A Thesis

Entitled

Molecular, morphological, and biogeographic resolution of cryptic taxa in the Greenside

Darter *Etheostoma blennioides* complex

By

Amanda E. Haponski

Submitted as partial fulfillment of the requirements for

The Master of Science Degree in Biology (Ecology-track)

Advisor: Dr. Carol A. Stepien

Committee Member: Dr. Timothy G. Fisher

Committee Member: Dr. Johan F. Gottgens

College of Graduate Studies

The University of Toledo

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An Abstract of
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DNA sequencing has led to the resolution of many cryptic taxa, which are especially prevalent in the North American darter fishes (Family Percidae). The Greenside Darter *Etheostoma blennioides* commonly occurs in the lower Great Lakes region, where two putative subspecies, the eastern “Allegheny” type *E. b. blennioides* and the western “Prairie” type *E. b. pholidotum*, overlap. The objective of this study was to test the systematic identity and genetic divergence distinguishing the two subspecies in areas of sympatry and allopatry in comparison to other subspecies and close relatives. DNA sequences from the mtDNA cytochrome *b* gene and control region and the nuclear S7 intron 1 comprising a total of 1,497 bp were compared from 294 individuals across 18 locations, including the Lake Erie basin and the Allegheny, Meramec, Obey, Ohio, Rockcastle, Susquehanna, and Wabash River systems. Results showed pronounced divergences among taxa ($\theta_{ST} = 0.92 - 0.97$; p -distance = 0.025 – 0.039) presently designated as *E. b. blennioides*, *E. b. newmanii*, and *E. b. pholidotum*, as well as identification of a fourth clade in the Meramec River. Most traditional morphological

characters were significantly different ($P= 0.0001$) in distinguishing between *E. b. blennioides* and *E. b. pholidotum*, including scale counts and degree of ventral squamation. However, the range of variation within these characters overlapped, obscuring accurate assignment of individuals to the taxa. The four significantly divergent taxa of the Greenside Darter complex should be evaluated further for potential elevation to species status.

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Chapter One

Introduction

Resolution of correct systematic relationships is essential to evolutionary, biogeographic, and ecological studies, enabling us to identify taxa, evaluate divergence patterns among them, and compare diversity across their range. However, the presence of cryptic taxa has confounded many traditional studies since they may appear morphologically similar yet be phylogenetically distinct. Advances in obtaining and analyzing DNA sequences now facilitate delineation of cryptic species and aid in evaluating their component genetic and ecological diversity. Approaches that combine data sets, such as mitochondrial and nuclear DNA sequences and evaluation of traditional morphological characters, are especially powerful for resolving systematic and biogeographic questions among cryptic species groups.

The darters (Percidae: Etheostomatinae) are one of the most diverse groups of North American freshwater fishes, comprising more than 200 species (Nelson, 2006). They have undergone remarkable taxonomic diversification, adaptive radiation, and differentiation. This group includes some of the most imperiled endemic freshwater taxa with 40 species listed on the 2006 International Union for the Conservation of Nature and Natural Resources' Red List of Threatened Species (IUCN, 2006). Species descriptions for the darters traditionally were based upon morphometrics, meristic characters, and male coloration patterns (Page, 1983) that often broadly overlap among taxa. During the

1800s, nearly 100 species of darters were described based on morphological characters (Collette, 1967) and were then reorganized into three genera (*Percina*, *Ammocrypta*, and *Etheostoma*; Bailey et al., 1954). The genus *Etheostoma* includes many newly identified cryptic taxa among an estimated 140 species today (Nelson, 2006), many of which were distinguished using DNA sequencing (e.g., Burr and Page, 1993; Porter et al., 2002; Page et al., 2003; Mayden et al., 2005).

Rafinesque (1819) originally described the Greenside Darter *Etheostoma blennioides*, whose name means “blenny-like”, referring to its cryptic color patterns, adhesive eggs in algal nests, and body form superficially similar to members of the Family Blenniidae. The Greenside Darter inhabits lake shores and the rocky riffles of creeks and rivers (Page and Burr, 1991), ranging from the lower Missouri and Ouachita River systems north through the Ohio River basin and the Great Lakes (Fig. 1; Boschung and Mayden, 2004). Rafinesque (1819) designated the type locality of the Greenside Darter as the falls of the Ohio River below Louisville, KY (Lee et al., 1980) that were destroyed when the McAlpine Locks and dam were built in 1975 (Army Corps of Engineers www.usace.army.mil/ accessed on 10-10-07).

Etheostoma blennioides is the largest species in its genus (reaching 132 mm standard length) and is distinguished by fusion of its maxilla skin to the snout skin (Boschung and Mayden, 2004), 5 to 8 square dark green saddles, and 6 to 10 V- or W-shaped lateral markings (Kuehne and Barbour, 1983). Greenside Darter breeding males differ from related species by their bright green to blue-green lateral bars (Boschung and Mayden, 2004). The Ohio Division of Natural Resources uses the Greenside Darter as a water quality indicator species since it is more tolerant of siltation and nutrient

enrichment than are other darters. An increase in abundance of the Greenside Darter coinciding with a decrease in other darter species is used to indicate polluted conditions (ODNR,

<http://www.dnr.state.oh.us/dnap/quarantine/copy%20of%20rivfish/greenside.html>, accessed on 10-10-07).

Etheostoma blennioides Rafinesque (1819) was reevaluated and split into subspecies by Miller (1968). These subspecies are morphologically difficult to distinguish, and have not been analyzed comprehensively using both nuclear and mitochondrial DNA sequences in combination with traditional morphological characters.

Subspecies of the Greenside Darter

Four subspecies of the Greenside Darter *Etheostoma blennioides* Rafinesque (1819) have been recognized based on meristic characters (Miller, 1968), including *E. b. blennioides* Rafinesque (1819), *E. b. newmanii* (Agassiz, 1854), *E. b. gutselli* (Hildebrand, 1932), and *E. b. pholidotum* (Miller, 1968). In addition, Miller (1968) noted Missouri and Great Lakes morphological races of *E. b. pholidotum*. Three of these subspecies (*E. b. blennioides*, *E. b. newmanii*, and *E. b. gutselli*) were once regarded as separate species, but later were demoted to subspecies (Welter, 1938; Kuhne, 1939; Miller, 1968). The Tuckasegee Darter *E. b. gutselli* was elevated to species status based on observations of no hybridization in a restored section of Pigeon Creek in eastern Tennessee (Etnier and Starnes, 2001) and thus is not further discussed here as a member of the Greenside Darter complex. Unfortunately, no DNA sequences for *E. gutselli* have been published to date and samples were unavailable for use as an outgroup comparison in the present study.

Welter (1938) described the subspecies *Etheostoma b. blennioides*, which was termed the “Allegheny” type of Greenside Darter due to its hypothesized origin in the Allegheny Plateau Pleistocene glacial refugium (see Trautman, 1981). The Allegheny type now ranges throughout the Ohio River basin and north eastward into the Potomac and upper Genesee Rivers (Miller, 1968; Trautman, 1981; Fig. 1). Miller (1968) stated that *E. b. blennioides* can be morphologically distinguishable from the other subspecies by its shorter dorsal lip tip (illustrated in Fig. 2b) and a small naked ventral area posterior to the pelvic fins (Fig. 2e).

Etheostoma b. pholidotum is common in lower Great Lakes tributaries near Lake Erie, Lake Ontario, and westward (Fig. 1; Smith, 1985), with the type specimen described from Bean Creek of the Middle Fork River, IL (Miller, 1968). Trautman (1981) termed this subspecies as the “Prairie” type of Greenside Darter due to its proposed western origin in the Mississippian glacial refugium and its more westerly distribution today. It is difficult to morphologically differentiate between *E. b. blennioides* and *E. b. pholidotum* due to appreciable overlap in the meristic characters described by Miller (1968) and Trautman (1981). Miller (1968) and Trautman (1981) distinguished *E. b. pholidotum* from *E. b. blennioides* by its fewer dorsal scale rows (i.e., 6 to 8; Trautman, 1981), lack of a dorsal lip tip (Fig. 2c), and complete ventral squamation (Fig. 2d; Miller, 1968). Miller (1968) further distinguished *E. b. pholidotum* from *E. gutselli* by its complete ventral squamation and from *E. b. newmanii* by its lower scale counts. Two morphological races of *E. b. pholidotum* have been described, one from the Wabash River-Great Lakes system and another from the Missouri River, which differ in that the former has lower scale counts (i.e., 51 to 67 vs. 53 to 71; Miller, 1968).

Etheostoma b. newmanii inhabits the Tennessee and Cumberland River drainages (Fig. 1), as well as the Arkansas, Ouachita, and White Rivers (Page, 1983). *Etheostoma b. newmanii* is reported to be identifiable from the other subspecies by its pronounced dorsal lip tip and higher scale counts (Miller, 1968).

Biogeography of the Greenside Darter

The Greenside Darter subspecies are believed to have diverged in separate drainage systems and glacial refugia during the Pleistocene Ice Ages (Miller, 1968), which destroyed older connections and shaped new river systems (Dyke and Prest, 1987; Pielou, 1991). As waterways opened with the glacial retreat at the end of the Wisconsinan episode (10 – 20,000 ya), fishes and other aquatic taxa recolonized the Great Lakes region, resulting in new contact among populations that had been isolated in southerly refugia for thousands of years. Other genetic studies of Lake Erie fishes have discerned a mixture of descendants from the Mississippi and Atlantic refugia, including sunfish *Lepomis* spp. (Bermingham and Avise, 1986), Brown Bullhead *Ameiurus nebulosus* (Murdoch and Hebert, 1997), Walleye *Sander vitreus* (Stepien and Faber, 1998), Yellow Perch *Perca flavescens* (Ford and Stepien, 2004), and Smallmouth Bass *Micropterus dolomieu* (Stepien et al., 2007). Following the last glaciation, *E. b. blennioides* and *E. b. pholidotum* migrated northward, along with other taxa, to recolonize the Great Lakes (Miller, 1968; Bailey and Smith, 1981). The descendants of the two refugia overlap in the lower Great Lakes region, with the Prairie type (*E. b. pholidotum*) more prevalent in the west and the Allegheny type (*E. b. blennioides*) more common in the east (Trautman, 1981; Fig. 1).

