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Conservation Status Assessment of the Egg-mimic Darter (Percidae: *Etheostoma pseudovulatum*) Using a Multi-faceted Approach

Abstract

The imperiled Egg-mimic Darter (*Etheostoma pseudovulatum*) is a headwater-adapted fish restricted to an area less than 1000 km^2 in Tennessee. It is found in only six tributaries of the Duck River and the large, mainstem of this system may act as a barrier to dispersal, restricting population connectivity. The only status assessment of this species was over two decades ago; genetic diversity and the degree of population connectivity have never been evaluated. We conducted a conservation status assessment using a multi-faceted approach to better inform conservation management plans, including examining its current distribution, assessing habitat quality, estimating abundance, population size and haplotype diversity, and evaluating historical population connectivity. Surveys were conducted in spring and fall (2014) and population size was estimated using the Petersen mark-recapture method at a subset of localities, which were then regressed to obtain population estimates at all localities. Haplotype diversity and population connectivity were examined using the mitochondrial *ND2* gene. The Egg-mimic Darter was present at all localities and was relatively abundant, comparable to historical observations. Habitat quality did not appear to be substantially degraded. Overall haplotype and nucleotide diversity were low compared to widespread darters and comparable to other imperiled darters; however, demographic analyses indicated the species has remained stable over contemporary and historical timeframes. The Egg-mimic Darter has likely maintained gene flow historically at five of the six tributary systems, suggesting the mainstem Duck has not been a long-standing barrier to dispersal. One haplotype was shared across all tributary systems except Beaverdam Creek, which had a largely unique assemblage of haplotypes. Overall, the conservation status of the Egg-mimic Darter appears to be stable. However, we recommend regular monitoring with special consideration given to smaller tributary systems and the genetically distinct Beaverdam Creek population. Even though there was evidence of historical population connectivity, the risk of local extirpation remains, considering the small population sizes in several tributary systems. We also recommend assessments of contemporary genetic structure and population connectivity.

Keywords

darters, mitochondrial DNA, population size, population connectivity, conservation

Cover Page Footnote

Historical locality data of the Egg-mimic Darter was obtained from the Illinois Natural History Survey, University of Michigan Museum of Zoology, University of Tennessee – Etnier Ichthyological Research Collection, Yale University Peabody Museum, Auburn University Museum of Natural History, Cornell University Museum of Vertebrates, University of Kansas Biodiversity Institute – Specimens, Harvard University Museum of Comparative Zoology, North Carolina State Museum of Natural Sciences, Ohio State University – Fish Division, Tulane University Museum of Natural History – Royal D. Suttkus Fish Collection, University of Alabama Ichthyological Collection, National Museum of Natural History, and the Smithsonian Institution (accessed through the Fishnet2 Portal, www.fishnet2.net, 9/15/2013). Additional museums surveyed include Southern Illinois University Carbondale, University of Florida, University of Southern Mississippi, Field Museum of Natural History, American Museum of Natural History, Academy of Natural Sciences of Drexel University, Mississippi Museum of Natural Science, and Austin Peay State

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INTRODUCTION

The Southeastern United States has the highest diversity of freshwater fishes in North America (Burr and Mayden 1992; Warren et al. 1997, 2000). Unfortunately, an estimated 28% of fishes from this region are recognized as extinct, endangered, threatened, or vulnerable (Warren et al. 1997, 2000; Jelks et al. 2008). In 2010, the Center for Biological Diversity submitted a petition to the United States Fish and Wildlife Service (USFWS) to review over 400 southeastern aquatic and riparian species for federal protection under the Endangered Species Act (ESA; USFWS 2011), including the Egg-mimic Darter (*Etheostoma pseudovulatum*; Figure 1). Currently, the Egg-mimic Darter is recognized as endangered by the state of Tennessee, vulnerable by the American Fisheries Society and the International Union for Conservation of Nature, and "globally critically imperiled" by NatureServe (Jelks et al. 2008; NatureServe 2013, 2020; TWRA 2018).

Figure 1. Nuptial male Egg-mimic Darter from Mill Creek (photographed by Z. L. Wolf 5/15/2014).

The small native range of the Egg-mimic Darter was the primary reason for its priority conservation status. Distributional surveys documented the species at 38 localities in 6 tributary systems of the Duck River (Tennessee River Drainage) with a geographical footprint of 958 km^2 on the Western Highland Rim physiographic region (Ceas and Page 1995; Etnier and Starnes 1993; Page et al. 1992; Figure 2). At the time of these surveys, the status of the Egg-mimic Darter was classified as relatively stable. However, the Egg-mimic Darter is a benthic specialist dependent upon clean, loose substrates for refuge, feeding, and spawning, and like other benthic species, it may be particularly sensitive to excessive siltation that can alter the structural composition of these habitats (Ceas and Page 1995; Helfman et al. 2009). The threat of habitat degradation is exacerbated by a limited geographic range, making the species vulnerable to small-scale disturbances that could result in local extirpations, especially in the smallest tributary systems of the Duck River (Ceas and Page 1995). Additionally, variation in morphology has been noted among the Piney River, Beaverdam Creek, and Little Piney Creek populations indicating potential long-standing genetic isolation among these tributary systems (Page et al. 1992). Despite these concerns, few studies have focused on the Egg-mimic Darter, thereby limiting information available for making decisions on the conservation of the species.

Traditional conservation status assessments primarily consist of measuring occupancy relative to historical distribution and estimating population size. Although these aspects are central to conservation management, the lack of other useful information (such as the availability of

suitable habitat, genetic diversity, and population connectivity) leaves conservation managers unable to optimize resources efficiently (FMCS 2016). Contemporary conservation biology places increasing importance on broad-based approaches to conservation assessments that incorporate various factors that affect the persistence of a species (Ferreira-Rodríguez et al. 2019). For example, one of the criteria that the USFWS uses to determine the listing of a species is, "the present or threatened destruction, modification, or curtailment of its habitat or range" (ESA, Section 4). Identifying characteristics of suitable habitat for a species and its availability is key to effective conservation management (Warren et al. 1997; Albanese et al. 2013; Compton and Taylor 2013). By incorporating habitat assessments into traditional conservation assessments, researchers can provide resource managers with more robust data to enhance conservation strategies.

Similarly, an understanding of the patterns of genetic variation in a species is a valuable tool in developing conservation management plans for imperiled taxa (Powers et al. 2004; George et al. 2006, 2009; Turner and Robison 2006; Fluker et al. 2011). For example, genetic data can help inform conservation strategies by identifying populations with low genetic diversity (George et al. 2006) or recognizing distinctive populations that should be managed independently (Blanton and Jenkins 2008). For the Egg-mimic Darter, variation in morphology among tributary systems noted by Page et al. (1992) suggests populations from different tributaries may be isolated by past vicariant events or by the larger Duck River mainstem, which lacks an abundance of habitat typical of Egg-mimic Darter; collections of the mainstem have failed to detect Egg-mimic Darter occurrence, despite detection of other darter species, based on museum records (Page et al. 1992; Etnier and Starnes 1993). Large rivers can act as barriers or filters to dispersal restricting gene flow among populations of headwater-stream adapted fishes (Starnes and Etnier 1986; Turner and Trexler 1998; Powers et al. 2004; George et al. 2006; Turner and Robison 2006; Hollingsworth and Near 2009; Fluker et al. 2011; Sterling et al. 2012). Like other upland, small stream-adapted fishes, the Egg-mimic Darter has strict habitat requirements (especially for breeding) that are not available or are rare in large rivers, potentially limiting successful dispersal through these environments (Starnes and Etnier 1986; Sterling et al. 2012). Additionally, this species has life history factors known to limit dispersal (Turner and Trexler 1998; Turner and Robison 2006; Fluker et al. 2011, 2014). For example, larval drift is a major contributor to downstream dispersal for some fishes (Ross 2013). However, the Egg-mimic Darter has benthic larvae that do not drift far from nest sites (Simon and Wallus 2005). This has the potential to impact both gene flow and recolonization of sites where local populations have been extirpated. If the Duck River limits Egg-mimic Darter dispersal, the six tributary system populations could be isolated and require population-specific management practices.

Plans for managing at-risk species are most effective when equipped with a complete knowledge of the species, including distribution, abundance, habitat quality, and genetic diversity (Warren et al. 1997; George et al. 2006). Therefore, the objective of this study was to evaluate the current conservation status of the Egg-mimic Darter by: 1) describing its current distribution in relation to its historic range; 2) estimating abundance at survey sites to obtain a measure of local population variability and approximate population size; 3) providing an assessment of habitat quality at survey localities; and 4) describing phylogeographic structure and haplotype diversity across the species' range. This will allow us to further evaluate patterns in historical population connectivity and assess whether the Duck River mainstem has served as a long-standing barrier for populations distributed among different tributary systems.

METHODS

Localities Examined

Localities for the Egg-mimic Darter were identified from published literature (Page et al. 1992; Ceas and Page 1995) and museum records (see Cover Page Footnote). Thirty-eight historical localities were identified, 24 of which were sampled. Excluded sites lacked accessibility or were proximate to other sampled localities. A previously unidentified locality on the mainstem Piney River was included in this study, bringing the total to 25 localities examined (Figure 2; Appendix A).

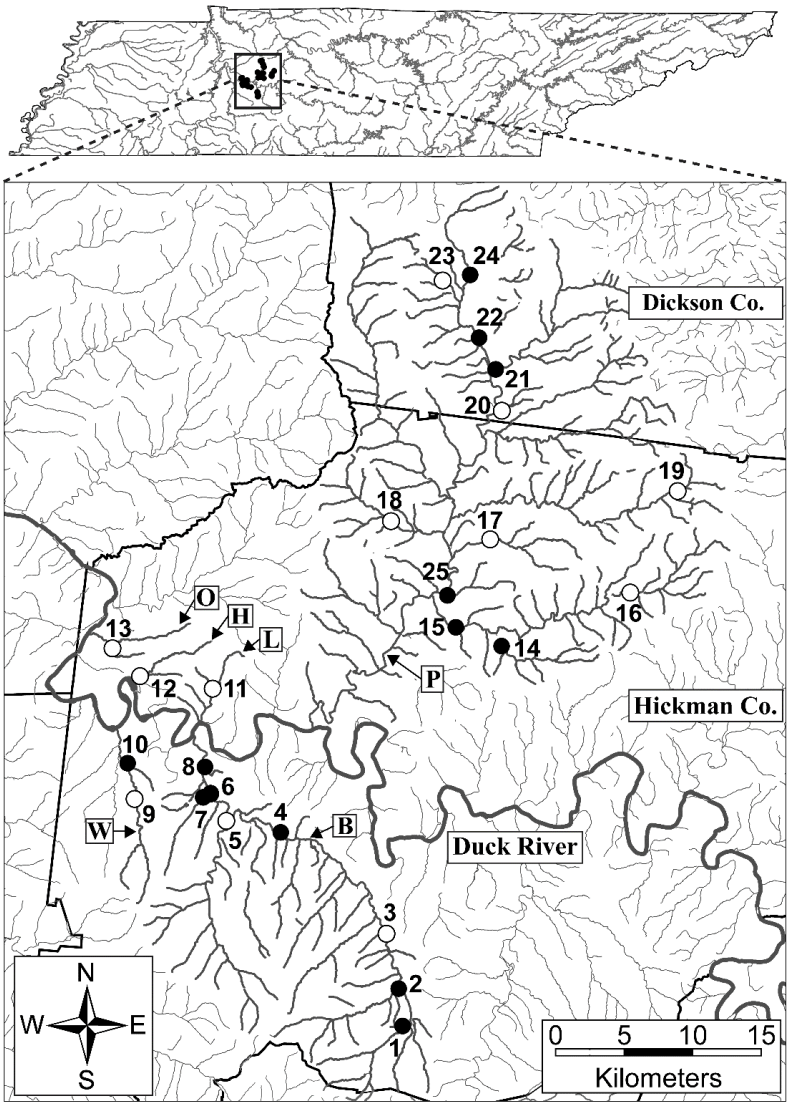

Figure 2. Localities sampled for the Egg-mimic Darter. Numbers are locality identifiers that correspond to specific location information in Appendix A. White circles represent localities where presence, abundance, and population size were estimated. Solid black circles represent localities where only Egg-mimic Darter presence was measured. Letters refer to tributary systems of the Duck River: P = Piney River; O = Only Creek; H = Happy Hollow Creek; L = Little Piney Creek; W = East Fork Wolf Creek; and B = Beaverdam Creek. Tissues for genetic samples were obtained from localities 1, 5, 7, 8, 9, 11, 12, 13, 16, 17, 18, 19, 20, and 23.

