 0.0–1.1%) among the widespread *E. b. newmanii* populations in the Tennessee River drainage and the *E. b. gutselli* population from the Upper Little Tennessee River system.

Divergence in mitochondrial DNA among *E. b. newmanii* populations within the entire Tennessee River basin, including populations from the Duck, Little, French Broad, Lower Pigeon, and Lower Little Tennessee River systems is also relatively low, ranging from 0.30% to 1.1% (mean = 0.60%). In contrast, the *E. b. newmanii*/*E. b. gutselli* (Upper Little Tennessee) clade and *E. b. gutselli* (Upper Pigeon River) on average are 2.02% divergent from one another, a pattern more supportive of the morphological divergence observed between *E. b. newmanii and E. b. gutselli*.

The remaining portion of the Tennessee River clade contains three strongly supported clades: (1) *E. b. gutselli* from the Upper Pigeon River system, (2) Hiwassee River system, termed Upper Hiwassee by Miller (1968), and (3) Toccoa River subsystem within the Hiwassee River system, which was termed lower Hiwassee River by Miller (1968). Miller (1968) identified the upper and lower Hiwassee River populations as intergrades between *E. b. newmanii* and *E. b. gutselli*, however, both populations possess unique haplotypes and are 2.6% divergent from one another. The average sequence divergence between the Toccoa River population and the Tennessee River–Upper Little Tennessee River system clade, inclusive of *E. b. newmanii* and *E. b. gutselli* is 1.7%.

The second major clade of greenside darters ($BPP = 100$) consists of populations from the Great Lakes, Cumberland, Wabash, Green-Barren, Osage, Gasconade, upper and lower Ohio, Arkansas, White, and Ouachita River drainages. The average level of genetic divergence within this clade is 2.9% (range 0.00% to 4.70%). The average divergence between this clade and the Tennessee River drainage clade is considerably higher (7.50%).

Populations of *E. b. blennioides*, *E. b. pholidotum*, and *E. newmanii* × *E. b. pholidotum* from the Great Lakes–Wabash–Lower Ohio River basins form a monophyletic group sister to western populations of *E. b. pholidotum* and *E. b. newmanii* × *E. b. pholidotum* from the Gasconade and Osage River systems in the Northern Ozarks with high support ($BPP = 94$). Both the Great Lakes–Wabash–