The study objective is to test the genetic divergence and morphological identity of *E. b. blennioides* and *E. b. pholidotum* in areas of sympatry and allopatry using sequences of the mitochondrial cytochrome *b* (cyt *b*) gene and control region, as well as the nuclear S7 intron 1. Genetic comparisons are made with *E. b. newmanii* and other species of *Etheostoma*, including putative sister species and close relatives. The possible congruence of DNA data with morphological characters described by Miller (1968) and Trautman (1981) is examined. Systematic status is evaluated based on criteria of the Phylogenetic Species Concept (PSC; *sensu* Mishler and Theriot, 2000) and the Evolutionary Species Concept (ESC; *sensu* Wiley and Mayden, 2000). Their approximate divergences are dated using the molecular clock calibration of Near and Benard (2004) for cytochrome *b* sequences of percids.

Chapter Two

Methods

Sampling

Representatives of the Greenside Darter (*Etheostoma blennioides*) taxa were collected under state collection permits using a combination of kick seining and electroshocking from 18 sampling sites within and outside of the Great Lakes watershed, totaling 294 individuals (Table 1, Fig. 1). A 2003 pilot study sampled Greenside Darter taxa from the adjacent Cuyahoga and Grand Rivers in northeast Ohio in the Lake Erie watershed, finding a marked genetic divergence corresponding to a possible species-level separation ($F_{ST} = 0.90$) that led to the present investigation. Sampling locations included major rivers in the Ohio River system (Great Miami, Hocking, and Scioto Rivers) and the Lake Erie watershed (Auglaize, Belle, Blanchard, Cuyahoga, Grand, Ottawa, and Portage Rivers), with outlying population samples from Ganargua Creek and the Allegheny, Meramec, Obey, Rockcastle, Susquehanna, and Wabash Rivers (Fig. 1). Samples from the type locality for *E. b. pholidotum* from the Wabash River, IL (Miller, 1968) also were analyzed. The type locality habitat for *E. b. blennioides* was destroyed decades ago and thus samples were not available (see Introduction).

Whole fish were preserved immediately in 95% EtOH and stored at room temperature in the Great Lakes Genetics Laboratory at the Lake Erie Center, University

of Toledo. Specimens were measured to the nearest mm and then sexed by examining the gonads under a Leica Microsystems MZ 12.5 stereomicroscope (Wetzlar, Germany). DNA was extracted from a right pectoral fin clip or ~25 mg of liver tissue using a QIAGEN extraction kit (Qiagen Inc., Valencia, CA), following manufacturer's directions. Extractions were assayed for quality and quantity on a 1% agarose mini-gel stained with ethidium bromide.

Gene amplification and DNA sequencing

The mitochondrial (mt) cytochrome *b* (cyt *b*) gene, mt control region, and nuclear S7 intron 1 were amplified using the polymerase chain reaction (PCR) and protocols adapted from Stepien and Faber (1998). Primers used for amplifications were L14724 and H15915 (Schmidt and Gold, 1993) for the cyt *b* gene, LW1-f (Gatt et al., 1992) and 12SARH (Martin et al., 1992) for the control region, and Eb-F and Eb-R (Morrison et al., 2006) for the S7 intron 1. PCR reactions contained 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, 50 µM of each deoxynucleotide, 0.5 µM each of the forward and reverse primers, 30 ng DNA template, and 1 unit of *Taq* polymerase in a 25 µl reaction. The amplification protocol was 42 cycles of 40 sec at 94°C, 40 sec at 52°C, and 1.5 min at 72°C. A 5 µl aliquot of each PCR product was visualized on a 1% agarose mini gel stained with ethidium bromide, and successful reactions were purified using a QIAGEN PCR Purification Kit. DNA sequencing was outsourced to the Cornell University Life Sciences Core Laboratories Center, which uses Applied Biosystems (ABI) Automated 3730 DNA Analyzers (Fullerton, CA, USA).

Greenside Darter mitochondrial and nuclear sequences were checked, identified, and aligned with the program BioEdit 7.05 (Hall, 1999) at the University of Toledo's

Great Lakes Genetics Laboratory, and sequences are deposited in the N.I.H GenBank (<http://www.ncbi.nlm.nih.gov/>; Appendix B). Aligned sequences total 1,076 bp for cyt *b*, 702 bp for the control region, and 419 bp for the S7 intron 1 (the entire intron). All individuals and taxa were sequenced for cyt *b* and morphologically analyzed. A subset of individuals from each clade identified in the cyt *b* analysis then were sequenced for the control region (N=123) and the S7 intron 1 (N=53).

Outgroups

Outgroup taxa included the Rock Darter *Etheostoma rupestre* Gilbert and Swain (1887; GenBank accession #AF288442 and AF404527), Blenny Darter *E. blennioides* Gilbert and Swain (1887; AY964698, AY964700, AF404529, and AF404528), Banded Darter *E. zonale* (Cope, 1863; AY964700 and U90621), and the Kentucky Darter *E. rafinesquei* Burr and Page (1982; AY374273 and AF404587). These taxa have been variously postulated to be the sister species of the Greenside Darter complex (Wood and Mayden, 1997; Piller, 2001 unpublished dissertation; Porter et al., 2002; and Sloss et al., 2004). Sequences of the Tuckasegee Darter *E. gutselli* were not available on GenBank and samples were not available to the present study. More distant relatives also were analyzed as outgroups, including the Orange-fin Darter *E. bellum* Zorach (1968; AY374260, AY572404, and AY573274), Bluebreast Darter *E. camurum* (Cope, 1870; AF045348, AY572403, and AY573273), and the Variegated Darter *E. variatum* Kirtland (1838; AY374278, AF404513, and AY573270). Samples of *E. rupestre* (EU118923), *E. blennioides* (EU118924), and *E. zonale* (EU118925-26) were obtained and sequenced using PCR protocols listed above for the nuclear S7 intron 1, as those sequences were not

available on GenBank. Samples of *E. rafinesquei* were not available, and thus this species was excluded solely from the nuclear S7 intron 1 analyses.

Genetic data analyses

Evolutionary relationships among the haplotypes for each gene region were evaluated using the neighbor-joining algorithm (Saitou and Nei, 1987) in Molecular Evolutionary Genetics Analysis (MEGA) 3.0 (Kumar et al., 2004), maximum likelihood analyses in PhyML (Guindon and Gascuel, 2003), and maximum parsimony analyses in PAUP* version 4.0b10 (Swofford, 2003). Support for nodes of the trees was evaluated using 1000 bootstrap pseudo-replications (Felsenstein, 1985).

Bayesian information criteria (BIC) from Modeltest 3.7 (Posada and Crandall, 1998) were used to determine the most appropriate model for the data sets for the neighbor-joining and maximum likelihood analyses. The Tamura-Nei model (Tamura and Nei, 1993) including invariable sites was selected as the most appropriate model for the *cyt b* data by Modeltest. For the neighbor-joining algorithm, those results were not informative, which may be due to the inclusion of invariable sites (see Waddell and Steel, 1997). In light of this finding, the Tamura-Nei model (Tamura and Nei, 1993) including the gamma distribution ($\alpha=0.162$) was selected, which was the next best model recommended by the BIC. For the mt control region data, the Hasegawa, Kishino, and Yano (HKY) model (Hasegawa et al., 1985) including invariable sites (0.772) was designated as the most appropriate model and the Kimura two-parameter model (Kimura, 1980) including invariable sites (0.700) was selected for the nuclear S7 intron 1 data set.

Maximum parsimony analysis in PAUP* was performed on the complete data set for each gene using a heuristic search with random stepwise addition, tree bisection-

reconnection (TBR; Swofford and Begle, 1993) branch swapping algorithm, and 1,000 bootstrap pseudo-replications.

A partition homogeneity test was conducted to compare the similarity of phylogenetic signal among sequence data from *cyt b*, control region, and the S7 intron 1 to evaluate whether the data sets could be reliably combined in a total evidence approach (Farris et al., 1994, 1995). Congruent data sets then were combined and a heuristic search performed with random stepwise addition, TBR branch swapping algorithm, and 1,000 bootstrap pseudo-replications in PAUP*. In addition, model selection for neighbor-joining and maximum likelihood analyses were repeated on the combined data set with Modeltest 3.7, which selected the Tamura-Nei model (Tamura and Nei, 1993) with a gamma distribution ($\alpha=0.1403$).

Population genetic data statistics were analyzed using Arlequin 3.11 (Excoffier et al., 2005), including measures of haplotype diversity and genetic divergence estimates for the major clades identified in the phylogenetic analyses. Uncorrected *p*-distances (proportion of different nucleotide sites among sequences; Kumar et al., 2004) within and among the primary clades were calculated in MEGA 3.0 (Kumar et al., 2004) for the *cyt b* data set. In order to compare the level of observed *p*-distances among taxa in this study, *cyt b* sequences were evaluated from GenBank for published sister species of darters. A molecular clock calibration of 2% *cyt b* sequence divergence per million years based on the related Logperch Darters *Percina* (Near and Benard, 2004) was used to evaluate possible separation times.

Pairwise relationships between Greenside Darter taxa sample sites using the *cyt b* data were evaluated in Arlequin using unbiased θ estimates of *F*-statistics (Weir and

Cockerham, 1984) as well as nonparametric exact tests of differentiation (χ^2 contingency table analyses; Raymond and Rousset, 1995). Use of F_{ST} estimates in the present study facilitated comparisons with other studies in the literature and provided levels of comparative genetic divergence. However, F -statistic estimates assume a normally distributed data set (Weir and Cockerham, 1984) and are affected by small sample sizes and rare alleles (Raymond and Rousset, 1995). In contrast, the exact test of differentiation uses a Markov Chain Monte Carlo (MCMC) procedure to randomly sample allele frequencies, is not affected by sample size and does not depend on a normally distributed data set (Raymond and Rousset, 1995). Probability values for both types of multiple pairwise comparisons were adjusted using the sequential Bonferroni method (Rice, 1989).