Distribution and Abundance

Collections were conducted during the spring (i.e., spawning season) and fall (i.e., non-spawning season) of 2014 to assess seasonal variation and deliver robust estimates of abundance and population size. At each locality, a 75-m stream reach was selected to include all observable habitat types and isolated using block-nets at the upstream and downstream ends of the reach.

A single pass was conducted at all 25 surveyed localities. If no Egg-mimic Darters were collected during the first pass, subsequent collections were made until at least one was collected or a maximum of three passes were made. Fish were collected using traditional kick-seining methods, using a 3.05 x 1.37 m wide, 0.32 cm mesh seine. To equalize sampling effort at each locality, the number of kick-sets was determined by the mean stream wetted-width (modified from Abernathy and Mattingly 2011) with 20, 40, 60, and 80 kick-sets conducted if mean wetted-width was < 6 m, 6 – 11 m, 11 – 16 m, and > 16 m, respectively. Mean wetted-width was calculated from three transects at the upstream, mid, and downstream portions of the reach. The location of each kick-set within the stream was chosen opportunistically to proportionally sample all available habitat types.

Surveys began at the downstream end of the reach and continued upstream until the minimum number of kick-sets was reached. Any Egg-mimic Darters collected were counted, measured for total length (TL), and sexed, if possible (sex was determined using visual cues, such as gravidity in females and coloration and egg mimics in males, and could only be completed in spring). Prior to release, all captured individuals were anesthetized using tricaine methane sulfonate (MS-222) and a portion of the caudal fin was removed and preserved in 95% non-denatured ethanol for DNA preservation and subsequent genetic analysis. Individuals were allowed to fully recover from anesthesia and fin biopsy and then released haphazardly within the reach. Voucher specimens were retained and accessioned into the Ichthyological Collection of the David H. Snyder Museum of Zoology at Austin Peay State University (APSU). Collection and euthanasia methods were reviewed and approved by the APSU Institutional Animal Care and Use Committee (IACUC Protocol #14.003).

Mark-recapture surveys were conducted at a subset of 12 of the 25 localities in both seasons (Figure 2). These sites were selected to cover the geographical extent of the range of the Egg-mimic Darter and included at least one locality from each occupied tributary system. Block nets were placed at the upstream and downstream ends of the targeted reach to create a closed system. At these sites, a second pass was made on the same day as the first pass. The second pass was conducted a minimum of 30 minutes after all individuals captured in the first pass were returned to the reach. This interval allowed individuals to disperse from the point of release, prior to the second pass sampling (pers obs.). Preliminary trials with longer time periods between samples resulted in failure of one or both block nets (due to debris accumulation) violating assumptions of a closed system (Krebs 1999). The sampling methodology followed that of the first pass except Egg-mimic Darters were identified as marked or unmarked based on the caudal fin clip. This two-pass process was practiced in both spring and fall sample events. However, during the fall, young-of-year individuals (YOY; approx. less than 42 mm TL) were not included in our population estimates because these individuals may violate the assumption of a closed system in two ways. First, fin-clipping fish of this size could potentially increase the risk of mortality (Krebs 1999). Second, these individuals were small enough to potentially move through the block nets.

Population estimates (POP_E) were calculated using the Chapman modification of the Petersen mark-recapture method from the mark-recapture surveys (Seber 1982; Krebs 1999). Due to the low number of recaptures during this study, 95% confidence intervals of POP_E were constructed using the Poisson distribution (Krebs 1999). To calculate predicted abundance (POP_P) based on first-pass capture rate (i.e., number of individuals captured in the first pass of collection), estimates from the mark-recapture surveys were used to construct a model by regressing $\log_{10}$ (POP_E) onto $\log_{10}$ (first-pass captures). Although these two variables are autocorrelated, this technique has been used in other studies to obtain range-wide population estimates based on a limited number of mark-recapture surveys within that range (e.g., Hall 1986, Black et al. 2013, Johansen et al. 2016). Mark-recapture surveys where the recapture rate was 0 were excluded from model construction. Prior to constructing the regression model, an equality-of-slopes method was used to assess if season affected the relationship between first-pass captures and POP_E (Black et al. 2013). Data were $\log_{10}$-transformed and the interaction between season and first-pass captures was evaluated. The interaction term was not significant ($P = 0.59$) indicating that there was no difference between slopes for the two seasons. Therefore, data from both seasons were pooled to build a combined model.

POP_P values were used to calculate local densities and capture efficiency for all collections made during this study. Density (individuals per 100 m^2) was calculated by dividing the POP_P by the wetted-surface area (m^2; mean wetted-width * 75) and multiplying that value by 100. Capture efficiency was calculated by dividing the first-pass captures by POP_P. Due to violations of assumptions for parametric tests, nonparametric univariate tests were performed to assess seasonal and geographic variation in POP_P, density, and capture efficiency. Seasonal variation was assessed using a Wilcoxon signed rank test and geographical variation was assessed with Kruskal-Wallis and Wilcoxon rank sums tests. Statistics for abundance and habitat quality were performed with JMP v.10 (SAS Institute Inc. 2012).

Habitat Quality

Following each survey, at least two people assessed reach-scale habitat quality using standardized habitat scoring methods from Barbour et al. (1999). Specific conductance (mS/cm) and water temperature (^oC) were measured using a YSI meter at the mid-point of the reach. Habitat scores were analyzed for variation among drainages with a Kruskal-Wallis test followed by Wilcoxon rank sums tests for each pair of drainages. The effect of stream size on local abundance and capture efficiency was assessed using a Kruskal-Wallis test followed by Wilcoxon rank sums tests for each pair of drainages. Strahler stream order and link magnitude were collected using the U.S. Geological Survey National Hydrology Dataset (available at https://nhd.usgs.gov/index.html) in ArcMap v.10.2 (ESRI 2013). Spearman's rank correlation coefficient was used to test significant correlations between habitat metrics and POP_P and capture efficiency. Nonparametric tests were employed as they are more appropriate for ordinal data.

Haplotype Diversity, Demographic History, and Phylogeographic Relationships

Whole genomic DNA was extracted from caudal fin clips of 59 individuals collected during the spring surveys using a GeneJET Genomic DNA Purification kit (Thermo-Scientific Inc.) or a 5% Chelex solution with 2 µL Proteinase K with overnight digestion. These individuals represented 14 localities from across the geographic range of the Egg-mimic Darter (Figure 2): six localities from Piney River (sites 16-20 and 23), four localities from Beaverdam Creek (1, 5, 7,

and 8), and one locality each from East Fork Wolf Creek (9), Happy Hollow Creek (12), Little Piney Creek (11), and Only Creek (13).

The mitochondrial NADH dehydrogenase subunit 2 gene (*ND2*) was amplified using previously published primers (Kocher et al. 1995): GLN (5'-CTACCTGAAGAGATCAAAAC-3') and ASN (5'-CGCGTTTAGCTGTTAAC TAA-3'); and polymerase chain reaction (PCR) with the following cycling protocol: 95° C for 1 min; 32 cycles of 95° C for 30 sec, 56° C for 1 min, then 72° C for 30 sec; followed by 72° C for 7 min. PCR products were sent to the Interdisciplinary Center for Biotechnology Research at the University of Florida for Sanger sequencing. Sequence data were edited and consensus sequences were made using CodonCode Aligner v.8.01 (CodonCode Corporation 2018). *ND2* sequences of all individuals examined were deposited in GenBank (accession numbers MK426222-MK426280; Appendix B).

Haplotype and nucleotide diversity and average sequence divergence within and between clades identified from our phylogeographic analyses were calculated using DnaSP v.5.10.1 (Librado and Rozas 2009). We also used this program to test for historic population expansions with Fu and Li's D* and F* (Fu and Li 1993; Fu 1997) and Tajimas's D (Tajima 1989) using 10,000 coalescent simulations. Because these tests assume a single population, tests were conducted independently for each of the two major clades identified from the phylogeographic analyses. We examined evidence for changes in relative, ancestral effective population size (N_e) using three independent runs of 30 million Markov chain Monte Carlo (MCMC) generations and the coalescent Bayesian skyline tree prior (Drummond et al. 2005) implemented in BEAST v.2.6.3 (Bouckaert et al. 2019) using all 59 Egg-mimic Darter *ND2* sequences. Because population structure can bias effective population size estimates under this tree prior (Heller et al. 2013), we also estimated changes in N_e separately for each of the two major clades of Egg-mimic Darter recovered in our phylogeographic analyses. All runs included 5 dimensions, the HKY model of nucleotide substitution and a relaxed uncorrelated lognormal clock to allow for rate variation; the clock rate was calibrated with the *ND2* substitution rate of 0.00929 s/s/my estimated for other darters using external Centrarchid fossil calibrations points of Near et al. (2005) (Fluker et al. 2014). Tracer v.1.71 (Rambaut et al. 2018) was used to ensure runs converged, all parameter ESS values were > 250, and to construct Bayesian skyline plots with a stepwise constant skyline variant from the resulting combined log file of the independent MCMC runs.