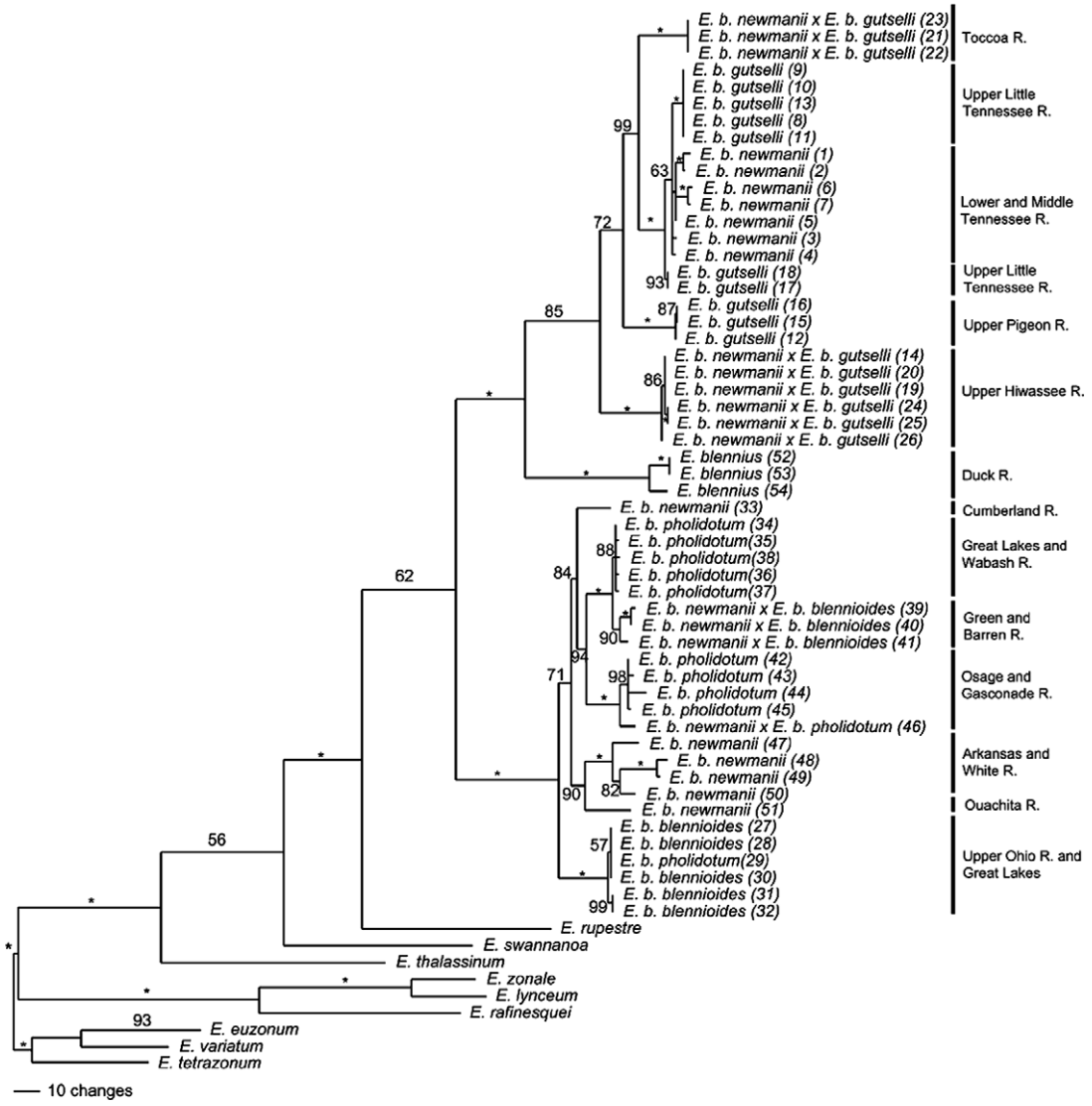

Fig. 2. Fifty-percent majority rule consensus tree from a partitioned mixed model Bayesian analysis of complete cytochrome *b* sequences of the *Etheostoma blennioides* complex. Taxonomic designations follow Miller (1968). Values above branches refer to posterior probabilities, and an "*" represents a value of 100. Numbers in parentheses following the taxonomic names refer to localities listed in Table 1.

Lower Ohio clade, and the Gasconade–Osage clades also were strongly supported ($BPP = 100$). Levels of divergence within each of these clades are minimal (average intracladal divergences 0.63% and 0.67% for the Great Lakes–Wabash–Lower Ohio River and Gasconade–Osage clades, respectively). A single individual, *E. b. newmanii*, from the Cumberland River drainage is sister to this entire clade. Western populations of putative *E. b. newmanii* are more than 7.0% divergent from *E. b. newmanii* from the Tennessee River drainage, but only 2.69% divergent, on average, from Great Lakes–Wabash–Green–Barren–Osage–Gasconade clade.

A strongly supported, monophyletic Southern Ozark–Ouachita clade consisting of populations in the White, Arkansas, and Saline River drainages was also recovered ($BPP = 90$). The single population of *E. blennioides* from the Ouachita Highlands is included in this clade and is 3.51% (range 3.33–3.68%) divergent on average from Southern Ozarkian populations of *E. b. newmanii*.

Finally, a monophyletic clade with high posterior probability support ($BPP = 100$) comprising multiple individuals from the Upper Ohio River basin plus a single individual from the Great Lakes basin (Ganarga Creek, Ontario Co., NY) forms the sister lineage to all populations in the second major clade, suggesting differentiation between upper and lower portion of the Ohio River basin.