Phylogeographic analyses

Phylogeographic patterns within primary taxa were analyzed using nested clade analysis (NCA; Templeton et al., 1995), as implemented in GEODIS version 2.5 (Posada et al., 2000). Based on a statistical parsimony procedure, haplotype networks were constructed using the program TCS (Clement et al., 2000) and NCA was applied to the networks. The nested clade input consisted of geographic distance (km) among locations, geographic coordinates, sample size for each haplotype, and nodal location of the clade on the tree (i.e., interior versus tip). GEODIS calculated the D_c (spatial spread of the clade) and D_n (distribution of a clade with respect to other clades) distance statistics. Association between the haplotype network and geography was tested with 10,000 permutations. Statistically significant patterns were interpreted using the GEODIS inference key (Templeton, 1998; updated by Posada, 2005), which suggests likely

cause(s) of associations (e.g., isolation by distance, range and population expansion/contraction, long distance dispersal, fragmentation, demographic connectivity, and shifts in hierarchical clade levels).

Morphological comparisons

Morphological variation among individuals comprising the genetic taxa was examined using characters defined by Miller (1968) and Trautman (1981). Counts of lateral line scales, transverse scale rows, dorsal scale rows, and least caudal peduncle scale rows (Fig. 2a) were analyzed, as well as the presence/absence of a dorsal lip tip (Fig. 2b and 2c) and complete versus incomplete ventral squamation (Fig. 2d and 2e). Scale counts were tested for correlation using an alpha test and then compared among different genetic groups using a Multivariate ANalysis Of VAriance (MANOVA) in SAS 9.1 (SAS Institute Inc., Cary, NC). The characters of presence/absence of a dorsal lip tip and complete vs. incomplete ventral squamation were tested using chi square analyses and odds ratio tests in SAS 9.1.

Chapter Three

Results

Phylogenetic analyses of cryptic taxa in the Greenside Darter complex

There are 57 mtDNA cyt *b* haplotypes in the Greenside Darter complex data set (GenBank accession #EF587846-48 and EU118843-96), along with 19 for the control region (EF587849-51 and EU118827-42), 26 for the nuclear S7 intron 1 (EU118897-922), and 46 combined cyt *b* and control region haplotypes (Appendix A). Four widely diverged Greenside Darter clades are discerned, comprising *Etheostoma b. blennioides*, *E. b. pholidotum*, *E. b. newmanii*, and a Meramec River clade, which are distinguished by unique haplotypes and sequence synapomorphies.

MtDNA cytochrome b data

Four distinct clades are recovered that are supported by 99-100% bootstrap pseudo-replications in neighbor-joining, maximum parsimony, and likelihood analyses (Fig. 3a) that have congruent topologies. The cyt *b* trees place *E. blennius* as the sister species to the Greenside Darter complex (Fig. 3a), diverging by *p*-distances of 0.076 to 0.080 and an estimated 3.8 to 4.0 million years (my). The *E. b. pholidotum* clade is the sister species to the other three taxa. Divergence comparisons using θ_{ST} and exact tests of differentiation reveal pronounced separation among the four clades ($\theta_{ST} = 0.92$ to 0.97 , $P < 0.0001$; Table 2). Uncorrected *p*-distances show little difference among haplotypes

within the clades, in contrast to the marked divergences among them ($p = 0.025$ to 0.039 ; Table 2) estimated as 1.25 to 1.85 my (Table 2).

Reciprocally monophyletic clades correspond to Miller's (1968) subspecies, including *E. b. blennioides*, *E. b. newmanii*, and *E. b. pholidotum*. A fourth reciprocally monophyletic clade contains samples from the Meramec River, MO, corresponding to the Missouri morphological race of *E. b. pholidotum* described by Miller (1968). This clade, however, forms a distinct taxon and is not the sister group of the Great Lakes race of *E. b. pholidotum*. It appears more closely related to *E. b. blennioides* and *E. b. newmanii* (Fig. 3a). Within the *E. b. blennioides* clade, samples from the Scioto River population are monophyletic (noted as clade "A" on Fig. 3a).

Cyt *b* sequence data shows that *E. b. blennioides* differs from the other three Greenside Darter taxa by 25 fixed nucleotide differences, *E. b. pholidotum* and the Meramec River clade by 13 synapomorphies each, and *E. b. newmanii* by 9 characters. *Etheostoma b. blennioides* diverges from *E. b. pholidotum* and the Meramec River clade by $p = 0.039$, and from *E. b. newmanii* by 0.036 . *Etheostoma b. newmanii* differs from *E. b. pholidotum* and the Meramec River clade by $p = 0.025$, whereas the Meramec River and *E. b. pholidotum* samples diverge by 0.029 .

MtDNA control region data

Congruent with the cyt *b* data, the control region sequence data reveals the same four reciprocally monophyletic clades using neighbor-joining, maximum parsimony, and likelihood analyses. The control region data likewise place *E. blennius* as the sister species to the Greenside Darter complex (Fig. 3b). However, *E. b. blennioides* appears as

the sister species of the other three taxa in place of *E. b. pholidotum* (Fig. 3b). No clear population-level patterns are discerned.

Nuclear S7 intron 1 data

Nuclear intron sequences do not show as clear a pattern among the four taxa, resolving only two of the clades, the Meramec River clade and *E. b. pholidotum* (except for a single specimen). The latter exception is a female specimen (haplotype 15S7; Fig. 3c) from the sympatric location at Ganargua Creek that is identified with mtDNA as *E. b. pholidotum*, but groups with *E. b. blennioides* in the nuclear intron tree. Using the nuclear S7 intron 1 data alone, *E. b. blennioides* and *E. b. newmanii* appear polyphyletic. *Etheostoma zonale* is depicted as the sister species to the Greenside Darter complex, replacing *E. blennioides* (from the mitochondrial data; Fig. 3c). Haplotype 11S7 is shared between samples from the Meramec River and a single individual from Ganargua Creek genetically identified as *E. b. pholidotum* in the mtDNA analyses.

Etheostoma b. blennioides appears divided in 3 clades, with haplotypes 7S7 and 14S7 from the Owego and Susquehanna River systems linked by 86% bootstrap support (Fig. 3c). Haplotypes belonging to *E. b. newmanii* (8S7, 10S7, and 26S7) are divided between two clades with low bootstrap support.

Combined mitochondrial data

A partition homogeneity test reveals that the mitochondrial DNA data sets (cyt *b* and control region) are congruent ($P=0.654$), but are incongruent with the nuclear S7 intron 1 data (1,000 replicates, $P=0.0001$). Dolphin et al. (2000) documented that the partition homogeneity test is susceptible to noise within a data set, and so the number of sequences per taxon was reduced and uninformative characters for the three data sets

were removed (Cunningham, 1997). However, the nuclear data set remains incongruent ($P=0.001$). Thus, only the mtDNA data sets are combined.

Four distinct clades comprising *E. b. blennioides*, *E. b. pholidotum*, *E. b. newmanii*, and the Meramec River samples are recovered in the combined analysis, with 89-99% bootstrap support in the neighbor-joining, maximum parsimony, and likelihood trees (Fig. 3d). Results depict *Etheostoma blennius* as the sister species of the Greenside Darter complex, supported by all analyses (Fig. 3d). Results do not resolve the order of ancestry among the four taxa (*E. b. blennioides*, *E. b. pholidotum*, *E. b. newmanii*, and the Meramec River clade; Fig. 3d), which appear to have differentiated about the same time. However, greater divergence distances distinguish *E. b. blennioides* from the other three taxa.

Population genetic and phylogeographic patterns of E. b. blennioides

Within the Ohio and Great Lakes basins, 20 *E. b. blennioides* haplotypes are identified. A common cyt *b* haplotype of *E. b. blennioides* (haplotype Cb 3; Fig. 4a) is recovered from all of its sampling localities and totaled 74%, increasing in frequency to the northeast (Fig. 4a). A common haplotype with a similar pattern also is resolved from the control region sequences (haplotype Cr 3).

Pairwise θ_{ST} comparisons reveal several significant divergences within the *E. b. blennioides* clade. *Etheostoma b. blennioides* samples from the Scioto River location significantly diverge from all other locations (Table 3a; designated as “A” on Figs. 3a and 3d), with haplotype Cb 16 being the most abundant (61%; Fig. 4a). Samples from the Great Miami River also differ significantly from most other sites, except from the Hocking River and Ganargua Creek samples (Table 3a). The most common cyt *b*

haplotype (Cb 3) dominates samples from the Great Miami and Hocking Rivers, and appears as monotypic in the Ganargua Creek, Cuyahoga, and Susquehanna River sites (Fig. 4a). Samples from the Great Miami and Scioto Rivers house the greatest haplotypic diversity levels for the *E. b. blennioides* clade (Table 1), and significantly diverge according to exact tests (Table 3a).

The nested clade analysis of *E. b. blennioides* cyt *b* sequences contains 5 levels, revealing two major clades and several significant subclades (Table 4). However, the GEODIS analysis for the *E. b. blennioides* total cladogram is not significant, showing no large-scale geographical association of haplotypes. Since significant subclades are recovered, localized structure within sampling sites is supported. Significant geographical associations are discerned for clades 2-1 and 4-2, with the latter comprising samples from the Hocking and Scioto Rivers. Samples from the Scioto River comprise an endemic clade (Fig. 5a), attributed to restricted gene flow with isolation by geographic distance (Table 4). Clade 4-1 contains all sampling sites of *E. b. blennioides* (Table 1) and its most common haplotype (Cb 3; Fig. 5a). Clade 2-1 contains significant subclade 1-3, comprising haplotypes from the Allegheny River (Fig. 5a) and showing restricted gene flow with isolation by distance. A geographical split occurs within *E. b. blennioides*, with samples from the Scioto River forming a distinct clade in the haplotype networks (3-3; Fig. 5a).