Phylogeographic structure was examined using statistical parsimony and Bayesian phylogeographic analyses. TCS v.1.21 (Clement et al. 2000) was used to build a statistical parsimony haplotype network from resulting sequences of all 59 individuals of Egg-mimic Darter using a 95% connection limit. TCS was also used to identify unique haplotypes. The resulting unique haplotypes were subjected to a partitioned mixed-model Bayesian analysis. Sequences for these haplotypes and outgroups were partitioned by codon position using Mesquite v.3.40 (Maddison and Maddison 2014). Each partition was analyzed with jModelTest (Posada 2008) and the Akaike Information Criterion (AIC) was used to determine the best model of nucleotide evolution for each codon position. These models were then incorporated into the partitioned, mix-model Bayesian analysis using MrBayes v.3.2 (Huelsenbeck and Ronquist 2001). Two runs of 12,000,000 generations each were conducted, and the resulting post-burnin trees from each run were examined for congruence in topology. The resulting maximum clade credibility tree was used to display haplotype and population relationships. *ND2* sequences of ten closely related darter

species were included in the Bayesian analyses as outgroups (Relict Darter (*Etheostoma chienense*), Crown Darter (*E. corona*), Fringed Darter (*E. crossopterum*), Fantail Darter (*E. flabellare*), Barrens Darter (*E. forbesi*), Lollipop Darter (*E. neopterum*), Blackfin Darter (*E. nigripinne*), Guardian Darter (*E. oophylax*), Striped Darter (*E. virgatum*), and Johnny Darter (*E. nigrum*); GenBank accession numbers JQ088521, JQ088530, JQ088531, JQ088540, JQ088543, JQ088558, JQ088560, JQ088565, JQ088598, and JQ088561, respectively). Lastly, the Bayesian skyline coalescent and coalescent constant population size tree models with an uncorrelated relaxed lognormal clock, calibrated with the estimated *ND2* substitution rate of 0.00929 s/s/my (Fluker et al. 2014), a HKY substitution model, and all 59 *ND2* sequences were used to estimate mean node ages for Egg-mimic Darters in BEAST v.2.6.3. Three independent runs of 30 million MCMC generations for each of the two coalescence-based tree models were conducted. Tracer v.1.7.1 was used to examine parameter convergence across runs and ensure ESS values were > 250. LogCombiner v.2.6.3 (Drummond and Rambaut 2007) was used to combine trees of independent run files for each analysis type. The combined tree files were annotated with mean node ages and associated values in TreeAnnotator v.2.6.3 (Drummond and Rambaut 2007) and reconstructed using FigTree v.1.4.3 (available: http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Distribution and Abundance

The Egg-mimic Darter was present at all 25 localities sampled in the spring and fall with a total of 795 individuals collected. The demographic breakdown of the 484 individuals in spring collections was 62 males, 188 females, and 234 juveniles. An additional 311 individuals were collected in the fall (these were not sub-divided based on difficulty of sexing non-reproductive individuals). Three age classes were observed based on TL in both spring (approx. 25–50, 50–70, and 70+ mm) and fall (approx. 20–40, 40–65, and 65+ mm).

First-pass captures varied among sites and seasons, ranging from 1–50 (mean ± SE = 10.33 ± 1.59) individuals per 75-m reach (Table 1). POP_E ranged from 5–258 individuals per 75-m reach (mean ± SE = 69.11 ± 16.67, excluding estimates with no recaptures). The combined regression model, $\log_{10} y = 0.4315 + (1.1726 * \log_{10} x)$, where y is the POP_E (individuals per 75-m) and x is the first-pass individuals captured, was significant ($P < 0.0001$) and explained 81% of the variation in population estimates (Figure 3). POP_P mean ± SE (individuals per 75-m reach) was 67.80 ± 14.60 (range = 3–265) in spring and 23.16 ± 4.93 (range = 3–91) in fall. Density mean ± SE (individuals per 100 m^2) was 13.86 ± 2.72 (range = 0.16–52.0) in spring and 7.47 ± 1.88 (range = 0.11–42.53) in fall. Both POP_P ($|S| = -103.50$; $P < 0.001$) and density ($|S| = -80.00$; $P < 0.01$) were significantly higher in spring. POP_P and density did not differ significantly among the tributary systems, but the highest estimates were primarily from localities within the largest system (Piney River), while the other five tributary systems generally had moderate-to-low estimates. Capture efficiency mean ± SE was 25% ± 0.95 in spring, 29% ± 0.90 in fall, and 27% ± 0.71 in total. This indicates that our survey crews collected an average of 27% of the Egg-mimic Darters present in an occupied reach. Seasonal variation in capture efficiency was noted and was significantly higher in fall ($|S| = 89.50$, $P < 0.005$).

Table 1. First-pass captures, recaptures and total second-pass captures, population estimate (POP_E) with 95% confidence interval, predicted abundance (POP_P), density, and capture efficiency for the Egg-mimic Darter from 25, 75-m reaches surveyed in 2014. POP_P was calculated by the regression model built using the mark-recapture data, density was calculated by dividing POP_P by the wetted-surface area and multiplying by 100, and capture efficiency was calculated by dividing the first-pass capture rate by POP_P.

Site	Season	First-pass captures (recaptures/total second-pass captures) (fish/75 m)	POP_E (95% CI) (fish/75 m)	POP_P (fish/75 m)	Density (fish/100 m^2)	Capture Efficiency
1	Spring	11	--	45	12.06	24
	Fall	7	--	26	10.82	26
2	Spring	10	--	40	8.64	25
	Fall	2	--	6	1.98	33
3	Spring	3 (0/1)	7 (0.9–7.0)	10	1.38	31
	Fall	9 (1/2)	14 (3.7–27.5)	36	9.66	25
4	Spring	20	--	91	8.23	22
	Fall	5	--	18	2.57	28
5	Spring	14 (0/9)	149 (34.0–149.0)	60	9.03	23
	Fall	6 (1/4)	16.5 (4.5–32.3)	22	5.32	27
6	Spring	33	--	163	9.26	20
7	Spring	8	--	31	12.89	26
	Fall	4	--	14	10.96	29
8	Spring	10	--	40	5.95	25
	Fall	3	--	10	1.37	31
9	Spring	7 (1/8)	35 (10.4–67.5)	26	6.05	26
	Fall	3 (0/6)	27 (4.5–27.0)	10	3.19	31
10	Spring	5	--	18	3.62	28
	Fall	2	--	6	1.82	33
11	Spring	5 (1/15)	47 (14.1–90.3)	18	4.66	28
	Fall	2 (1/3)	5 (1.0–10.4)	6	1.98	33
12	Spring	6 (1/3)	13 (3.4–25.6)	22	8.02	27
	Fall	6 (0/8)	62 (13.7–62)	22	11.64	27
13	Spring	10 (0/2)	32 (6.7–32.0)	40	35.72	25
	Fall	7 (1/3)	15 (4.1–29.4)	26	16.56	26
14	Spring	13	--	55	15.09	24
	Fall	3	--	10	3.41	31
15	Spring	8	--	31	3.63	26
	Fall	1	--	3	0.29	37
16	Spring	44 (8/32)	164 (92.3–345.6)	228	29.65	19
	Fall	20 (2/17)	125 (48.2–278.0)	91	15.65	22
17	Spring	17 (1/6)	62 (18.9–118.9)	75	25.6	23
	Fall	15 (3/16)	67 (28.9–148.6)	65	14.54	23
18	Spring	36 (4/34)	258 (121.2–546.3)	180	28.99	20
	Fall	11 (1/14)	89 (27.5–170.3)	45	6.84	24

Table 1 cont.

Site	Season	First-pass captures (recaptures/total second-pass captures) (fish/75 m)	POP_E (95% CI) (fish/75 m)	POP_P (fish/75 m)	Density (fish/100 m^2)	Capture Efficiency
19	Spring	24 (4/28)	144 (67.4–305.4)	112	32.52	21
	Fall	18 (6/19)	53 (26.5–360.6)	80	42.53	22
20	Spring	1 (0/3)	7 (0.9–7.0)	3	0.16	37
	Fall	2 (1/5)	8 (1.8–16.1)	6	0.37	33
21	Spring	1	--	3	0.38	37
	Fall	1	--	3	0.42	37
22	Spring	7	--	26	3.79	26
	Fall	3	--	10	1.59	31
23	Spring	11 (1/9)	59 (18.0–113.2)	45	15.36	24
	Fall	6 (0/4)	34 (7.2–34.0)	22	12.27	27
24	Spring	50	--	265	52.02	19
	Fall	5	--	18	3.29	28
25	Fall	1	--	3	0.11	37

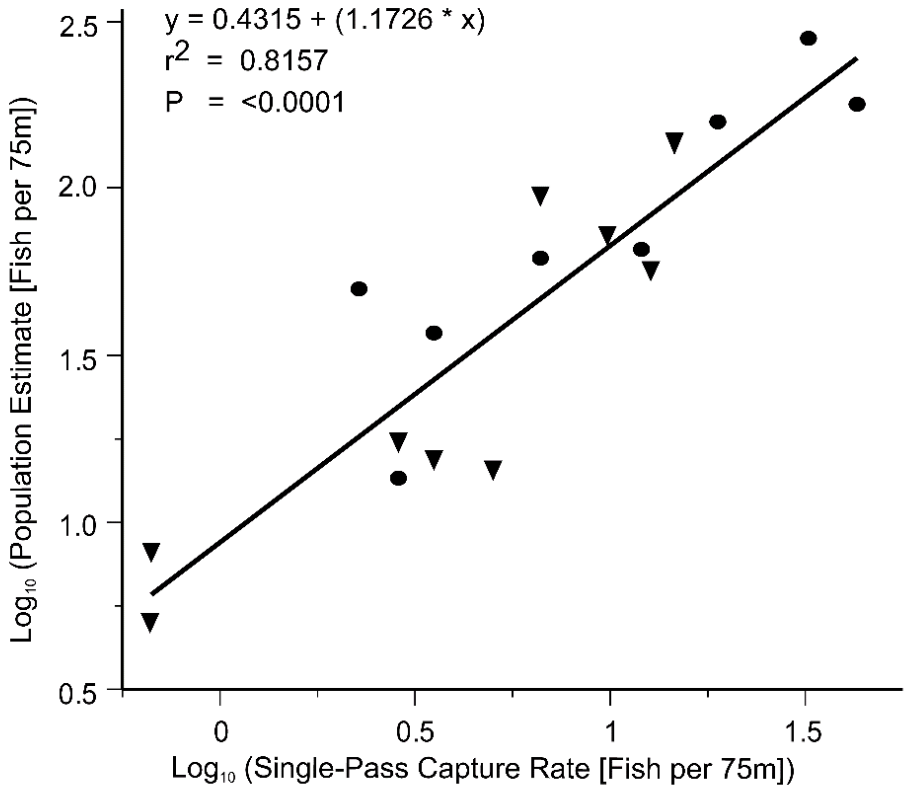

Figure 3. Combined regression model constructed by regressing log10 (POP_E) onto log10 (first-pass captures). This model was constructed using mark-recapture data seasonally collected (circles: spring; triangles: fall) from 12, 75-m reaches across the range of the Egg-mimic Darter in 2014 (Table 1). Note that 7 of the 24 abundance estimates were excluded from this regression due to a recapture rate of zero.

Habitat Quality

Sites ranged in size from 1st to 4th order streams with link magnitudes ranging from 1–52 upstream first-order tributaries. The mean ± SE total habitat score (THS) in the spring was 129.6 ± 3.55 (range = 97 – 177.5) and the mean ± SE THS in the fall was 148.7 ± 3.13 (range = 102.5 – 183.5). There were no significant differences in physiochemical variables measured (i.e., depth, flow, wetted-width, temperature, and specific conductance) or THS across drainages.

Stream size measured as Strahler stream order or link magnitude did not have a significant effect on POP_P or density. However, mean ± SE POP_P was highest in 3rd order streams for both the spring (99.36 ± 33.29 individuals per 75-m) and the fall (27 ± 9.21 individuals per 75-m). Mean ± SE predicted densities were highest in 1st order streams in both spring (18.0 ± 6.10 individuals per 100 m^2) and fall (12.86 ± 1.26 individuals per 100 m^2).

In spring, POP_P was negatively correlated with Riffle Frequency Score (r = -0.4693; P = 0.0207). In fall, POP_P was positively correlated with mean depth (r = 0.6147; P = 0.0442). Capture efficiency mirrored the results of POP_P: significant positive correlation with Riffle Frequency Score (r = 0.4693; P = 0.0207) in spring; and significant negative correlation with mean depth (r = -0.6147; P = 0.0442) in fall. All other habitat metrics had no significant correlation with POP_P or capture efficiency.