The S7 nuclear data supports many of the results recovered in the mitochondrial analysis of the mitochondrial sequences, but there are also several differences (Fig. 3). The nuclear data recovered a monophyletic *E. blennioides* complex ($BPP = 100$), as *E. blennius* is basal to the entire clade, rather than rooted within the Tennessee River drainage clade of *E. blennioides* as it is in the mitochondrial tree. *E. b. newmanii* was paraphyletic, as was recovered in the

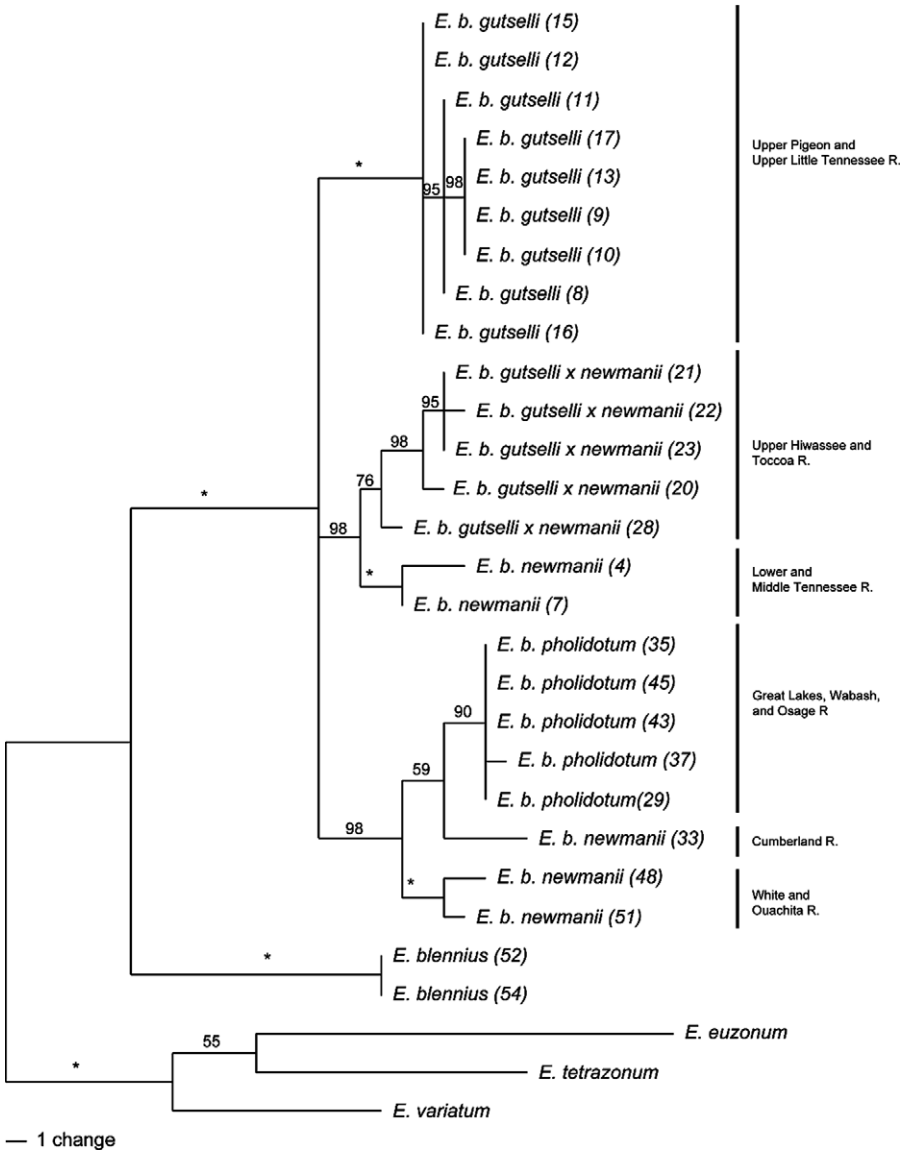

Fig. 3. Consensus phylogeny from a Bayesian analysis based on sequences of intron 1 of the S7 ribosomal protein from a subset of the taxa in the *E. blennioides* complex. Numbers above the branches represent posterior probabilities and an "*" represents a value of 100. Taxonomic designations follow Miller (1968) and numbers in parentheses following the taxonomic names refer to localities listed in Table 1.

mtDNA tree. In addition, *E. b. gutselli* from both the Pigeon and Upper Little Tennessee River systems and populations from the Hiwassee River system were each recovered as monophyletic with the nuclear data, whereas neither set of populations were monophyletic in the cytochrome *b* phylogeny.