Population genetic and phylogeographic patterns of E. b. pholidotum

Twenty-five haplotypes are identified for *E. b. pholidotum* from the Great Lakes region. Pairwise θ_{ST} comparisons reveal fewer significant divergences within the *E. b. pholidotum* clade compared to the *E. b. blennioides* clade. Haplotype Cb 1, the most

common haplotype, is shared among all sampling sites (Fig. 4b), and its frequency increases to the northeast (like haplotype Cb 3 within the *E. b. blennioides* clade; Fig. 4b). Haplotype Cb 1 is monotypic in Ganargua Creek (Fig. 4b); the sole sympatric sampling location, whereas samples from the Auglaize, Blanchard, and Wabash (IL) Rivers have the highest haplotypic diversities (Table 1). Samples from the Portage River diverge significantly from the other *E. b. pholidotum* sampling localities (Table 3b). Exact tests of differentiation (Raymond and Rousset, 1995) discern that most *E. b. pholidotum* sampling locales do not differ genetically (Table 3b), except those from the Portage and Grand Rivers.

The nested clade for *E. b. pholidotum* consists of 2 levels, with 2 major clades and 3 significant subclades (Table 4; Fig. 5b). Like the *E. b. blennioides* clade, the overall GEODIS result is not significant. However, significant geographical associations for *E. b. pholidotum* sampling sites are found in clades 1-1 and 1-2. As in *E. b. blennioides* sampling sites, the historical process among *E. b. pholidotum* sampling sites is restricted gene flow with isolation by distance (Table 4). In clade 1-1, haplotype Cb 43 is significant and the sampling design is inadequate for differentiating between isolation by distance or long-distance dispersal (Table 4; Fig. 5b). Clade 1-2 is significant due to restricted gene flow with isolation by distance and contains samples from the Auglaize, Blanchard, Ottawa, and Wabash (IL) Rivers (Table 4).

Morphological comparisons among cryptic taxa of the Greenside Darter complex

Counts for lateral line scales, dorsal scale rows, transverse scale rows, and least caudal peduncle scale rows for all four genetic clades are similar to the original subspecies values described by Miller (1968) and Trautman (1981) for *E. b. blennioides*,

E. b. pholidotum, and *E. b. newmanii* (Table 5). An alpha test for correlation reveals that the four counts are uncorrelated with one another and thus are appropriately considered as separate characters in statistical analyses. Scale counts for individuals from the Meramec River also match those originally described by Miller (1968) for the subspecies *E. b. pholidotum*. Due to small sample sizes, samples from the Meramec River and *E. b. newmanii* could not be included in further statistical analyses. Sex data from 294 individuals belonging to the Greenside Darter complex reveal a sampling bias towards females. No statistical tests could be performed on males vs. females due to the small representation of males (~40 individuals of 294).

Significant differences are found between *E. b. blennioides* and *E. b. pholidotum* for the four scale counts using MANOVA (Wilk's λ $P < 0.0001$; Zar, 1999), as well as pairwise comparisons for the measures individually (Table 5). However, the four scale counts for the four genetic clades overlap (Table 5). In addition, chi-square analyses reveal that *E. b. blennioides* and *E. b. pholidotum* differ significantly in degree of ventral squamation ($P < 0.0001$; Table 5). An odds ratio test (Zar, 1999) shows that *E. b. blennioides* is five times as likely to have incomplete ventral squamation (Fig. 2e) and *E. b. pholidotum* is five times as likely to be completely scaled (Fig. 2d). The presence or absence of a dorsal lip tip does not differ significantly between *E. b. blennioides* and *E. b. pholidotum* (Table 5), showing that it is an unreliable character.

Chapter Four

Discussion

Phylogenetic relationships and resolution of cryptic taxa

Phylogenetic analyses using combined mitochondrial gene regions for Miller's (1968) subspecies of Greenside Darter resolve four distinct reciprocally monophyletic taxa: *Etheostoma blennioides blennioides*, *E. b. pholidotum*, *E. b. newmanii*, and the Meramec River clade. The four clades are distinguished by marked divergences that are over ten times greater than the variation found within them; and θ_{ST} divergences among the four taxa are ~ 1.00 , corresponding to species-level differences and absence of gene flow (Wright, 1978).

The genetic distributions of the four taxa correspond to ranges described by Miller (1968; Fig. 1). Specimens from the Allegheny, Ohio, and Susquehanna River systems form the *E. b. blennioides* clade. *Etheostoma b. blennioides* also is found within the Lake Erie watershed in the Cuyahoga River, which may be due to its ancestral ties to the Ohio River via the Tuscarawas River (Szabo, 1987). Ganargua Creek in western New York is a site of sympatry, containing both *E. b. blennioides* and *E. b. pholidotum*. Samples from the Wabash River and the Great Lakes basin (except the Cuyahoga River samples) are *E. b. pholidotum* and those from the Meramec River constitute the Missouri morphological race of *E. b. pholidotum*, which forms a fourth taxon. This

taxon is not the sister group of *E. b. pholidotum* according to the cyt *b* data and combined mtDNA trees. Additional locations should be analyzed and this taxon may be named to recognize the pioneering morphological work of Miller (1968). *Etheostoma b. newmanii* comprises all samples from the Rockcastle and Obey Rivers in central Kentucky and Tennessee, respectively.

In comparison to the present study, Berendzen (2005, unpublished dissertation) examined a geographic range for *E. blennioides* that focused in the south and used only cyt *b* data. He recovered only two major clades of the *E. blennioides* complex, including an *E. blennioides* group that extended from the Missouri and Meramec Rivers into the Wabash and Ohio Rivers and the Great Lakes region, and *E. newmanii* in the Tennessee River system. Berendzen (2005) discerned a similar *p*-distance (0.04) between the two clades to that found here, however, the sequences are not provided in his dissertation and are not in GenBank or any public databases. In contrast, the present study discerns four clades due to more extensive sampling to the north. Berendzen (2005) results supported rapid diversification and expansion showing little geographical structure within his *E. blennioides* and *E. newmanii* clades. The present study likewise supports the interpretation of rapid expansion of *E. b. blennioides*, as well as *E. b. pholidotum*, and additionally resolves appreciable geographic structure in *E. b. blennioides* from the Scioto and Great Miami Rivers.

The cytochrome *b* gene genetic distances distinguishing four taxa of the Greenside Darter complex ($p = 0.025$ to 0.039) are similar to those that differentiate known sister species of other darters ($p = 0.009$ to 0.129 ; Table 6). Notably, similar divergence levels differentiate the sister species pairs of *Etheostoma smithi* from *E.*

striatulum ($p= 0.028$), *E. caeruleum* from *E. burri* ($p= 0.031$), and *Percina smithvanizi* from *P. kusha* ($p= 0.038$). Divergences among taxa in the Greenside Darter complex are higher than those determined for the sister species pairs of *Percina caprodes* from *P. suttkusi* ($p= 0.009$) and *E. bellum* from *E. camurum* ($p= 0.015$; Table 6). These comparative data suggest that the four Greenside Darter complex taxa may be recognizable as species.

However, the nuclear S7 intron 1 sequence data clearly resolve only the *E. b. pholidotum* and Meramec River clades. Nuclear S7 DNA results group a single female from Ganargua Creek identified as *E. b. pholidotum* in the mtDNA data more closely to an *E. b. blennioides* clade, indicating probable mtDNA introgression at that locality. A female *E. b. pholidotum* may have reproduced with a male *E. b. blennioides*, resulting in “mitochondrial capture” (Avice, 2004).

Lower resolution for *E. b. blennioides* and *E. b. newmanii* in the nuclear DNA data may be due to its slower evolutionary rate ($\sim 1/4$ that of haploid mtDNA; see Avice, 2004). Since the Greenside Darter complex evolved during the early Pleistocene Epoch, it is likely that not enough time has passed to clearly discern changes in the nuclear S7 intron 1 and for lineage sorting to occur (Avice, 2004).

Similarly, Morrison et al. (2006) found lower genetic resolution using the S7 intron in the Crystal Darter *Crystallaria asprella* (Jordan, 1878), whereas mtDNA sequences resolved four distinct reciprocally monophyletic clades (Wood and Raley, 2000). The p -distances for cyt *b* gene sequences of the Crystal Darter had a wide range (0.014 to 0.119; Wood and Raley, 2000), with middle values similar to those distinguishing the Greenside Darter taxa here (0.025 to 0.039). The highest levels of

genetic divergence observed for the Crystal Darter (Wood and Raley, 2000) were comparable to those recovered by Song et al. (1998) among darter genera. The Crystal Darter has a highly disjunct distribution, which may contribute to its high divergence levels (Wood and Raley, 2000).

Ancestry of the Greenside Darter complex

The present study discerns that the Greenside Darter complex and its sister species *E. blennioides* diverged ~3.8 to 4.0 million years ago (mya) during the mid-Pliocene Epoch from a common ancestor that once was distributed widely throughout the Teays-Mississippi River system (Burr and Page, 1986). This sister relationship also was discerned by Wood and Mayden (1997) and Morrison et al. (2006) using allozymes and mtDNA control region sequence data, respectively. Their divergence likely stemmed from a stream capture that changed the courses of the Duck and lower Tennessee Rivers to flow southward (Starnes and Etnier, 1986), thereby restricting *E. blennioides* to its present range in the central Tennessee and northern Alabama region of the Tennessee River system (Page and Burr, 1991). Meanwhile, the Cumberland River's northward flow into the outlet of the Ohio River (Starnes and Etnier, 1986) isolated the ancestor of the Greenside Darter complex to the north in the Teays-Mississippi River system (Fig. 6a).

Separation of the four Greenside Darter clades

The Pliocene drainage systems of the Teays and Mississippi Rivers were significantly altered by glacial advances and retreats during the Pleistocene Epoch, eradicating some lineages of fishes and isolating others (Robison, 1986). The first divergence within the Greenside Darter complex split the *E. b. blennioides* lineage from the ancestral clade that led to the *E. b. pholidotum*, *E. b. newmanii*, and Meramec River

groups ~ 1.85 mya (Fig. 6b). About that time, the advancing glaciers are believed to have dammed the headwaters of the Teays River, which changed course to flow along the path of the present Ohio River (Mayden, 1988) and isolated *E. b. blennioides* in the Eastern Highlands. The sister clade, which was the ancestor of the remaining three Greenside Darter taxa (*E. b. pholidotum*, *E. b. newmanii*, and the Meramec River clade) then was isolated to the west and south in the Interior Highlands and the Interior Low Plateaus (Fig. 6b).