Haplotype Diversity and Demographic Analyses

Eleven haplotypes were recovered from 59 individuals of Egg-mimic Darter (Figure 4A). In total, haplotype diversity (Hd) was 0.624 and nucleotide diversity (π) was 0.0054 (Table 2). The Bayesian skyline plots showed an increase in historical effective population size (N_e) beginning approximately 20,000 years before 2014 when examining all individuals (n = 59) of Egg-mimic Darter (Figure 5A). Within each of the two major clades recovered in the Bayesian analysis (Figure 4B), N_e has remained fairly constant over the last several thousand years, showing a slight increasing trend over time in both (Figure 5B and 5C). All measures of population expansion were negative and significant ($P < 0.05$) for the Beaverdam Creek Clade (Tajima's D = -1.84; F* = -2.41; D* = -2.47) indicating deviation from a constant population size. Although two metrics for the Piney River Clade were negative, none were significant ($P > 0.05$) (Tajima's D = 0.00; F* = -1.59; D* = -1.31) indicating this clade has not undergone population expansion and does not deviate from expectations of neutral evolution.

Phylogeographic Relationships

Results from the 95% statistical parsimony analysis represented by the haplotype network (Figure 4A) showed that one haplotype (H1) was shared among 34 individuals found in all tributary systems except Beaverdam Creek. Two divergent haplotype clusters that differed by 13 or more nucleotide substitutions were recovered. One of the clusters consisted of individuals from Piney River, Little Piney Creek, Happy Hollow Creek, Only Creek, and East Fork Wolf Creek; the other consisted of individuals from Beaverdam Creek and 2 of the 10 individuals examined from Little Piney Creek.

Table 2. Summary statistics of variation in the mitochondrial *ND2* gene for Egg-mimic Darters sampled in this study (n = 59), overall and by tributary system, and summary statistics of variation in mitochondrial genes for other benthic species.

Taxa	Sample size (n)	Haplotype diversity (Hd)	Nucleotide diversity (π)
Egg-mimic Darter	59	0.624	0.0054
Piney River	19	0.45	0.0006
Beaverdam Creek	15	0.371	0.0004
Little Piney Creek	10	0.533	0.0046
Happy Hollow Creek	5	0.6	0.0005
Only Creek	5	0	0
E. Fk. Wolf Creek	5	0	0
Fantail Darter (*ND2*; Blanton 2007)	--	0.995	0.037
Okaloosa Darter (cyt *b*; Austin et al. 2011)	--	0.38-0.67	0.00045-0.00256
Yazoo Darter (cyt *b*; Sterling et al. 2020)	--	0.11-0.66	--
Rainbow Darter (cyt *b*; Haponski et al. 2009)	--	0.791	--
Rainbow Darter (cyt *b*; Ray et al. 2006)	--	--	0.032
Blotchside Logperch (combination of cyt *b* and *ND2*; George et al. 2006)	--	0.891	0.013
Roanoke Logperch (combination of cyt *b* and *ND2*; George et al. 2010)	--	0.919	0.00367
Conasauga Logperch (combination of cyt *b* and *ND2*; George et al. 2010)	--	0.889	0.00485

Haplotypes sorted into two well-supported clades in the Bayesian phylogenetic analysis (Figure 4B) that were consistent with clusters recovered by the haplotype network. One clade contained all individuals from Beaverdam Creek and the same two individuals from Little Piney Creek identified above (referred to as the Beaverdam Creek clade), while the other clade contained all individuals from Piney River, East Fork Wolf Creek, Only Creek, Happy Hollow Creek, and eight individuals from Little Piney Creek (referred to as the Piney River clade). Average sequence divergence was 0.040% within the Beaverdam Creek clade, 0.041% within the Piney River clade, and 1.24% between the two clades.

Bayesian skyline and coalescent constant-population analyses recovered the same set of phylogeographic relationships and similar node age estimates (Table 3), thus, only the tree from the Bayesian coalescent, constant-population model is shown (Figure 6). Phylogeographic relationships recovered from these analyses were consistent with those from the TCS and Bayesian

phylogenetic analysis of unique haplotypes. Divergence from the most recent common ancestor (MRCA) of the Piney River clade and Beaverdam Creek clade was relatively old, having occurred approximately 570,000 years ago (95% HPD 363,300 - 964,100 ya; Table 3; Figure 6). In fact, age estimates for all moderately (> 0.75 pp) or strongly supported clades (> 0.95) had mean age estimates from the Pleistocene, including evidence for potential long-standing isolation of Mill Creek (Piney River system) from other members of the Piney River Clade. However, sample sizes for Mill Creek were low (n = 3) and this result should be interpreted with caution.

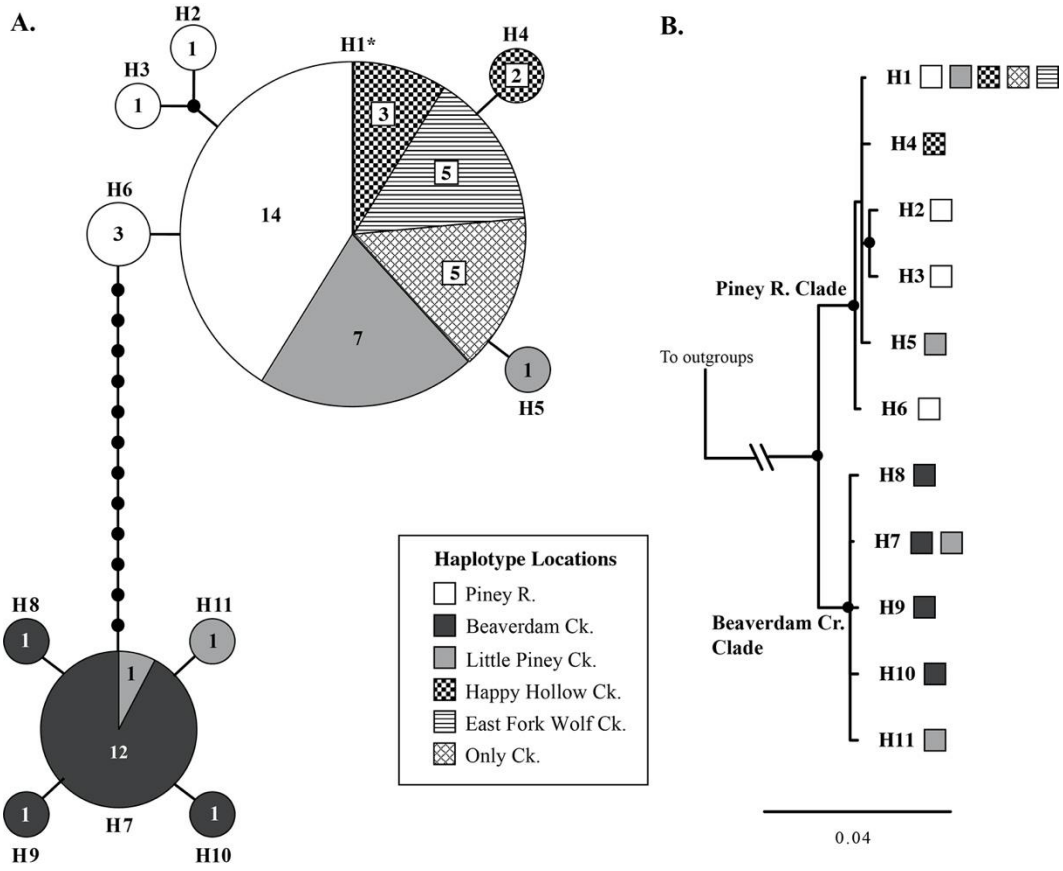

Figure 4. (A) Ninety-five percent statistical parsimony haplotype network for the Egg-mimic Darter representing 11 unique *ND2* haplotypes from 59 individuals from 14 localities. Haplotypes are represented by pie charts and labeled in bold. Pie size and the number in each pie reflects frequency of individuals from a tributary system with that haplotype. Haplotype 1 (H1) had the highest outgroup probability, and thus, was estimated to be the most ancestral haplotype (*). Each line separated by a black circle represents one nucleotide difference between haplotypes. Small black circles along lines represent unsampled haplotypes in the population. (B) Maximum clade credibility tree resulting from the Bayesian analysis of the 11 unique haplotypes of Egg-mimic Darter identified from the TCS analysis. Nodes with black circles had > 0.98 posterior probabilities. Labels are haplotype numbers and location from which a given haplotype was found as described in 4A. Outgroups not shown.

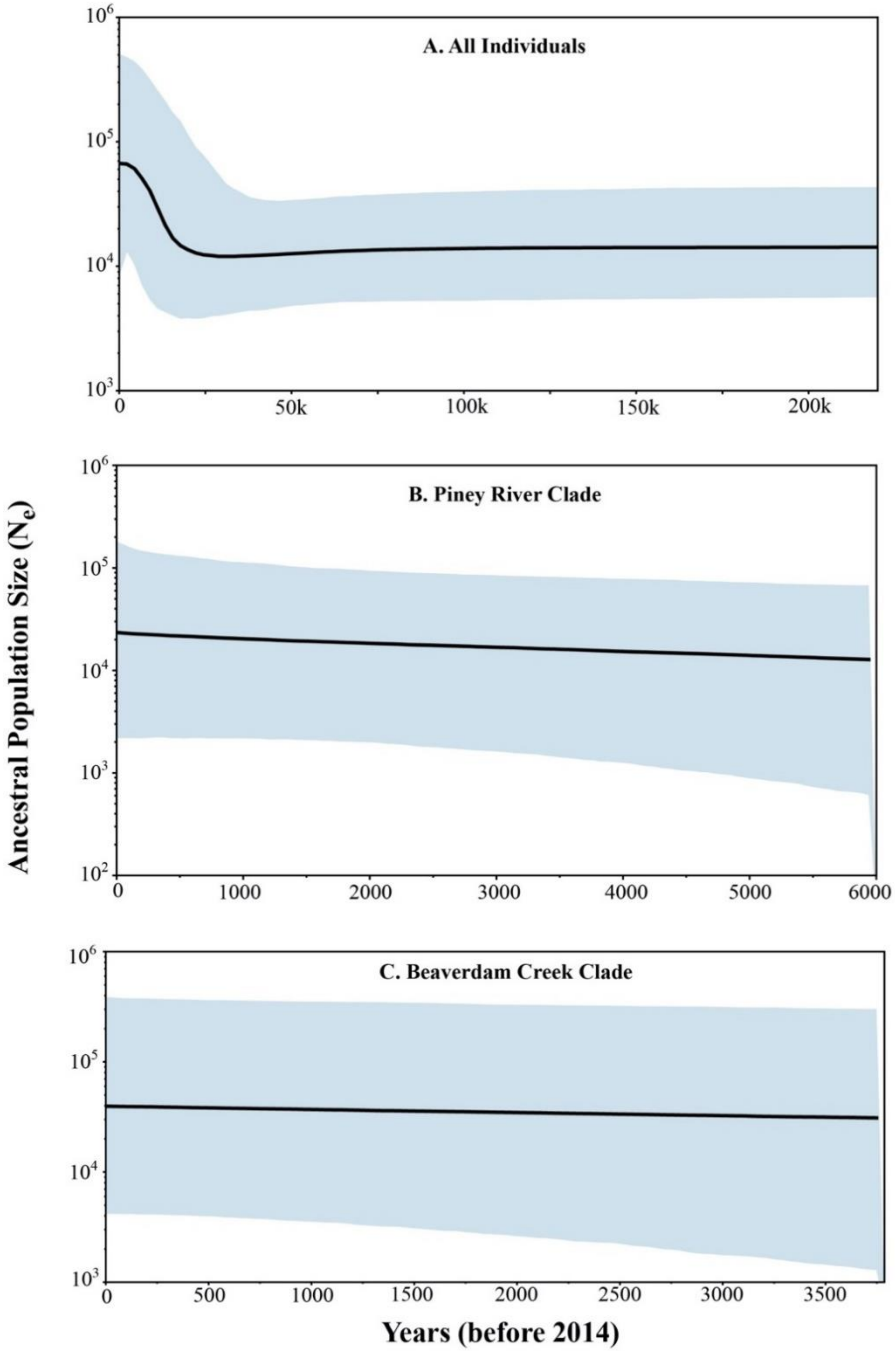

Figure 5. Bayesian skyline plots showing change in effective populations size (N_e) over time (years before 2014) for: (A) all individuals of Egg-mimic Darter (n = 59); (B) Piney River Clade individuals (n = 42); and (C) Beaverdam Creek Clade individuals (n = 17). Clades correspond to those in Figure 4B.