3.3. Hypothesis testing

For the mitochondrial data set, none of the constraint tree searches or statistical analyses supported the monophyly of the putative subspecies of Miller (1968) or *E. blennioides* (*sensu stricto*). All maximum parsimony constraint tree searches resulted in trees that were longer (1097 to 1167 steps) than the most parsimonious tree (1031 steps, Table 3). Constraint searches of post-burn in trees from the BI also did not support the monophyly of any of the putative subspecies. In each of the analyses, none of the 40,000 post-burn in trees that were recovered contained monophyletic clades of *E. b. newmanii*, *E. b. pholidotum*, *E. b. gutselli*, or *E. b. blennioides*. The maximum parsimony tree was recovered in 1890 of the 40,000 post-burn in trees, which equates to 4.75%. Furthermore, a monophyletic *E. blennioides* complex was not recovered in any of the post-burn in trees. Finally, 5 of the 6 alternative phylogenetic hypotheses were significantly different from the BI tree based with the SH tests ($P < 0.05$). The SH test failed to support a significant difference between the BI and MP trees ($P = 0.657$).

Table 3
Alternative topologies tested using constraint parsimony, post-burn searches (40,000 trees), and SH tests within the *Etheostoma blennioides* complex using mitochondrial (cyt *b*) and nuclear (S7 intron-1) sequences. Statistical significance is noted with an*

Hypothesis	Cytochrome *b*					S7 Intron-1				
	Maximum parsimony tree length	Post-burn in tree search	ln *L*	Differences in ln *L*	P	Maximum parsimony tree length	Post-burn in tree search	ln *L*	Differences in ln *L*	*P*
Bayesian	—	—	7488.04	Best	—	—	—		Best	—
Maximum parsimony consensus	1031	1890	7514.34	26.30	0.657	121	14,925	1500.70	30.20	0.614
Monophyletic *E. b. newmanii*	1165	0	16885.04	9397.00	0.001*	125	0	2347.28	876.76	0.001*
Monophyletic *E. b. blennioides*	1128	0	15794.66	8306.61	0.001*	—	—	—	—	—
Monophyletic *E. b. gutselli*	1167	0	15125.32	7637.28	0.001*	121	40,000	2088.55	618.02	0.001*
Monophyletic *E. b. pholidotum*	1163	0	14816.56	7328.52	0.001*	121	4717	2207.07	736.54	0.001*
Monophyletic *E. blennioides (sensu stricto)*	1097	0	17160.69	9672.64	0.001*	121	40,000	2238.96	768.44	0.001*

Constraint tree searches of the S7 data resulted in trees that ranged from 121 to 125 steps in length (Table 3). However, only the *E. b. newmanii* constraint resulted in a tree that was significantly longer than the maximum parsimony tree. Examination of the 40,000 post-burn in trees resulted in a significant proportion of the alternative hypotheses being recovered. *E. b. gutselli*, *E. b. pholidotum*, and *E. blennioides* (*sensu stricto*) were recovered in greater than 5% of the post-burn in trees, and could therefore, not be rejected. A monophyletic *E. b. gutselli* and *E. blennioides* were recovered in all of the post-burn in trees. When compared to the Bayesian tree, only one of the four alternative hypotheses, maximum parsimony tree, could not be rejected using the S–H test.

4. Discussion

The shortcomings of recognizing subspecies as biological entities have long been argued on both theoretical and practical grounds (Frost et al., 1992; Burbrink et al., 2000; Zink, 2004). Much of the argument centers on the subjectivity involved in delineating subspecies. Subspecies have traditionally been recognized where otherwise distinct entities come together and produce morphologically intermediate populations (intergrades). Miller (1968) identified three morphologically intermediate populations within the *E. blennioides* complex based on meristics, designated them as intergrades, and stated that several of his subspecies would warrant specific recognition were it not for the these morphologically intermediate populations.