The three taxa then diverged ~1.3 mya during an interglacial period when the glaciers released large quantities of water laden with sediments into the Mississippi River system, creating inhospitable habitat for most fish species (Burr and Page, 1986). The Mississippi River proper has remained poor habitat for these Greenside Darter taxa, leading to long-term separation of these clades. Near and Keck (2005) similarly found that the Central Highlands were a historically isolated area reflecting a long-term vicariant separation for *Nothonotus* darters. During this time tectonic activity uplifted Crowley's Ridge to ~60 m above the adjacent lowlands (Van Arsdale et al., 1995), forcing the Mississippi River to flow along its western side and the ancestral Ohio River along the eastern side (Robison, 1986). These events isolated the Meramec River clade, which was prevented from expanding eastward due to Crowley's Ridge and northward by siltation from the melting glaciers (Fig. 6c).

At about the same time, the *Etheostoma b. pholidotum* clade became isolated in the unglaciated portion of the Wabash River system in southern Illinois on the eastern side of Crowley's Ridge, north of the Mississippi embayment (Fig. 6c). That taxon, in turn, was restricted by the sediment released from the glaciers to the north. *Etheostoma*

b. pholidotum may have been prevented from colonizing eastward into the Ohio River due to competition with *E. b. blennioides*, which should be investigated further. About 1.3 mya, *E. b. blennioides*, along with other fishes (see Mayden, 1988), likely expanded its range westward from the Eastern Highlands through the newly formed Ohio River to colonize the Scioto and Great Miami Rivers. This hypothesis is supported by greater genetic diversity and significant divergences in those southerly populations as compared to those in the north.

During this time, *Etheostoma b. newmanii* was isolated to the south in the Eastern Highlands in the Tennessee and Cumberland River headwaters that occupied the channel of the present-day Mississippi River (Robison, 1986), as hypothesized by Miller (1968). Miller (1968) described an additional morphological race of *E. b. newmanii* in the Arkansas, Ouachita, St. Francis, and White Rivers (Fig. 1), which should be investigated in future studies.

Congruent divergence of other Interior and Eastern Highlands fishes

The present study discerns distinct Greenside Darter taxa in the Interior Highlands (Meramec River clade), the Eastern Highlands (*E. b. blennioides* and *E. b. newmanii*), and northeast of the Mississippi River (*E. b. pholidotum*) (Fig. 6d). Similar to this evolutionary pathway, disjunct distributions were described across the Mississippi River in distinct Interior and Eastern Highlands populations of the Northern Hogsucker *Hypentelium nigricans* (Berendzen et al., 2003), Gilt Darter *Percina evides* (Near et al., 2001), Rainbow Darter *Etheostoma caeruleum* (Ray et al., 2006), and the Rosyface Shiner *Notropis rubellus* complex (Berendzen et al., 2007) using *cyt b*. This disjunct

pattern also was observed by Strange and Burr (1997) for the *Etheostoma* subgenus *Litocara* and the *Percina* subgenus *Odontopholis* using allozyme data.

Post-glacial spread of the Greenside Darter complex

Following the retreat of the Wisconsinan glaciers 10 – 20,000 ya, *E. b. blennioides* likely traveled northeastward through the Allegheny and Susquehanna River systems, colonizing the lower Great Lakes through Ontario and Eastern Lake Erie as suggested by Miller (1968; Fig. 6d). *Etheostoma b. pholidotum* presumably spread northward from its southwestern refugium via the Wabash River and entered western Lake Erie through its Maumee River/glacial Lake Maumee outlet (Fig. 6d). It appears that *E. b. pholidotum* quickly expanded into numerous local watersheds, as its populations are little differentiated today among Lake Erie tributaries. As the water levels in paleo-Lake Erie lowered, *E. b. pholidotum* likely migrated across the south shore of Lake Erie and into eastern Lake Erie and Lake Ontario (Fig. 6d), meeting *E. b. blennioides*. Genetic results support this colonization pattern as greater haplotypic diversity of *E. b. pholidotum* occurs in the west, suggesting its longer history and proximity to the western refugium. Currently, *E. b. pholidotum* is sympatric with *E. b. blennioides* in some northeastern locations, such as Ganargua Creek in the present study, and *E. b. blennioides* is found in the Cuyahoga River of the central Lake Erie watershed.

According to these genetic data, *E. b. newmanii* did not migrate northward and the Meramec River clade did not migrate eastward following the retreat of the Wisconsinan glaciers (10 – 20,000 ya; Fig. 6d). Today, the Greenside Darter complex has a disjunct distribution and is absent from southern Illinois, western Kentucky and Tennessee, and eastern Missouri and Arkansas (Figs. 1, 6d). This absence may be due to outburst floods

from glacial Lakes Agassiz and Chicago routed through the Mississippi and Illinois Rivers, respectively, which transported large quantities of suspended material (Kehew et al., in press; Fig. 6d).

Post-glacial spread of other fishes

Similar to the *E. b. blennioides* clade, genetic studies showed that *Hypentelium nigricans* (Berendzen et al., 2003), *Percina evides* (Near et al., 2001), *Etheostoma caeruleum* (Ray et al., 2006), and *Notropis rubellus* (Berendzen et al., 2007) spread into the Great Lakes region from the Eastern Highlands after the retreat of the Wisconsin glaciers 10 – 20,000 ya. These four species dispersed northward from the Eastern Highlands into the modern Ohio River system and eastern Great Lakes (Near et al., 2001; Berendzen et al., 2003, 2007; Ray et al., 2006), as did *E. b. blennioides*. Unlike the results of the present study, little genetic differentiation occurs in the Eastern Highlands clades of *H. nigricans*, *P. evides*, *E. caeruleum*, and *N. rubellus*. These populations show no geographical structure, implying rapid population expansion into the Ohio River and Great Lakes (Near et al., 2001; Berendzen et al., 2003, 2007; Ray et al., 2006), which may be an artifact of incomplete sampling in the Great Lakes region. In contrast, the present study discerns greater geographical structure in *E. b. blennioides* from the Eastern Highlands, with samples from the Scioto and Great Miami Rivers diverging significantly, likely due to increased resolution from more intensive sampling.

Also differing from the present study, analyses of *H. nigricans*, *P. evides*, and *E. caeruleum* from the Ohio River and Wabash River/Great Lakes (Near et al., 2001; Berendzen et al., 2003; Ray et al., 2006) grouped in a single Eastern Highlands clade without differentiation, likely due to limited sampling of their respective northern ranges.

Those fish species are believed to have used the Wabash River to disperse northward after the retreat of the Wisconsin glaciers (Near et al., 2001; Berendzen et al., 2003; Ray et al., 2006), along the route used by *E. b. pholidotum*. In contrast to the other studies, a marked divergence distinguishes the *E. b. pholidotum* and *E. b. blennioides* clades ($p=0.039$). Berendzen et al. (2007) similarly discerned a distinct Wabash River clade of the Rosyface Shiner *Notropis rubellus* using *cyt b* data, which ranges from Illinois into Indiana and western Lake Erie up through Ontario, congruent with *E. b. pholidotum*.

Several other fishes are characterized by genetic differences across Lake Erie, with eastern populations hypothesized to have descended from an eastern glacial refugium and those in the west from the Mississippian refugium, as indicated here for *E. b. blennioides* and *E. b. pholidotum*. Sympatric patterns are found in the Brown Bullhead *Ameiurus nebulosus* (Murdoch and Hebert, 1997), Walleye *Sander vitreus* (Stepien and Faber, 1998), Yellow Perch *Perca flavescens* (Ford and Stepien, 2004), Banded Killifish *Fundulus diaphanus* (April and Turgeon, 2006), and Smallmouth Bass *Micropterus dolomieu* (Stepien et al., 2007). Similar to the present study, Walleye (Stepien and Faber, 1998) and Smallmouth Bass (Stepien et al., 2007) from the Ohio River system today are quite divergent from those in the Great Lakes, retaining their Teays River ancestry.

Morphological comparisons among cryptic taxa of the Greenside Darter complex

The present study discerned that Miller's (1968) four subspecies of the Greenside Darter complex are genetically distinct and differ significantly in the meristic counts described by Miller (1968) and Trautman (1981). Those counts are too variable to be clearly diagnostic among individuals. Morphological values in this study are within the

range described by Miller (1968) and Trautman (1981). Least caudal peduncle scale rows are in the lower portion of Miller's (1968) range (Table 5). Ventral squamation and the presence/absence of a dorsal lip tip differ from the observations of Miller (1968). The present study found both complete and incomplete ventral squamation characterizing individuals of *Etheostoma b. pholidotum* and *E. b. newmanii*, whereas Miller (1968) observed only complete squamation for them. Individual samples of *E. b. blennioides* and *E. b. newmanii* either had or lacked a dorsal lip tip, whereas Miller (1968) described only presence of the dorsal lip tip for these two subspecies.

Piller (2001, unpublished dissertation) found some similar results for the Greenside Darter complex using a single gene (cytochrome *b*) and based only on data from 3 individual *E. b. blennioides*, 3 *E. b. pholidotum*, and a single outgroup (*E. rupestre*). Those sequences remain unpublished and are not available on GenBank, in any public database, or even in his dissertation. Thus his study could not be used for comparison with the present results. Piller's (2001) study additionally examined morphometrics (body shape measures) for 400+ individuals of the Greenside Darter complex, focusing on southern sampling sites. He discerned some statistical differences, although those characters overlapped, rendering correct field identification improbable. Both Piller's (2001) and the present study did not reveal clear morphological characters that could be used on their own to identify the cryptic Greenside Darter taxa. The publication of Piller's (2001) data would provide a valuable addition to the present findings.