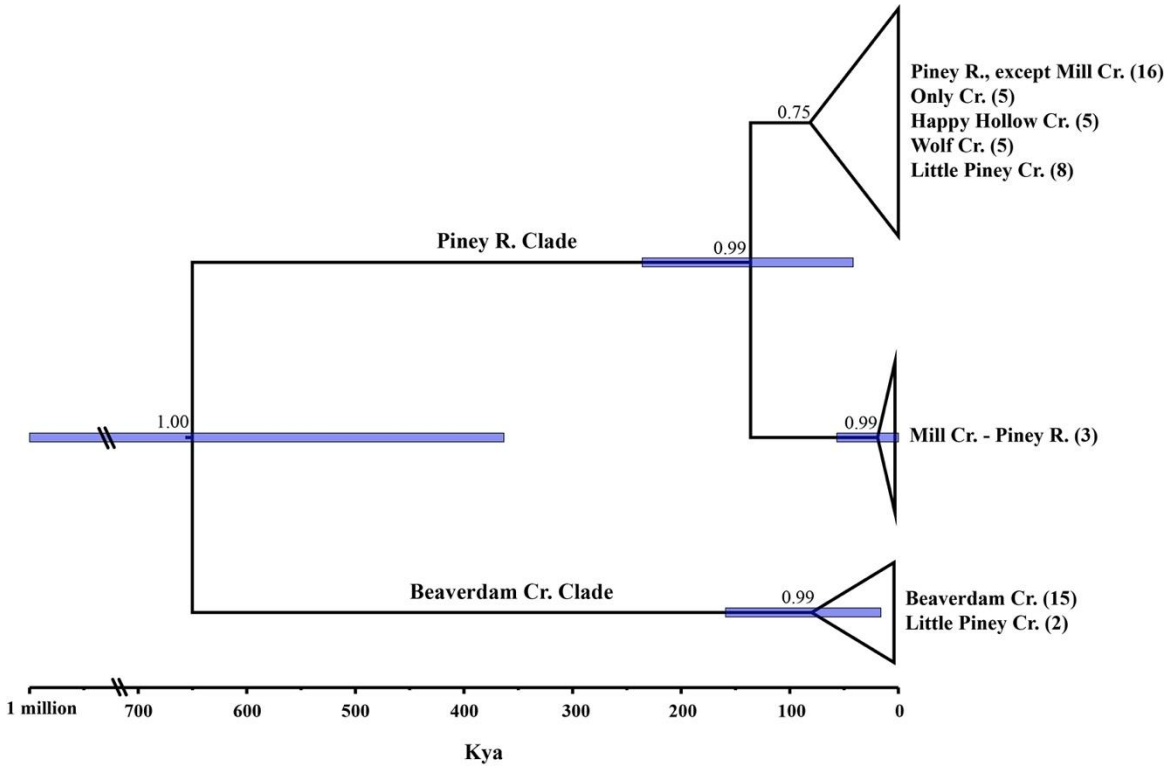

Figure 6. Phylogeographic relationships and node age estimates for the Egg-mimic Darter (n = 59) using the coalescent constant-population size model and the externally fossil-calibrated mutation rate estimated for *ND2* (Fluker et al. 2014). Numbers in parentheses are numbers of individuals included from a given river system recovered in each clade. Only clades with posterior probabilities of 0.75 or higher are shown; others were collapsed. Age estimates are provided only for clades with > 0.98 posterior probabilities. Numbers at nodes are posterior probabilities and bars at nodes are 95% highest posterior densities (HPD) for each node mean age estimate. The scale bar is thousands of years (kya). Mean age and HPDs are provide in Table 3.

Table 3. Node age estimates (thousands of years before present) for the Egg-mimic Darter (n = 59) using the coalescent constant-population size and Bayesian skyline models with the externally fossil-calibrated substitution rate estimated for *ND2* (Fluker et al. 2014). Values are posterior mean age with the 95% highest posterior density in parentheses. Clades correspond to those in Figure 6.

Clade	Coalescent Constant Population	Coalescent Bayesian Skyline
Egg-mimic Darter root	650,100 (363,300 – 964,100)	570,663 (209,722 – 913,874)
Piney R. Clade	136,100 (41,700 – 235,900)	111,440 (27,986 – 198,156)
Mill Cr. – Piney R.	19,600 (37 – 56,700)	26,401 (137 – 45,430)
Beaverdam Cr. Clade	79,800 (16,148 – 159,266)	67,607 (12,774 – 124,505)

DISCUSSION

As threats to imperiled species continue to increase, management agencies must prioritize limited resources in ways that are both efficient and effective. This requires a basic understanding of the different attributes that contribute to a species' imperilment including patterns of distribution and abundance, habitat quality, and genetic diversity (Warren et al. 1997; George et al. 2006). Our results suggest the Egg-mimic Darter has remained relatively stable with respect to ancestral population size and contemporary patterns of occurrence and abundance. These observations, in conjunction with evidence for historic population connectivity and sustainable habitat quality, indicate this species is likely to persist pending no major stochastic events or anthropogenic changes in land-use that alter current conditions. Such disturbances could have large impacts due to its small range (4 out of the 6 tributary systems are less than 50 km^2 in drainage area) and could lead to local extirpations, particularly in the smallest tributary systems. Although dispersal and recolonization potential of benthic, headwater-stream adapted fishes can be restricted by large rivers (Starnes and Etnier 1986; Turner and Trexler 1998; Powers et al. 2004; Simon and Wallus 2005; George et al. 2006; Turner and Robison 2006; Hollingsworth and Near 2009; Fluker et al. 2011; Sterling et al. 2012), we found evidence of historical connectivity within and among populations of the different Duck River tributary systems indicating an absence of long-standing isolation among these systems, excluding Beaverdam Creek. The lack of long-standing isolation suggests the mainstem has not served as a historic barrier to dispersal and local extirpations may be recovered by emigrants from neighboring tributary systems, and thus, the species potentially has a more resilient metapopulation than previously thought. The long-standing isolation of haplotypes found in Beaverdam Creek and the absence of the widespread, common haplotype found elsewhere implies this genetically distinctive population may warrant special monitoring or management. Overall, our multi-faceted approach provides a more complete knowledge base for making decisions about how to best manage the Egg-mimic Darter to support its long-term persistence.

Distribution and Abundance

We collected the Egg-mimic Darter at all localities sampled, indicating that local occupancy patterns have not changed since the last survey in 1995 (Ceas and Page 1995). This is promising for its conservation, as range-reduction and habitat fragmentation are classic symptoms of threatened and endangered species (Warren et al. 2000). Although additional sampling in adjacent tributary streams could reveal new localities outside of its known range, previous surveys of other localities have not yielded Egg-mimic Darters and authors have noted many of these adjacent localities lack suitable habitat for the species (Page et al. 1992; Ceas and Page 1995). Despite this, it may prove beneficial to survey for undetected populations, if resources allow.

Overall, the Egg-mimic Darter appears to be relatively abundant throughout most of its distribution (Tables 1 and 4). Although abundances observed in this study are not directly comparable to those of previous studies due to sampling differences, a general comparison to Ceas and Page (1995) provides historical context for our findings. Ceas and Page (1995) collected in April with effort ranging from 15 minutes to 2 hours using seines and dip nets and reported relative abundance categorically: abundant (> 20 individuals), common (11-20 individuals), present (< 11 individuals), and absent (0 individuals). In our spring sampling period, most of the sites sampled (87.5%) had first-pass capture numbers consistent with or higher than those reported by Ceas and

Page (1995; Table 4). This suggests abundance patterns have remained relatively stable over the past two decades.

Table 4. Comparison of spring first-pass captures of Egg-mimic Darters with spring collection results of Ceas and Page (1995). Collections from Ceas and Page (1995) were made on 3 April, 1995, by sampling for 15 minutes to 2 hours using seines and dip nets; data were reported as categories: abundant (greater than 20 individuals), common (10-20), present (less than 10), and absent.

Site	Tributary System	First-pass captures	
		1995	Spring 2014
1	Beaverdam Ck.	10-20	11
2	Beaverdam Ck.	<10	10
4	Beaverdam Ck.	<10	20
7	Beaverdam Ck.	>20	8
8	Beaverdam Ck.	<10	10
10	E. Fk. Wolf Ck.	<10	5
11	Little Piney Ck.	0	5
12	Happy Hollow Ck.	<10	6
13	Only Ck.	<10	10
16	Piney R.	>20	44
17	Piney R.	<10	17
18	Piney R.	<10	36
19	Piney R.	>20	24
22	Piney R.	0	7
23	Piney R.	>20	11
24	Piney R.	10-20	50

Ceas and Page (1995) noted that the Piney River system most likely harbored the most stable population of Egg-mimic Darter, while the smaller tributary systems were more vulnerable to extirpation due to lower population sizes. Although there were no significant differences between tributary systems in POP_P and density in our study, abundance patterns generally agree with Ceas and Page (1995). For both seasons, the highest POP_P estimates were from sites within the largest tributary systems, Piney River and Beaverdam Creek, followed by smaller tributary systems; a few of the Piney River and Beaverdam Creek sites had the lowest POP_P estimates (Table 1). A similar pattern was observed in population density with the exception of Only Creek, which had the second highest density in both spring and fall despite having the smallest mean wetted-width (1.8-m averaged between spring and fall). Compared to the other sites with small mean

wetted-widths, Only Creek appears to have a relatively high density for such a small system, which may be an important consideration in conservation planning.

Temporal variation in abundance has been found in other stream fishes (Schlosser and Toth 1984; Schlosser 1985; Matthews 1990). Although we observed seasonal variation in POP_P, density, and capture efficiency, this likely reflects the YOY age-class which was not counted in the fall estimates but were counted as juveniles in the spring estimates. To confirm this, statistical comparisons of POP_P, density, and capture efficiency between seasons were redone with the removal of the juvenile class from spring estimates. We found no significant seasonal variation in adults for POP_P ($P = 0.07$), density ($P = 1.0$), or capture efficiency ($P = 0.13$), supporting that the observed seasonal variation is likely driven by our methodology. However, because seasonal variation has been observed in these metrics for other fishes it is possible that there is significant seasonal variation in Egg-mimic Darters, but more data are needed to discern whether this is a trend for our focal taxon.