During the period in which Miller conducted his study, the biological species concept (BSC, Mayr, 1957) was the dominant species concept, which posited that any perceived evidence of hybridization or intergradation between otherwise differentiated populations should be interpreted as evidence of subspecific differentiation (*sensu* Mayr et al., 1953). Taxonomic revisions and geographic variation studies of this era took what is by today's standards a non-rigorous, unscientific approach to interpreting evidence of intergradation. As a result, countless subspecies of many kinds of organisms, including many subspecies of Etheostomatine darters, were formally recognized (Hubbs and Black, 1941; Knapp, 1964; Distler, 1968; Howell, 1968; Tsai and Raney, 1974) and most of these subspecies are still recognized today.

We hypothesized that the evolutionary history we recovered from analysis of DNA sequence data would be generally concordant with Miller's (1968) taxonomic groups. However, this was not the case.

Cytochrome *b* sequence data are more variable than S7 intron data, and support multiple, unique greenside darter lineages. However, most of the lineages do not correspond to taxa recognized by Miller (1968) and lineages constrained to correspond to Miller's (1968) taxa are significantly different than those supported by cyt *b* data, based on topology tests and post-burn in tree searches.

Some aspects of the cytochrome *b* tree confound the current understanding of darter taxonomy. *E. blennius*, a morphologically divergent Tennessee River drainage endemic species, is nested within the Tennessee River drainage clade of *E. blennioides* based on cytochrome *b* data. Such an anomalous relationship has not been obtained in any previous study involving *E. blennius* and *E. blennioides*. Previous morphological studies concluded that *E. blennius* is closely related either to members of the *E. variatum* species group (Hubbs and Black, 1940; Richards, 1966; Wiley and Mayden, 1985) or to *E. swannanoa* (Burr, 1979; Bailey and Etnier, 1988). Porter et al. (2002) recovered a monophyletic greenside darter clade sister to a group consisting of *E. blennius blennius* and *E. blennius sequatchiense* based on mitochondrial control region sequences. However, Porter et al. (2002) did not include any greenside darter populations from the Tennessee River drainage.

Etheostoma blennius lies outside the *E. blenniodes* clade in the tree based on S7 intron 1 data, suggesting that the two species do indeed have separate histories based on this independent nuclear marker. The unusual position of *E. blennius* within the *E. blennioides* species complex in the cytochrome *b* tree suggests that the similarity in cytochrome *b* gene sequences in Tennessee River basin populations of *E. blennioides* and *E. blennius* reflects an ancestral introgressive hybridization event. A less likely explanation is that the position of sympatric *E. blennius* populations within the *E. blennioides* clade reflects incomplete lineage sorting of an ancestral polymorphism. The interspecific branches between *E. blennius* and Tennessee River populations of *E. blennioides* are long, relative to the intraspecific branches in the Tennessee River clade, which suggests that there has been a substantial amount of time for coalescence and reciprocal monophyly for the mitochondrial DNA (Moore, 1995). This cursory examination of branch lengths suggests that ancestral polymorphism is unlikely. If the discrepancies between the gene trees are due to ancestral hybridization then it is likely that introgression occurred long ago, given that the average cytochrome *b* divergence between *E. blennius* and Tennessee River clade of *E. blennioides* is more than 4% and the phylogenetic placement of *E. blennius* is at the base of the Tennessee River clade rather than nested within it. The fact that these taxa may have hybridized in the past should not be too surprising, considering they occupy nearly identical riffle habitats in the Tennessee River basin and spawn at roughly the same time of year (Burr, 1979, Etnier and Starnes, 1993).

The low level of mitochondrial sequence divergence between populations of *E. b. newmanii* and *E. b. gutselli* from the Upper Little Tennessee River basin also is suggestive of ancestral hybridization, since the S7 intron tree recovered reciprocally monophyletic *E. b. newmanii* and *E. b. gutselli*. Populations of *E. b. newmanii* and *E. b. gutselli* both occupy the Little Tennessee River system, however, the two taxa do not occur sympatrically at present. *E. b. gutselli* is confined to higher elevation, Blue Ridge reaches of the Little Tennessee River system, whereas *E. b. newmanii* is limited to lower Ridge and Valley reaches. The transition zone between these reaches is an area of extreme topographic relief, which makes contemporary upstream dispersal of *E. b. newmanii* into the Blue Ridge unlikely. Downstream dispersal of *E. b. gutselli* into the range of *E. b. newmanii* is possible, particularly during flood events or other periods of elevated flow. There is some evidence of this in the Pigeon River system. *E. b. gutselli* and *E. b. newmanii* have never been collected synchronously from the same locality in the Pigeon River, however, a single collection (UT 91.4796) of *E. b. gutselli* is known from the middle Pigeon River, downstream of the Blue Ridge, a locality typically occupied by *E. b. newmanii*. No other syntopic collections of these taxa are known.