Molecular genetics and the resolution of morphologically cryptic taxa

Molecular genetics have led to the correct identification of many cryptic species from a broad range of taxa, including fishes (Perdices et al., 2002; Feulner et al., 2006; Robalo et al., 2007), birds (Olsson et al., 2005), horseshoe crabs (Avise et al., 1994), and bats (Kiefer et al., 2002). A classic example of species separated by a large genetic divergence, but very little morphological variation is the North American Horseshoe Crab *Limulus polyphemus* from the three Asiatic Horseshoe Crab species (*Carcinoscorpius rotundicauda*, *Tachypleus tridentatus*, and *T. gigas*), which diverged ~45 – 60 mya (Avise et al., 1994). Similar to the present study of the Greenside Darter complex, Page et al. (2003) recovered three new species belonging to the morphologically cryptic Barcheek Darter *Etheostoma virgatum* complex through phylogenetic analyses of a nuclear (S7 intron) and three mitochondrial (cyt *b*, ND2, and ND4) DNA regions. Like the present study, Page et al. (2003) found the Barcheek Darter species group to be phenotypically similar, but distinguishable by scale counts, number of dorsal and anal fin rays, and color patterns of breeding males. Similarly, the madtom catfishes *Noturus albater* and *N. maydeni* lacked diagnostic morphological characters, but were divergent and reciprocally monophyletic using cyt *b* ($p = 0.036$; Egge and Simons, 2006).

Members of the Greenside Darter complex, including *E. b. blennioides*, *E. b. pholidotum*, *E. b. newmanii*, and the Meramec River clade occupy similar habitats in riffle areas and feed on similar prey items (Page and Burr, 1991), which likely conserved morphological characters despite their significant genetic divergence. Duftner et al. (2006) reported a lack of morphological differentiation among Lake Tanganyika cichlid fishes that occupied similar habitats, but were genetically distinct. The present results

show that genetic characters are the sole reliable means to differentiate among *E. b. blennioides*, *E. b. pholidotum*, *E. b. newmanii*, and the Meramec River clade.

Are the Greenside Darter taxa separate species?

The question of whether or not to elevate taxa and which species concept to use has been debated extensively and well reviewed in the literature (see Zink and McKittrick, 1995; Mayden, 1997; Coyne and Orr, 2004). Many studies have discerned morphologically cryptic species using DNA sequences with recommendations for elevating the recovered taxa. For example, morphologically cryptic species of the Madtom Catfish *Noturus* spp. (Egge and Simons, 2006), the Woodrat genus *Neotoma* (Edwards and Bradley, 2002), and the Green Python *Morelia viridis* (Rawlings and Donnellan, 2003) were recommended as newly detected species under the Phylogenetic Species Concept (PSC; Cracraft, 1983). The four taxa of Greenside Darter discerned in this study also meet the criteria of the PSC (*sensu* Mishler and Theriot, 2000), in being reciprocally monophyletic, having diagnosable synapomorphies, and demarcated by high bootstrap support. The Evolutionary Species Concept (ESC; *sensu* Wiley and Mayden, 2000), in turn defines a species as “an entity that keeps its identity from other such entities over time and space and that has its own independent evolutionary fate and historical tendencies”, which also is true for the four taxa here. Moreover, the present study discerns that each clade is separated by marked genetic divergence that is ten times or greater than that found within each clade, denoting their long-term separation with negligible gene flow.

The present study thus recovers four reciprocally monophyletic taxa showing a common ancestry, a large number of diagnosable synapomorphies (including 25 cyt *b*

substitutions for *E. b. blennioides*, 13 each for *E. b. pholidotum* and the Meramec River clade, and 9 for *E. b. newmanii*), and high bootstrap support, ~100% for all 4 taxa in the neighbor-joining, maximum parsimony and likelihood analyses. The present distribution and divergences of the Greenside Darter taxa indicate that they are distinct evolutionary entities and have been isolated since the early Pleistocene Epoch, meeting the criteria of the ESC. However, Mishler and Brandon (1987) also cite that the branching order should be resolved for the PSC. The branching order for the mitochondrial DNA sequence data is not well resolved here, since three of the taxa diverged about the same time. Moreover, only two of the taxa are resolved using the nuclear S7 intron 1 data (*E. b. pholidotum* and the Meramec River clade) and evidence for some introgression may indicate they are along the path of becoming separate species, but are not yet there.

Conclusions

The present study finds four reciprocally monophyletic taxa that are highly genetically divergent from one another based on mtDNA sequences. The nuclear S7 intron 1 sequences do not resolve all four taxa, which may be due to its slower evolutionary rate. The four taxa likely separated due to vicariant events during the Pleistocene glaciations. Morphological characters examined here do not reveal four significantly different clades, since individuals overlap precluding their correct field identification. *Etheostoma b. blennioides* and *E. b. pholidotum* significantly differ based on scale counts and ventral squamation. *Etheostoma b. blennioides* and *E. b. pholidotum* also have unique population genetic patterns with sampling sites in the west having higher haplotypic diversity than those in the east. *Etheostoma b. blennioides* and *E. b. pholidotum* likely dispersed into the Great Lakes region during the retreat of the

Wisconsinan glaciers. *Etheostoma b. blennioides* may have used the Ohio River system to move into the Allegheny and Susquehanna River systems, whereas *E. b. pholidotum* most likely used the Wabash River as a dispersal route into western Lake Erie. Future studies thus should assess additional samples from other locations, including *E. b. newmanii* in the Tennessee and Cumberland Rivers, and the Meramec River clade in the Interior Highlands. The four monophyletic taxa recovered in this study then should be reevaluated for possible elevation to species level.

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VI. Tables

Table 1. Sampling location and size, genetic taxon identity, number of haplotypes, and haplotype (gene) diversity of the Greenside Darter *Etheostoma blennioides* taxa based on mitochondrial cytochrome *b* sequences.

Genetic Taxon Identity	Location	Latitude	Longitude	<i>N</i> individuals	<i>N</i> haplotypes	Haplotypic diversity
<i>E. b. blennioides</i>	Great Miami River, OH	39.514381	-84.713230	11	6	0.82
	Scioto River, OH	39.768330	-82.995785	18	7	0.63
	Hocking River, OH	39.461552	-82.312984	21	6	0.56
	Cuyahoga River, OH	41.144678	-81.438273	53	1	0.00
	Allegheny River, NY	41.998071	-78.244045	21	3	0.19
	Susquehanna River, NY	42.108825	-76.273823	23	1	0.00
<i>E. b. pholidotum</i>	Wabash River, IL	39.773704	-87.603568	8	4	0.75
	Wabash River, IN	40.839698	-85.438040	11	3	0.35
	Auglaize River, OH	40.678657	-84.259573	19	10	0.78
	Ottawa River, OH	40.755208	-84.036779	14	3	0.38
	Blanchard River, OH	41.036229	-83.576721	32	10	0.82
	Portage River, OH	41.476112	-83.295837	38	2	0.05
	Belle River, MI	42.941847	-82.828679	9	2	0.22
	Grand River, OH	41.734939	-81.047452	24	2	0.26
<i>E. b. blennioides</i> (39%)* and <i>E. b. pholidotum</i> (61%)*	Ganargua Creek, NY	43.068899	-77.298166	18	2	0.50
<i>E. b. newmanii</i>	Obey River, TN	36.528483	-85.440139	2	2	1.00
	Rockcastle River, KY	37.306665	-84.148008	5	2	0.40
Meramec River clade	Meramec River, MO	38.503970	-90.590975	14	8	0.87

* Percentage of population that was identified as *E. b. blennioides* or *E. b. pholidotum*.

Table 2. Pairwise divergences from cytochrome *b* sequence data for the Greenside Darter *Etheostoma blennioides* complex using θ_{ST} (below diagonal; Weir and Cockerham, 1984) and uncorrected *p*-distances within (diagonal, bold) and among groups (above diagonal; Kumar et al., 2004).

Taxa	<i>E. b. blennioides</i>	<i>E. b. pholidotum</i>	Meramec River	<i>E. b. newmanii</i>
<i>E. b. blennioides</i>	0.005	0.039	0.038	0.036
<i>E. b. pholidotum</i>	0.968**	0.002	0.029	0.025
Meramec River	0.945**	0.973**	0.003	0.025
<i>E. b. newmanii</i>	0.942**	0.973**	0.921**	0.002

* significant difference ** remains significant following sequential Bonferroni correction

(Rice, 1989)

Table 3. Population level pairwise divergences within the Greenside Darter taxa (a) *Etheostoma blennioides blennioides* and (b) *E. b. pholidotum* based on cytochrome *b* sequence data using θ_{ST} (below diagonal; Weir and Cockerham, 1984) and exact tests of differentiation (above the diagonal; Raymond and Rousset, 1995)

3a. *Etheostoma b. blennioides*

Site	Great Miami River	Scioto River	Hocking River	Cuyahoga River	Allegheny River	Ganargua Creek	Susquehanna River
Great Miami River	--	**	N. S.	*	*	N. S.	**
Scioto River	0.57**	--	**	**	**	**	**
Hocking River	0.02	0.58**	--	N. S.	N. S.	N. S.	*
Cuyahoga River	0.02**	0.67**	0.02	--	N. S.	N. S.	N. S.
Allegheny River	0.05**	0.71**	0.04*	0	--	N. S.	N. S.
Ganargua Creek	0	0.62**	0	0	0	--	N. S.
Susquehanna River	0.07**	0.74**	0.06**	0	0.01	0	--

3b. *Etheostoma b. pholidotum*

Site	Wabash River, IL	Wabash River, IN	Auglaize River	Ottawa River	Blanchard River	Portage River	Belle River	Grand River	Ganargua Creek
Wabash River, IL	--	N. S.	N. S.	N. S.	N. S.	**	N. S.	*	*
Wabash River, IN	0	--	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
Auglaize River	0.01	0	--	N. S.	N. S.	**	N. S.	**	N. S.
Ottawa River	0	0.04	0.01	--	N. S.	*	N. S.	*	N. S.
Blanchard River	0	0.02	0.02	0	--	**	N. S.	**	N. S.
Portage River	0.29**	0.08	0.05**	0.16*	0.10**	--	N. S.	*	N. S.
Belle River	0.06	0	0	0.03	0.02	0.08	--	N. S.	N. S.
Grand River	0.13	0.06	0.05*	0.10*	0.08**	0.13*	0.05	--	N. S.
Ganargua Creek	0.11*	0	0	0.04	0.01	0	0.02	0.05	--

* significant difference between sampling sites. ** remains significant after sequential Bonferroni correction (Rice, 1989). N.S.=not significant

Table 4. Nested Cladistic Analysis (Templeton et al., 1995) significant results ($P < 0.05$) for *Etheostoma blennioides blennioides* and *E. b. pholidotum* and corresponding historical inferences following Posada and Templeton (2005).