Habitat Quality

Several localities exhibited anthropogenic disturbances, primarily related to agriculture (either for row crops or pasture) and residential development, both of which reduce the riparian buffer. However, habitat quality across the range of the Egg-mimic Darter does not appear to be substantially degraded. For both spring and fall, sites had a mean THS that met suboptimal conditions, which suggests that the quality of physical habitat at localities appears to be sufficient, yet warrants future monitoring. We have no recommendations for specialized monitoring of tributary systems based on habitat quality alone given there were no significant differences in THS between tributary systems.

The significant correlations between specific habitat quality metrics and POP_P are not surprising considering the ecology of the Egg-mimic Darter. For a benthic species that is found primarily in pool and marginal habitats with low flow (Page et al. 1992; Ceas and Page 1995; unpublished data from Master's thesis [Wolf 2015]), a negative correlation with the frequency of riffles was expected. Although higher riffle frequency scores are generally associated with higher habitat quality (Barbour et al. 1999), it also reflects a decrease in occurrence of pool and other low flow habitats used by Egg-mimic Darters. Metrics associated with low flow, such as sediment deposition, can have a negative impact on the THS. The significant positive correlation with POP_P and mean depth in fall supports this hypothesis. These results show that it is important to consider the ecology of a species when conducting habitat assessments; a high habitat score may not reflect improved habitat quality for all species, even for those species groups (e.g., darters) considered as indicators of high habitat quality.

Haplotype Diversity and Demographic History

Overall haplotype and nucleotide diversity in the Egg-mimic Darter was generally lower than widespread darters (Fantail Darter and Rainbow Darter (*Etheostoma caeruleum*)) and comparable to other imperiled darters (Yazoo Darter (*E. raneyi*), Okaloosa Darter (*E. okaloosae*), Roanoke Logperch (*Percina rex*), and Conasauga Logperch (*P. jenkinsi*); Table 2). Populations from Happy Hollow Creek and Little Piney Creek have haplotype and nucleotide diversity comparable to the larger Piney River and Beaverdam Creek populations despite having lower estimates of POP_P and density (Table 1). The proportionally smaller populations of East Fork Wolf

Creek and Only Creek had no observable genetic diversity. However, our sample sizes were relatively small for these latter sites and likely contributed to these findings. Additionally, Beaverdam Creek, Little Piney Creek, Happy Hollow Creek, and the Piney River contain unique haplotypes. These results indicate that both the species and each tributary system should be regularly monitored for Egg-mimic Darter persistence and abundance. If any one of these populations were extirpated, then a portion of the already low overall genetic diversity would be lost. This is especially important for the Beaverdam Creek population because of its particularly unique haplotype assemblage.

Although haplotype diversity was relatively low, demographic analyses indicate Egg-mimic Darters have either not deviated from a constant population size throughout most of its range (represented by the Piney River Clade) or have experienced a recent population expansion (in the case of the Beaverdam Creek Clade). These results were reflected by ancestral N_e estimates for each clade that suggest several thousand years of population size stability with slight increasing trends in N_e observed in each. The increase in N_e for all individuals (approximately 20,000 years ago) is consistent with observations from many other studies of North American freshwater organisms that show periods of population contraction and expansion associated with climatic fluctuations of the Pleistocene (Hewitt 2000; Fluker et al. 2014). However, this result may be biased by the occurrence of population structure in our overall dataset that includes two divergent geographically definable clades (Heller et al. 2013). While the significant, negative Tajima's D and Fu and Li's F* and D* likely indicates population expansion after a bottleneck event in Beaverdam Creek, these results could alternatively reflect a selective sweep or the relatively small sample size for this clade, which may inflate the frequency of rare alleles; both can also lead to deviations from expectations of neutral evolution and negative, significant values for these statistics (Tajima 1989; Fu and Li 1993; Simonson et al. 1995). Regardless, the N_e estimates for each clade, which are not biased by the presence of population structure, and those from recent surveys of occurrence and abundance indicate the species has remained relatively stable over both contemporary and historical timeframes. From a conservation perspective, the consistent ancestral N_e estimates suggest that human actions have not led to detectable declines in diversity in this species over the past several thousand years.

Phylogeographic Relationships

Numerous small, headwater-stream adapted fishes (similar to the Egg-mimic Darter) exhibit phylogeographic structure indicative of reduced gene flow among different river systems across their range (Echelle et al. 1975; Starnes and Etnier 1986; Bernatchez and Wilson 1998; Turner and Trexler 1998; Near et al. 2001; Berendzen et al. 2003; Powers et al. 2004; George et al. 2006; Ray et al. 2006; Turner and Robison 2006; Hollingsworth and Near 2009; Sterling et al. 2012; Blanton et al. 2013; Bossu et al. 2013; Fluker et al. 2014; Echelle et al. 2015). For many of these, historical vicariance and/or life history characteristics that may reduce dispersal potential (e.g., low fecundity, large eggs, reduced larval dispersal) and strict habitat requirements have been considered as possible explanations for reduced gene flow leading to increased genetic differentiation among populations in different tributaries (e.g., Turner and Trexler 1998; Hollingsworth and Near 2009; Fluker et al. 2014). Despite sharing life history and habitat requirement traits typically associated with reduced dispersal, the Egg-mimic Darter did not display a high degree of genetic structure among tributary systems as expected, except for Beaverdam Creek (Figure 4A). Haplotype 1 was shared among all tributary systems except

Beaverdam Creek, which supports that gene flow has likely been maintained historically at some level among these five populations (Avise 2004; George et al. 2006; Hughes et al. 2009). Although the lack of genetic structure observed among most tributaries was unexpected, other upland-adapted fishes appear to traverse large rivers and maintain gene flow despite intrinsic and extrinsic features that would suggest otherwise. The Tuxedo Darter (*Etheostoma lemniscatum*) is closely related to the Egg-mimic Darter and shares many of the same characteristics that would indicate limited dispersal potential. Despite being a habitat specialist residing in a river system with various potential barriers and large gaps in suitable habitat, the Tuxedo Darter was found to have little genetic structure and high population connectivity (Washburn et al. 2020). Lang and Echelle (2011) recovered clades of Cypress Darter (*Etheostoma proeliare*) which included individuals from populations on both sides of the lower Mississippi River, suggesting that the species has maintained gene flow across the Mississippi River. Studies of Roanoke Logperch have found the species exhibits extensive dispersal including individuals traveling up to 3.2 km (Roberts et al. 2008, 2016). These studies demonstrate that not all small-bodied, benthic fishes are equally limited with respect to dispersal and gene flow by larger-river mainstem habitats.

Although we did not observe phylogeographic structure corresponding to each of the different tributaries of the Duck River, haplotypes were recovered in two geographically definable and highly divergent clades in all phylogeographic analyses: the Piney River Clade (Piney River and all other Duck River tributaries except Beaverdam Creek) and Beaverdam Creek Clade (including two individuals from Little Piney Creek). Node age estimates show these clades diverged from a MRCA during the Pleistocene. We also found support for potential Pleistocene-age isolation of Mill Creek (Piney River system) from other Piney River clade members, but sample sizes were low for Mill Creek and require further investigation; no other well-supported geographically definable structure was observed in the species.

The long-standing isolation of Beaverdam Creek (and possibly Little Piney Creek) from other populations is likely attributed to historical vicariance associated with Pleistocene climatic fluctuations and associated geofluvial instability. The influence of pre-Pleistocene river drainage patterns and fragmentation of the Central Highlands on phylogeography of North American freshwater fishes is well-studied. Vicariance, such as through drainage re-arrangements associated with Pleistocene glacial cycles is a commonly invoked mechanism contributing to speciation and phylogeographic structure across Central Highlands fishes (Pflieger 1971; Mayden 1988; Near et al. 2001; Berendzen et al. 2003, 2008; Blanton et al. 2013). Paleogeographic factors contributing to diversification of lineages within the unglaciated Eastern Highlands (to which the Egg-mimic Darter is restricted), a region that experienced fewer drainage reconfigurations during the Pleistocene (Thornbury 1965; Mayden 1988) are less well understood. However, several studies have found evidence of long-standing isolation among lineages of species distributed in this region, with Pleistocene age estimates similar to that observed for Egg-mimic Darter clades (e.g., Ashy Darter, *Etheostoma cinereum*, Powers et al. 2004; Blotchside Logperch, *Percina burtoni*, George et al. 2006). For example, George et al. (2006) found that haplotypes of Blotchside Logperch sorted into two clades representative of two drainage areas (Duck River + lower Tennessee River clade and the middle + upper Tennessee River clade) supporting isolation between these populations. Near et al. (2017) described a new species from the Duck River + lower Tennessee River clade of Blotchside Logperch and estimated divergence among the two species at approximately 2.0 mya (Hollingsworth and Near 2009). Powers et al. 2004 identified three

clades of Ashy Darter, each from a different river system, as the result of haplotype sorting and concluded gene flow had been reduced between these populations and described the Cumberland River clade of Ashy Darter as the Redlips Darter (*Etheostoma maydeni*); divergence of these lineages was approximately 1.5 mya (Hollingsworth and Near 2009). Hollingsworth and Near (2009) found evidence for micro-allopatric divergence within barcheek darters of the Cumberland river system, similar to the observation of isolation of the Beaverdam Creek clade of Egg-mimic Darter. Species of barcheek darters occupying the lower Tennessee River and Duck River were among the youngest diversification events and had ages similar to those observed for Egg-mimic Darter clades. Other species in the region such as Spotted Darter (*Nothonotus maculatus*) exhibit similar recent, Pleistocene-age divergence times (Near and Keck 2005). These studies have invoked dispersal and vicariance such as that associated with the paleogeographic disturbance of these areas at the onset of the Pleistocene and increased opportunities for isolation across long periods of relative geological and climatic stability as factors contributing to isolation and diversification of lineages of fishes in the Eastern Highlands. We assume similar factors have contributed to the phylogeographic history of the Egg-mimic Darter.

There are no known physical barriers, such as a waterfall, drainage divide, or physiographic break present between Beaverdam Creek and the Duck River that would explain isolation from other populations, in general. Hollingsworth and Near (2009) also found allopatric divergence in barcheek darters in the absence of traditionally recognized barriers to gene flow and that isolation and divergence occurred at smaller spatial scales than previously recognized. As noted, one of the most common explanations for phylogeographic breaks in freshwater fishes is vicariance resulting from past geologic events. Particularly, historical patterns of eastern North American fluvial geomorphology are commonly used to explain the distribution of fishes, and vice versa (Starnes and Etnier 1986; Mayden 1988; Bernatchez and Wilson 1998; Near et al. 2001; Berendzen et al. 2003; Near and Keck 2005; George et al. 2006; Ray et al. 2006; Lang and Echelle 2011; Blanton et al. 2013; Bossu et al. 2013). The underlying geology within the range of the Egg-mimic Darter shows a large section of sand surrounded by a trail of limestone in Beaverdam Creek, which extends 4 km upstream from its confluence with the Duck River (Figure 7). Other tributary systems where Egg-mimic Darters occur primarily exhibit limestone at their confluences with no or only a small segment of sand. Bedrock substrata, such as limestone, are formed by long-term erosion caused by the persistently-strong flow of a stream, which suggests a correlation between bedrock substrata and the position of a stream's thalweg (Charlton 2007). Fine substrata, such as sand, are deposited by areas in a stream with less flow, such as the inside of a bend in the stream (Charlton 2007). Therefore, the pattern seen at the confluence of Beaverdam Creek suggests that the channel of the Duck River once traveled through the position of the limestone that borders the large sand deposit and at some point was captured at the northern tips of the bend, resulting in a shift in the Duck River 4 km north of its former position. If accurate, this event could have effectively formed a barrier for fish dispersal, either by creating unsuitable habitat or by physical disconnection from the Duck River, thereby isolating Egg-mimic Darters in Beaverdam Creek. Subsequently, the remaining long stretch of mostly sand deposits in the lower reaches of Beaverdam Creek may continue to limit movement into Beaverdam Creek, resulting in the accumulation of divergent haplotypes in this system (13 mutational differences from the other tributary systems). This hypothesis is consistent with the observation of recent population expansion, possibly after a bottleneck event, stemming from isolation of Beaverdam Creek under this scenario of a fluvial shift in the Duck River mainstem. The occurrence of shared haplotypes with Little Piney Creek is

interesting, but this tributary may have been similarly affected given its proximity to Beaverdam Creek and these two systems may have maintained some degree of connectedness throughout this event or at least for a longer period of time relative to their connections with other streams. Although shifts in the fluvial geomorphology of the Duck River may have occurred, our hypothesis is based solely on the population connectivity results of the Egg-mimic Darter and the geologic map of the area. Investigating the phylogeography of fishes ecologically similar to the Egg-mimic Darter within its range and a more in-depth study of the geological history of the area could provide better context for the explanation of the pattern we see in the Egg-mimic Darter.