Overall, S7 intron is much less variable than cytochrome *b* and it provides much less phylogenetic resolution within the *E. blennioides* species complex than cytochrome *b*. The marker is informative and indicates that the monophyly of *E. b. gutselli*, *E. b. pholidotum*, and *E. blennioides* (*sensu stricto*) could not be rejected under constraint parsimony tree searches and examination of post-burn in trees with S7 intron data. Both nuclear and mitochondrial markers failed to support a monophyletic *E. b. newmanii*, a taxon clearly in need of a taxonomic reassessment. The inconsistencies between trees based on mtDNA and nuclear DNA are largely due to ancestral hybridization suggested in the mtDNA tree. Mitochondrial introgression is a more common phenomenon than previously believed and has been documented in numerous other studies (Wilson and Bernatchez, 1998; Ballard, 2000; Ballard and Whitlock, 2004).

Gene trees and levels of sequence divergence have often been used to delimit species (Sites and Marshall, 2004). The levels of intraspecific cytochrome *b* sequence divergence recovered for populations of *E. blennioides* is as high or higher than that observed in other interspecific comparisons of Etheostomatine darters (Kinziger et al., 2001; Switzer and Wood, 2002). In fact, comparison of levels of genetic divergence observed in this study with those observed in previous studies of cytochrome *b* variation (Johns and Avise, 1998), including divergence levels among other described Etheostomatine species deposited in Genbank (September 2006), supports a more diverse taxonomy of the *E. blennioides* complex from that proposed by Miller (1968).

Revisionary studies often utilize subspecies as starting points for the identification of evolutionary lineages (McKitrick and Zink, 1988). However, recognizing subspecies as the endpoints of the taxonomic continuum is problematic on many levels. Subspecies assumes the existence of hybrid zones, intergrade populations with no taxonomic status, and the possibility of introgression and reticulate evolution, both of which confuse interpretation of evolutionary history.

Regardless of whether one recognizes populations at specific or subspecific levels, the taxonomy of any group should reflect its evolutionary history (Wiley, 1981). In this

study, two molecular markers indicate that there is more diversity within the *E. blennioides* species complex than is currently recognized. A thorough taxonomic re-evaluation of diversity within the complex, inclusive of genetic and morphological characters, is presently underway.

Darter taxonomy, like taxonomy in general, is in the midst of a paradigm change. The use of subspecies has fallen out of favor. In fact, no new subspecies of darters have been described since *E. blennius sequatchiensis* in 1979 (Burr, 1979). Previously recognized subspecies have been elevated to species in a number of recent studies (Etnier and Starnes, 1986; McCormick, 1990; Layman, 1994; Ceas and Page, 1997; Piller et al., 2001). The combination of new species concepts (e.g., Evolutionary Species Concept and Phylogenetic Species Concept vs. Biological Species Concept) and modern methodologies (e.g., molecular systematics) has resulted in a flurry of new darter descriptions. In fact, the rate of new darter description over the past twenty years surpasses that of the so-called "Golden Era" of darter description (Collette, 1967; Page, 2000). Thus, it is clear that the result of paradigm shift is higher diversity of darters and other organisms than recognized in the past.

Acknowledgments

We thank J. M. Guill and J. A. Tipton for assistance in the field. Numerous individuals including T. Near, R. Mayden, D. Carlson, S. Reid, W. Poly, and R. Blanton provided tissues/sequences from additional populations not sampled directly by the authors. This work was supported, in part, by a Theodore Roosevelt Memorial Award (AMNH) and an Edward C. Raney Award (ASIH) made to K.R.P. Additional funds were provided by the U. S. Fish and Wildlife Service provided to K.R.P. and H.L.B. All samples were collected under scientific collecting permits in the states of Missouri, Kentucky, Tennessee, North Carolina, Indiana, Illinois, and Georgia.

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