Species	Clades	Significant subclades	D_c	D_n	Chain of inference	Historical process inferred
<i>E. b. blennioides</i>	2-1	1-3		(L) $P = 0.022$	1-2-11-17-4- No	Restricted gene flow with isolation by distance
	4-2	3-3 3-4 I-T	(S) $P=0.005$	(S) $P = 0.005$ (L) $P = 0.005$ (L) $P = 0.005$	1-2-3-4-No	Restricted gene flow with isolation by distance
<i>E. b. pholidotum</i>	1-1	Haplotype Cb 43 I-T	(L) $P=0.007$	(L) $P = 0.035$	1-2-3-5-6-7-8-No	Sampling design inadequate to discriminate between isolation by distance vs. long distance dispersal
	1-2	I-T	(L) $P=0.034$		1-2-3-4-No	Restricted gene flow with isolation by distance

Within clade (D_c) and within nested clade (D_n) values that were significantly small are denoted with an “S” and those that were significantly large are denoted with an “L”.

Table 5. Morphological characters and counts for Greenside Darter taxa, with tests for statistical differences between *Etheostoma blennioides* *blennioides* and *E. b. pholidotum*.

Character	<i>E. b. blennioides</i>		<i>E. b. pholidotum</i>		Meramec River		<i>E. b. newmanii</i>		MANOVA or χ^2 result
	Mean $\pm$ S.D.(range)	Miller's (1968) mean (range)	Mean $\pm$ S.D. (range)	Miller's (1968) mean (range)	Mean $\pm$ S.D. (range)	Miller's (1968) mean (range)	Mean $\pm$ S.D. (range)	Miller's (1968) mean (range)	
Lateral line	63.66 $\pm$ 3.99 (55–79)	65.42 (56–78)	57.77 $\pm$ 3.39 (48–66)	58.61 (51–67)	62.57 $\pm$ 2.77 (58–65)	61.85 (53–71)	71.57 $\pm$ 4.50 (66–79)	70.69 (59–82)	$P<0.0001$
Dorsal scales*	7.44 $\pm$ 0.62 (6–9)	8.50 (7–10)	6.52 $\pm$ 0.55 (5–8)	7.00 (6–8)	7.43 $\pm$ 0.51 (7–8)	NA	9.14 $\pm$ 0.69 (8–10)	NA	$P<0.0001$
Transverse scales	16.57 $\pm$ 1.70 (13–21)	17.74 (14–22)	14.54 $\pm$ 1.12 (11–18)	15.17 (13–18)	16.07 $\pm$ 1.33 (13–18)	16.36 (14–19)	19.00 $\pm$ 1.00 (18–21)	19.38 (16–23)	$P<0.0001$
Least caudal peduncle	19.50 $\pm$ 2.37 (12–24)	23.32 (19–28)	18.29 $\pm$ 2.08 (14–28)	21.10 (18–24)	19.71 $\pm$ 1.20 (18–22)	21.87 (17–25)	22.86 $\pm$ 1.57 (21–26)	26.32 (21–31)	$P<0.0001$
Overall count									$P<0.0001$
Ventral squamation	+/-	+/-	+/-	+	+	+	+/-	+	$P<0.0001$
Dorsal lip tip	+/-	+	+/-	+/-	+/-	+/-	+/-	+	$P=0.6697$ N.S.

*Described by Trautman (1981), NA= not available, += complete ventral squamation/dorsal lip tip presence, -=incomplete ventral squamation/dorsal lip tip absence

Table 6. Uncorrected p -distances between selected darter sister species based on cytochrome b sequences, with their GenBank accession numbers and publication sources.

Species	GenBank accession number	Hypothesized sister species	GenBank accession number	p -distance	Sister species relationship source
<i>Percina caprodes</i>	AY770841*	<i>P. suttkusi</i>	AF386551*	0.009	Near and Benard (2004)
<i>Etheostoma bellum</i>	AY374260 (Sloss et al., 2004)	<i>E. camurum</i>	AF045348 (Song et al., 1998)	0.015	Morrison et al. (2006)
<i>Etheostoma smithi</i>	AF412531*	<i>E. striatulum</i>	AF123042 (Porterfield et al., 1999)	0.028	Page et al. (2003)
<i>Etheostoma caeruleum</i>	AY374263*	<i>E. burri</i>	AY374262*	0.031	Sloss et al. (2004)
<i>Percina smithvanizi</i>	EF613215*	<i>P. kusha</i>	EF613218*	0.038	Williams et al. (2007)
<i>Etheostoma nianguae</i>	AY964693 (Switzer and Wood, unpublished)	<i>E. sagitta</i>	AY94696 (Switzer and Wood, unpublished)	0.050	Strange and Burr (1997)
<i>Ammocrypta bifascia</i>	AF183940 (Near et al., 2000)	<i>A. clara</i>	AF183941 (Near et al., 2000)	0.129	Lang and Mayden (2007)

*Published by same authors in GenBank

VII. Figures

Figure 1. Distribution of the four taxa belonging to the Greenside Darter *Etheostoma blennioides* complex across sampling sites based on their cytochrome *b* sequence identity (symbols) from this study in relation to their morphological identity (shading; adapted from Denoncourt, 1980, *sensu* Miller, 1968).

Figure 2. Morphological characters that vary among taxa of the Greenside Darter complex (fish drawing adapted with permission of artist Joseph Tomelleri). (a) Scale counts (A= lateral line, B= dorsal scale rows, C= transverse scale rows, and D= least caudal peduncle scale rows). (b) Dorsal lip tip presence (indicated by arrow) and (c) absence. (d) Complete ventral squamation and (e) incomplete (indicated by circle). Scale bar represents 2mm.

Figure 3. Neighbor-joining haplotype trees for the Greenside Darter complex showing percent (%) bootstrap support based on DNA sequences from the (a) mitochondrial (mt) DNA cytochrome *b* gene, (b) mtDNA control region, (c) nuclear DNA intron S7, and (d) combined mtDNA haplotype tree. Clade “A” = haplotypes from the Scioto River. * = high bootstrap support agreement for neighbor-joining, maximum parsimony, and likelihood analyses. Note: Maximum parsimony trees revealed similar topologies for (a) 32 most parsimonious trees of 760 steps, with 289/1,076 parsimony informative characters, CI = 0.62, RI = 0.84, RC = 0.52, (b) 758 trees, 194 steps, 72/702 parsimony informative characters, CI = 0.69, RI = 0.74, RC = 0.51, (c) 20,981 trees, 119 steps, 46/419 parsimony informative characters, CI = 0.72, RI = 0.81, RC = 0.58, and (d) 52 trees, 930 steps, 358/1,778 parsimony informative characters, CI = 0.639, RI = 0.822, RC = 0.525.

3a Cytochrome *b* Tree

3b Control Region Tree

3c Nuclear S7 Intron 1 Tree

3d Combined mtDNA Tree

a

b

Figure. 4. Cytochrome *b* haplotype frequency distribution among sites for (a) *Etheostoma blennioides blennioides* and (b) *E. b. pholidotum*.

Figure 5. Network analyses for (a) *Etheostoma blennioides blennioides* and (b) *E. b. pholidotum* mtDNA cytochrome *b* haplotypes from this study. Number within each circle is the haplotype identified in Figure 3a and Appendix B. Labeled clades are from Table 4. Circle sizes are proportional to the number of individuals per haplotype. Unfilled circles = hypothetical intermediate haplotypes.

Figure 6. Reconstructed Pliocene drainage maps overlaying the present-day drainage patterns (Mayden, 1988), showing the hypothetical distribution of Greenside Darter taxa during the late Pliocene and Pleistocene Epochs, based on present genetic results. (a) Widely distributed ancestral species within the Teays and Mississippi River basins during the Pliocene (~2.5 mya). (b) Divergence of *Etheostoma blennioides blennioides* (*E. b. b.*) from the ancestor of the other 3 taxa within the headwaters of the Teays River (~1.8 – 1.9 mya). (c) Divergence of *E. b. pholidotum* (*E. b. p.*), *E. b. newmanii* (*E. b. n.*), and the Meramec River clade (MC) (~1.3 mya), Orange line = Crowley's Ridge. Clade “?” =

Miller's *E. b. newmanii* race. (d) Post-glacial dispersal of *E. b. blennioides* (*E. b. b.*) and *E. b. pholidotum* (*E. b. p.*) (10 – 20,000 ya). LA= glacial Lake Agassiz, LC= glacial Lake Chicago.

VIII. Appendices

Appendix A. Combined mitochondrial DNA sequence haplotypes of the Greenside Darter complex per sampling site with GenBank accession numbers (<http://www.ncbi.nlm.nih.gov/>).