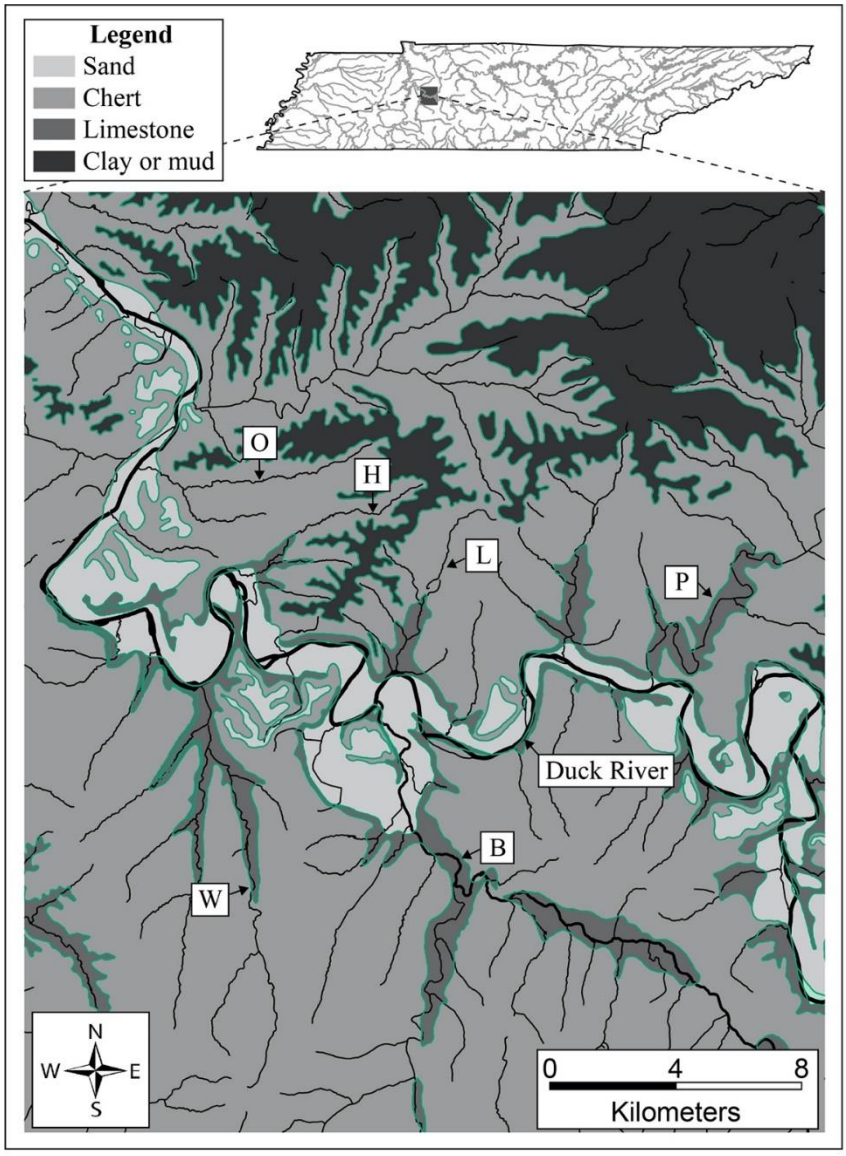

Figure 7. Underlying geology within the range of the Egg-mimic Darter, highlighting the confluence of Beaverdam Creek with the Duck River system. The Duck River is shown in its current channel position. Letters refer to tributary systems of the Duck River: P = Piney River; O = Only Creek; H = Happy Hollow Creek; L = Little Piney Creek; W = East Fork Wolf Creek; and B = Beaverdam Creek. Tennessee geologic layer downloaded from the US Geological Survey website http://mrdata.usgs.gov/geology/state/state.php?state=TN.

Lastly, Page et al. (1992) noted considerable morphological variation across the range of the Egg-mimic Darter. In particular, they highlighted several differences observed in Beaverdam Creek individuals relative to those from the Piney River including modal dorsal-fin spines (9 vs. 8 in the Piney River), mean pored lateral-line scales (31.0 vs. 21.9 in Piney River), and the presence of an uninterrupted infraorbital canal (vs. interrupted in the Piney River). They also commented on variation in Little Piney Creek, which shared some features with both Beaverdam Creek and Piney River individuals including 8 or 9 dorsal-fin spines, 32.3 mean pored lateral-line scales, and an interrupted infraorbital canal. The authors concluded no subspecific designations were warranted due to a lack of concordance in character variation between meristics and pigmentation patterns. In light of results that demonstrate Beaverdam Creek also has a unique haplotype assemblage with evidence for Pleistocene-age divergence of this lineage, further study is needed to evaluate its potential taxonomic distinctiveness. Additional evaluation of the Little Piney Creek population is also warranted given the mixed ancestry of haplotypes found in this system and its potential intermediate morphological traits.

Implications for Conservation

The criteria used by US Fish and Wildlife Service to determine the listing of a species are: 1) the present or threatened destruction, modification, or curtailment of its habitat or range; 2) overutilization for commercial, recreational, scientific, or educational purposes; 3) disease or predation; 4) the inadequacy of existing regulatory mechanisms; or 5) other natural or manmade factors affecting its continued existence (Endangered Species Act, Section 4). When considering these criteria, we conclude that the Egg-mimic Darter does not currently warrant federal protection. When comparing results to those of Ceas and Page (1995), the abundance and distribution of the species have remained relatively stable over the past 20 years. Despite the finding that localities of the Egg-mimic Darter were, on average, of suboptimal conditions based on habitat assessments, there were no major threats observed to the habitat or range of the species. We also found that conventional methods of assessing habitat may not be beneficial for measuring suitable habitat for species that utilize pools. Conservation managers should consider Egg-mimic Darter ecology when evaluating habitat availability. There was no evidence of the Egg-mimic Darter being overutilized for commercial, recreational, scientific, or educational purposes, and there were no indications of disease or predation threatening the persistence of the species.

Our results for the last two criteria for listing, including a small range (and associated risk of local extirpations) and relatively low-to-moderate genetic diversity, suggest concern for the species' persistence. However, the Egg-mimic Darter appears to be relatively abundant throughout its range and our data suggest that gene flow was historically maintained between most of the tributary systems, with the exception of Beaverdam Creek (and possibly the Little Piney Creek). Assuming the potential for dispersal through the Duck River persists for all other populations, the potential for recolonization from neighboring tributary systems would help buffer the threat of local extirpation. Additionally, gene flow among the different populations would increase the likelihood of maintaining genetic diversity, which is important to species or population viability (Frankel 1974; Avise 2004). We recommend that the species be regularly monitored with special consideration given to the smaller tributary systems (Happy Hollow Creek, Little Piney Creek, Only Creek, and East Fork Wolf Creek) and the genetically distinct Beaverdam Creek population. Because our genetic data provide a snapshot of historical population structure and not of contemporary population connectivity or isolation, we also recommend future studies examine

genetic diversity using markers such as microsatellites or SNPs to capture contemporary gene flow and to more fully understand recolonization potential and population dynamics. Such studies could also provide robust estimates of important measures of population persistence such as allelic richness, heterozygosity, and contemporary effective population size.

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APPENDICES – LOCALITIES AND MATERIALS EXAMINED

Appendix A. Locality information for all historical and new localities of the Egg-mimic Darter (Figure 2). H-E = historical examined; H-U = historical unable to examine due to stream conditions or access; H-A = historical not examined due to adjacent proximity to other localities; and New = newly examined. Institutional abbreviations follow Sabaj Perez (2014).

Site	Type	County	Locality	Museum Record	Latitude	Longitude
1	H-E	Hickman	Piney Fork Creek near TN-48, 0.5 mi NE of Aetna.	USNM 231310.509	35.66204	-87.4999
2	H-E	Hickman	Brushy Fork Creek at TN-48 of Aetna.	USNM 230491.5089	35.68102	-87.5024
3	H-E	Hickman	Beaverdam Creek 4 mi N of Aetna off East Beaverdam Creek Road, 0.5 mi from TN-48.	INHS 77593	35.70666	-87.5093
4	H-E	Hickman	Beaverdam Creek at bridge of Backside Beaverdam Creek Road, 3 mi E of Coble.	INHS 77817	35.76482	-87.5798
5	H-E	Hickman	Sulphur Fork Creek at Mitchell bridge on Backside Beaverdam Creek Road and Sulphur Fork Creek Road.	UT 91.5796	35.77103	-87.6152
6	H-E	Hickman	Beaverdam Creek, 1 mi E of Coble at bridge of TN-438.	INHS 82756	35.78693	-87.6272
7	H-E	Hickman	Cow Hollow Creek at Coble on TN-438 near crossing of Briar Pond Road.	INHS 58414	35.7839	-87.6302
8	H-E	Hickman	Beaverdam Creek at TN-50, 1 mi N of Coble.	UT 91.1629	35.800	-87.6293
9	H-E	Hickman	East Fork Wolf Creek, 3 mi W of Coble off Wolf Creek Road.	INHS 91989	35.77654	-87.674
10	H-E	Hickman	East Fork Wolf Creek on Mathis Loop Road, 3 mi NE of Coble.	INHS 33682	35.80285	-87.6828
11	H-E	Hickman	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	INHS 91948	35.83049	-87.6291
12	H-E	Hickman	Happy Hollow Creek 1.5 mi SE of Only near TN-50 bridge via TWRA WMA.	INHS 36030	35.85014	-87.6659
13	H-E	Hickman	Only Creek tributary at intersection of Only Road and Dyer Road.	INHS 91987	35.86308	-87.692
14	H-E	Hickman	Bell Branch, 6.5 mi NNE of Centerville, at powerline clearing of Bell Branch Trace.	INHS 91987	35.86449	-87.434
15	H-E	Hickman	Mill Creek at TN-48, 1 mi N of Nunnelly, 0.5 mi upstream of bridge.	UT 91.5796	35.87312	-87.4634
16	H-E	Hickman	Mill Creek, 1.6 km S of Wrigley at TN-100 bridge.	INHS 58630	35.89307	-87.3506
17	H-E	Hickman	Little Spring Creek, 2 mi NE of Pinewood at Pinewood Road.	INHS 36024	35.92043	-87.4424
18	H-E	Hickman	Beaver Creek, 2.2 mi W of TN-48 off Old Beaver Creek Road.	INHS 62771	35.93054	-87.5061

Appendix A cont.

Site	Type	County	Locality	Museum Record	Latitude	Longitude
19	H-E	Hickman	Big Spring Creek at Missionary Ridge Road, 0.75 mi SE of Bon Aqua, Bon Aqua Springs.	INHS 36029	35.94586	-87.3204
20	H-E	Dickson	Piney River at Double Branch Road 0.5 mi from I-40, 2 mi SW of Mount Sinai.	UMMZ 104881	35.9893	-87.4349
21	H-E	Dickson	East Piney River at North Mount Sinai Road.	UT 91.5797	36.01035	-87.4382
22	H-E	Dickson	West Fork Piney River at TN-48 0.2 km downstream of bridge, private access.	UT 91.7629	36.02695	-87.4493
23	H-E	Dickson	Coon Creek at Coon Creek Road, 3 mi SE of Tennessee City.	INHS 35923	36.05801	-87.4735
24	H-E	Dickson	West Fork Piney River at Eno Road, 0.1 mi W of Eno.	INHS 62760	36.06058	-87.4556
25	New	Hickman	Piney River at TN-48, 2 mi N of Nunnelly.	APSU 1045.03	35.89066	-87.4706
26	H-U	Hickman	Only Creek tributary along Nunnelly-Only Road.	INHS 35980	35.86727	-87.6801
27	H-U	Hickman	Mill Creek, 1 mi N of Wrigley.	INHS 63484	35.91385	-87.3434
28	H-U	Hickman	Little Piney Cr. at co. rd. 6173.	UT 91.2580	35.87083	-87.5012
29	H-U	Hickman	Piney River at Pinewood on Co. Rd., ~1 mi from jct. with Rt. 48, 9 air mi N of Centerville.	CU 52472	35.91072	-87.4678
30	H-A	Hickman	Beaverdam Creek, Aetna.	INHS 61767	35.65514	-87.5048
31	H-A	Hickman	Piney Fork Creek at TN-48.	USNM 231310.509	35.66455	-87.4977
32	H-A	Hickman	Tributary to Beaverdam Creek at TN-48, about 1.0 miles S junction with TN-100.	NCSM 28827	35.6817	-87.5025
33	H-A	Hickman	Beaverdam Creek; at TN-48 and 100 intersection, ~10 km SW Centerville.	YPM ICH 023883	35.70384	-87.5102
34	H-A	Hickman	Beaverdam Creek at Beaverdam Creek Road.	UT 91.5796	35.78564	-87.6258
35	H-A	Hickman	trib. Duck River, Only, Rt. 229.	INHS 33651	35.86331	-87.6918
36	H-A	Hickman	Mill Creek, 1 mi. S Wrigley.	KU 14399, KU 16216	35.887	-87.345
37	H-A	Hickman	Little Spring Creek along Pinewood Road ~3 air km E Pinewood; TJN08-19.	YPM ICH 018560	35.91797	-87.456
38	H-A	Hickman	Piney River at I-40.	UT 91.5797	35.99455	-87.4396

Appendix B. GenBank accession numbers with associated metadata. Each accession is for a single individual fish. Metadata includes sex (M = male; F = female; U = unknown), collection date, site number (Figure 2), and locality information.

GenBank Accession	Sex	Collection Date	Site	Locality	Latitude	Longitude
MK426222	F	3/23/2014	18	Beaver Creek, 2.2 mi W of TN-48 off Old Beaver Creek Road.	35.93054	-87.5061
MK426223	U	3/13/2014	9	East Fork Wolf Creek, 3 mi W of Coble off Wolf Creek Road.	35.77654	-87.674
MK426224	F	5/3/2014	19	Big Spring Creek at Missionary Ridge Road, 0.75 mi SE of Bon Aqua, Bon Aqua Springs.	35.94586	-87.3204
MK426225	M	3/30/2014	23	Coon Creek at Coon Creek Road, 3 mi SE of Tennessee City.	36.05801	-87.4735
MK426226	F	4/4/2014	17	Little Spring Creek, 2 mi NE of Pinewood at Pinewood Road.	35.92043	-87.4424
MK426227	U	3/21/2014	12	Happy Hollow Creek 1.5 mi SE of Only near TN-50 bridge via TWRA WMA.	35.85014	-87.6659
MK426228	F	3/11/2014	5	Sulphur Fork Creek at Mitchell bridge on Backside Beaverdam Creek Road and Sulphur Fork Creek Road.	35.77103	-87.6152
MK426229	M	3/30/2014	23	Coon Creek at Coon Creek Road, 3 mi SE of Tennessee City.	36.05801	-87.4735
MK426230	U	3/21/2014	12	Happy Hollow Creek 1.5 mi SE of Only near TN-50 bridge via TWRA WMA.	35.85014	-87.6659
MK426231	U	3/13/2014	9	East Fork Wolf Creek, 3 mi W of Coble off Wolf Creek Road.	35.77654	-87.674
MK426232	U	3/12/2014	1	Piney Fork Creek near TN-48, 0.5 mi NE of Aetna.	35.66204	-87.4999
MK426233	U	5/3/2014	19	Big Spring Creek at Missionary Ridge Road, 0.75 mi SE of Bon Aqua, Bon Aqua Springs.	35.94586	-87.3204
MK426234	F	4/2/2014	7	Cow Hollow Creek at Coble on TN-438 near crossing of Briar Pond Road.	35.7839	-87.6302
MK426235	F	3/12/2014	1	Piney Fork Creek near TN-48, 0.5 mi NE of Aetna.	35.66204	-87.4999
MK426236	F	3/11/2014	5	Sulphur Fork Creek at Mitchell bridge on Backside Beaverdam Creek Road and Sulphur Fork Creek Road.	35.77103	-87.6152
MK426237	M	4/2/2014	7	Cow Hollow Creek at Coble on TN-438 near crossing of Briar Pond Road.	35.7839	-87.6302
MK426238	U	4/2/2014	7	Cow Hollow Creek at Coble on TN-438 near crossing of Briar Pond Road.	35.7839	-87.6302
MK426239	U	4/2/2014	7	Cow Hollow Creek at Coble on TN-438 near crossing of Briar Pond Road.	35.7839	-87.6302

Appendix B cont.

GenBank Accession	Sex	Collection Date	Site	Locality	Latitude	Longitude
MK426240	M	3/11/2014	5	Sulphur Fork Creek at Mitchell bridge on Backside Beaverdam Creek Road and Sulphur Fork Creek Road.	35.77103	-87.6152
MK426241	F	3/11/2014	5	Sulphur Fork Creek at Mitchell bridge on Backside Beaverdam Creek Road and Sulphur Fork Creek Road.	35.77103	-87.6152
MK426242	U	4/4/2014	17	Little Spring Creek, 2 mi NE of Pinewood at Pinewood Road.	35.92043	-87.4424
MK426243	F	3/30/2014	23	Coon Creek at Coon Creek Road, 3 mi SE of Tennessee City.	36.05801	-87.4735
MK426244	F	3/30/2014	23	Coon Creek at Coon Creek Road, 3 mi SE of Tennessee City.	36.05801	-87.4735
MK426245	U	3/28/2014	13	Only Creek tributary at intersection of Only Road and Dyer Road.	35.86308	-87.692
MK426246	U	3/28/2014	13	Only Creek tributary at intersection of Only Road and Dyer Road.	35.86308	-87.692
MK426247	U	3/23/2014	18	Beaver Creek, 2.2 mi W of TN-48 off Old Beaver Creek Road.	35.93054	-87.5061
MK426248	M	3/13/2014	9	East Fork Wolf Creek, 3 mi W of Coble off Wolf Creek Road.	35.77654	-87.674
MK426249	F	3/13/2014	9	East Fork Wolf Creek, 3 mi W of Coble off Wolf Creek Road.	35.77654	-87.674
MK426250	U	4/2/2014	7	Cow Hollow Creek at Coble on TN-438 near crossing of Briar Pond Road.	35.7839	-87.6302
MK426251	F	3/7/2014	16	Mill Creek, 1.6 km S of Wrigley at TN-100 bridge.	35.89307	-87.3506
MK426252	U	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426253	F	3/21/2014	12	Happy Hollow Creek 1.5 mi SE of Only near TN-50 bridge via TWRA WMA.	35.85014	-87.6659
MK426254	F	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426255	F	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426256	U	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426257	F	3/28/2014	13	Only Creek tributary at intersection of Only Road and Dyer Road.	35.86308	-87.692
MK426258	F	3/28/2014	13	Only Creek tributary at intersection of Only Road and Dyer Road.	35.86308	-87.692
MK426259	F	3/21/2014	12	Happy Hollow Creek 1.5 mi SE of Only near TN-50 bridge via TWRA WMA.	35.85014	-87.6659
MK426260	U	5/2/2014	8	Beaverdam Creek at TN-50, 1 mi N of Coble.	35.800	-87.6293

Appendix B cont.

GenBank Accession	Sex	Collection Date	Site	Locality	Latitude	Longitude
MK426261	U	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426262	U	5/2/2014	8	Beaverdam Creek at TN-50, 1 mi N of Coble.	35.800	-87.6293
MK426263	U	5/2/2014	8	Beaverdam Creek at TN-50, 1 mi N of Coble.	35.800	-87.6293
MK426264	U	3/21/2014	12	Happy Hollow Creek 1.5 mi SE of Only near TN-50 bridge via TWRA WMA.	35.85014	-87.6659
MK426265	F	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426266	M	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426267	F	3/7/2014	16	Mill Creek, 1.6 km S of Wrigley at TN-100 bridge.	35.89307	-87.3506
MK426268	U	5/2/2014	8	Beaverdam Creek at TN-50, 1 mi N of Coble.	35.800	-87.6293
MK426269	F	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426270	F	3/7/2014	16	Mill Creek, 1.6 km S of Wrigley at TN-100 bridge.	35.89307	-87.3506
MK426271	F	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426272	U	3/23/2014	18	Beaver Creek, 2.2 mi W of TN-48 off Old Beaver Creek Road.	35.93054	-87.5061
MK426273	F	4/4/2014	17	Little Spring Creek, 2 mi NE of Pinewood at Pinewood Road.	35.92043	-87.4424
MK426274	U	4/9/2014	20	Piney River at Double Branch Road 0.5 mi from I-40, 2 mi SW of Mount Sinai.	35.9893	-87.4349
MK426275	U	4/9/2014	20	Piney River at Double Branch Road 0.5 mi from I-40, 2 mi SW of Mount Sinai.	35.9893	-87.4349
MK426276	U	3/28/2014	13	Only Creek tributary at intersection of Only Road and Dyer Road.	35.86308	-87.692
MK426277	F	3/13/2014	9	East Fork Wolf Creek, 3 mi W of Coble off Wolf Creek Road.	35.77654	-87.674
MK426278	F	5/3/2014	11	Little Piney Creek off Little Piney Road, 3.5 mi SW of Spot.	35.83049	-87.6291
MK426279	U	5/3/2014	19	Big Spring Creek at Missionary Ridge Road, 0.75 mi SE of Bon Aqua, Bon Aqua Springs.	35.94586	-87.3204
MK426280	U	4/9/2014	20	Piney River at Double Branch Road 0.5 mi from I-40, 2 mi SW of Mount Sinai.	35.9893	-87.4349