Taxa	Combined haplotype	River site	Cytochrome <i>b</i> haplotype, GenBank #	Control region haplotype, GenBank #
<i>E. b. blennioides</i>	CM15	Scioto	Cb 14, EU118853	Cr 3, EF587851
	CM16	Allegheny, Cuyahoga, Great Miami, Susquehanna	Cb 3, EF587848	Cr 3, EF587851
	CM17	Scioto	Cb 16, EU118855	Cr 8, EU118831
	CM18	"	Cb 39, EU118878	Cr 12, EU118835
	CM19	Allegheny	Cb 24, EU118863	Cr 3, EF587851
	CM20	Scioto	Cb 15, EU118854	Cr 8, EU118831
	CM21	"	Cb 3, EF587848	"
	CM22	Great Miami	Cb 28, EU118867	Cr 3, EF587851
	CM23	"	Cb 30, EU118869	"
	CM24	"	Cb 3, EF587848	Cr 9, EU118832
	CM25	"	Cb 27, EU118866	Cr 8, EU118831
	CM26	"	Cb 26, EU118865	Cr 3, EF587851
	CM27	"	Cb 3, EF587848	Cr 10, EU118833
	CM28	"	"	Cr 11, EU118834
	CM29	"	Cb 29, EU118868	Cr 3, EF587851
	CM30	"	Cb 25, EU118864	"
		Auglaize, Blanchard, Grand, Ottawa, Portage	Cb 1, EF587846	Cr 1, EF587849
	CM31			
	CM32	Blanchard	"	Cr 5, EU118828

Taxa	Combined haplotype	River site	Cytochrome <i>b</i> haplotype, GenBank #	Control region haplotype, GenBank #
<i>E. b. pholidotum</i>	CM33	Blanchard	Cb 23, EU118862	Cr 1, EF587849
	CM34	"	Cb 22, EU118861	"
	CM35	"	Cb 6, EU118845	Cr 7, EU118830
	CM36	"	Cb 20, EU118859	Cr 1, EF587849
	CM37	Blanchard, Ottawa	Cb 1, EF587846	Cr 7, EU118830
	CM38	Blanchard	Cb 19, EU118858	"
	CM39	"	Cb 17, EU118856	Cr 1, EF587849
	CM40	"	Cb 6, EU118845	Cr 4, EU118827
	CM41	"	Cb 21, EU118860	Cr 6, EU118829
	CM42	"	Cb 8, EU118847	Cr 7, EU118830
	CM43	"	Cb 18, EU118857	Cr 1, EF587849
	CM44	Grand	Cb 2, EF587847	"
	CM45	"	Cb 1, EF587846	Cr 2, EF587850
	CM46	Auglaize	Cb 4, EU118843	Cr 1, EF587849
Meramec River	CM6	Meramec	Cb 51, EU118890	Cr 14, EU118837
	CM7	"	Cb 48, EU118887	"
	CM8	"	Cb 47, EU118886	"
	CM9	"	Cb 53, EU118892	Cr 15, EU118838
	CM10	"	"	Cr 14, EU118837
	CM11	"	Cb 49, EU118888	"
	CM12	"	Cb 50, EU118889	Cr 13, EU118836
	CM13	"	Cb 52, EU118891	Cr 14, EU118837
<i>E. b. newmanii</i>	CM14	"	Cb 46, EU118885	"
	CM1	Obey	Cb 56, EU118895	Cr 18, EU118841
	CM2	"	Cb 57, EU118896	Cr 19, EU118842

Taxa	Combined haplotype	River site	Cytochrome <i>b</i> haplotype, GenBank #	Control region haplotype, GenBank #
<i>E. b. newmanii</i>	CM3	Rockcastle	Cb 54, EU118893	Cr 17, EU118840
	CM4	"	Cb 55, EU118894	"
	CM5	"	"	Cr 16, EU118839

Appendix B. Greenside Darter sequence haplotypes discerned with gene/region, taxa, haplotype number, sampling site, and GenBank accession number.

Gene/region	Taxa	Haplotype	<i>N</i> individuals	River site (<i>N</i>)	GenBank #
Cytochrome <i>b</i>	<i>E. b. blennioides</i>	Cb 3	86	Ganargua Creek (7), Allegheny (18), Cuyahoga (17), Great Miami (5), Hocking (14), Scioto (2), Susquehanna (23)	EF587848
		Cb 14	1	Scioto	EU118853
		Cb 15	1	"	EU118854
		Cb 16	11	"	EU118855
		Cb 24	1	Allegheny	EU118863
		Cb 25	1	Great Miami	EU118864
		Cb 26	1	"	EU118865
		Cb 27	1	"	EU118866
		Cb 28	1	"	EU118867
		Cb 29	1	"	EU118868
		Cb 30	1	"	EU118869
		Cb 31	1	Hocking	EU118870
		Cb 32	1	"	EU118871
		Cb 33	1	"	EU118872
		Cb 34	2	"	EU118873
		Cb 35	2	"	EU118874
		Cb 36	1	Allegheny	EU118875
		Cb 39	1	Scioto	EU118878
		Cb 40	1	"	EU118879
		Cb 41	1	"	EU118880

Gene/region	Taxa	Haplotype	<i>N</i> individuals	River site (<i>N</i>)	GenBank #
Cytochrome <i>b</i>	<i>E. b.</i> <i>pholidotum</i>	Cb 1	123	Ganargua Creek (11), Auglaize (9), Belle (8), Blanchard (9), Grand (25), Ottawa (11), Portage (37), Wabash, IL (4), Wabash, IN (9)	EF587846
		Cb 2	3	Grand	EF587847
		Cb 4	1	Auglaize	EU118843
		Cb 5	1	"	EU118844
		Cb 6	2	Auglaize (1), Blanchard (1)	EU118845
		Cb 7	1	Auglaize	EU118846
		Cb 8	4	Auglaize (1), Blanchard (2), Wabash, IL (1)	EU118847
		Cb 9	1	Auglaize	EU118848
		Cb 10	1	"	EU118849
		Cb 11	1	"	EU118850
		Cb 12	2	"	EU118851
		Cb 13	1	Belle	EU118852
		Cb 17	1	Blanchard	EU118856
		Cb 18	1	"	EU118857
		Cb 19	1	"	EU118858
		Cb 20	1	"	EU118859
		Cb 21	1	"	EU118860
		Cb 22	3	Blanchard (2), Ottawa (1)	EU118861
		Cb 23	3	Blanchard	EU118862
		Cb 37	2	Ottawa	EU118876
		Cb 38	1	Portage	EU118877
		Cb 42	1	Wabash, IL	EU118881
		Cb 43	2	"	EU118882

Gene/region	Taxa	Haplotype	N individuals	River site (N)	GenBank #
Cytochrome <i>b</i>	<i>E. b.</i> <i>pholidotum</i>	Cb 44	1	Wabash, IN	EU118883
		Cb 45	1	"	EU118884
	Meramec R.	Cb 46	1	Meramec	EU118885
		Cb 47	1	"	EU118886
		Cb 48	1	"	EU118887
		Cb 49	1	"	EU118888
		Cb 50	1	"	EU118889
		Cb 51	4	"	EU118890
		Cb 52	1	"	EU118891
		Cb 53	4	"	EU118892
	<i>E. b.</i> <i>newmanii</i>	Cb 54	1	Rockcastle	EU118893
		Cb 55	4	"	EU118894
		Cb 56	1	Obey	EU118895
		Cb 57	1	"	EU118896
Control Region	<i>E. b.</i> <i>blennioides</i>	Cr 3	34	Allegheny (5), Cuyahoga (17), Great Miami (7), Scioto (1), Susquehanna (5)	EF587851
		Cr 8	6	Great Miami (1), Scioto (5)	EU118831
		Cr 9	1	Great Miami	EU118832
		Cr 10	1	"	EU118833
		Cr 11	1	"	EU118834
		Cr 12	1	Scioto	EU118835
	<i>E. b.</i> <i>pholidotum</i>	Cr 1	46	Auglaize (5), Blanchard (13), Grand (21), Ottawa (4), Portage (3)	EF587849
		Cr 2	3	Grand	EF587850
		Cr 4	1	Blanchard	EU118827

Gene/region	Taxa	Haplotype	N individuals	River site (N)	GenBank #
Control region	<i>E. b.</i>				
	<i>pholidotum</i>	Cr 5	2	Blanchard	EU118828
		Cr 6	1	"	EU118829
		Cr 7	5	Blanchard (4), Ottawa (1)	EU118830
	Meramec R.	Cr 13	1	Meramec	EU118836
		Cr 14	11	"	EU118837
		Cr 15	2	"	EU118838
	<i>E. b.</i>				
	<i>newmanii</i>	Cr 16	2	Rockcastle	EU118839
		Cr 17	3	"	EU118840
		Cr 18	1	Obey	EU118841
		Cr 19	1	"	EU118842
	<i>E. b.</i>				
	<i>blennioides</i>	1S7	3	Great Miami (2), Scioto (1)	EU118897
		2S7	3	Belle (1), Great Miami (1), Scioto (1)	EU118898
		3S7	1	Great Miami	EU118899
		4S7	5	Allegheny (2), Cuyahoga (1), Hocking (2)	EU118900
		5S7	7	Belle (1), Cuyahoga (1), Hocking (2), Scioto (3)	EU118901
		7S7	2	Susquehanna	EU118903
		12S7	1	Allegheny	EU118908
		13S7	1	"	EU118909
		14S7	1	Susquehanna	EU118910
		18S7	1	Great Miami	EU118914
		20S7	1	Hocking	EU118916
		21S7	1	"	EU118917

Gene/region	Taxa	Haplotype	<i>N</i> individuals	River site (<i>N</i>)	GenBank #
Nuclear S7 intron 1	<i>E. b.</i> <i>pholidotum</i>	6S7	19	Ganargua Creek (2), Auglaize (3), Belle (2), Blanchard (5), Grand (2), Ottawa (2), Portage (1), Wabash, IL (1), Wabash, IN (1)	EU118902
		15S7	1	Ganargua Creek	EU118911
		16S7	1	Blanchard	EU118912
		17S7	3	Auglaize (1), Blanchard (1), Ottawa (1)	EU118913
		19S7	1	Wabash, IN	EU118915
	Meramec R.	9S7	2	Meramec	EU118905
		11S7	5	Ganargua Creek (1), Meramec (4)	EU118907
		22S7	1	Meramec	EU118918
		23S7	4	"	EU118919
		24S7	2	"	EU118920
		25S7	1	"	EU118921
	<i>E. b.</i> <i>newmanii</i>	8S7	2	Obey (1), Rockcastle (1)	EU118904
		10S7	1	Rockcastle	EU118906
		26S7	1	"	EU118922