Final Report

Conservation Genetics of Spring Associated Darters in Alabama

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Contents

Project Overview

Organisms inhabiting freshwater springs present unique challenges to conservationists and natural resource managers. The naturally fragmented distribution of spring species render them extremely vulnerable to fine-scale disturbance and springs are among the most anthropogenically exploited freshwater habitats (Meffe and Vrijenhoek 1988; Hubbs 1995; Etnier 1997; Timpe et al. 2009; Fluker et al. 2010; Martin 2010). Spring endemic species are often confined to spring pools and short stretches of spring runs, with interconnecting streams and rivers acting as major barriers to dispersal (Starnes and Etnier 1986). Consequently, spring endemic species should share several characteristics with island endemic species, i.e. small genetically structured populations with low genetic diversity, making them more susceptible to local extinction compared to their mainland or widespread relatives (Frankham 1997; Wilson et al. 2009). Recent studies of spring taxa support this hypothesis in terms of genetic structure (Martin and Wilcox 2004; Hurt and Hedrick 2004; Wilmer and Wilcox 2007) and small population sizes with low genetic diversity (Duvernell and Turner 1999; Fluker et al. 2010). Most genetic studies of North American spring endemic species have focused on taxa from arid lands (Vrijenhoek et al. 1985; Meffe and Vrijenhoek 1988; Echelle et al. 1989; Thompson et al. 2002; Martin and Wilcox 2004; Hurt and Hedrick 2004; Bernardi et al. 2007) where demands from municipal and agricultural users for groundwater have long conflicted with biodiversity conservation (Deacon et al. 2007). Relatively few genetic studies have been conducted in the southeastern United States, however, where recent groundwater demands due to rapid human population growth threaten the rich diversity of coldwater spring endemics (Hubbs 1995; Etnier 1997; Mirarchi et al. 2004).

Within the state of Alabama, seven darter species (Percidae: *Etheostoma*) either permanently inhabit springs or require springs and spring seeps for reproduction. The Watercress Darter (*Etheostoma nuchale*) and Tuscumbia Darter (*E. tuscumbia*) are permanent residents of springs and their associated spring runs. The Coldwater Darter (*E. ditrema*), Goldstripe Darter (*E. parvipinne*), and Rush Darter (*E. phytophilum*) inhabit small headwater streams, springs, spring runs, and seeps. In contrast, the Slackwater Darter (*E. boschungi*) and Trispot Darter (*E. trisella*) normally occupy small to moderately large streams, but migrate into ephemeral seeps during winter months for reproduction. These spring associated darters are some of Alabama's most critically imperiled fishes and are listed as either S1 or S2 conservation status: *E. nuchale* (S1), federally endangered; *E. boschungi* (S2), federally threatened; *E. phytophilum* (S1), federally endangered; *E. ditrema* (S1) and *E. tuscumbia* (S2), state protected; and *E. trisella* was considered extirpated in Alabama until rediscovered in 2008 (Johnson et al. 2011).

The stringent habitat requirements and restricted geographic distributions of these species render them extremely vulnerable to local extirpation and extinction (Etnier 1997). Further, many of the native springs and spring runs occupied by these darters have been capped for industrial and/or residential development, stripped of vegetation, transformed into fishing ponds, or otherwise modified in ways harmful to native species (Mirarchi et al. 2004). Previous genetic studies indicated that the spring endemic *E. nuchale* consists of three highly structured populations, each of which were recommended as distinct management units in future conservation planning (Mayden et al. 2005; Fluker et al. 2010). Further, Fluker et al. (2010) showed that populations of *E. nuchale* exhibited low genetic diversity, and thus increased extinction risk, compared to its widespread stream-dwelling relative, *E. swaini*. Although the genetic characteristics of the federally endangered *E. nuchale* are now better understood, little is

known about the population genetic structure and genetic health, as it relates to conservation practices, for other spring-adapted species throughout the state (Warren et al. 2000; Boschung and Mayden 2004; Mirarchi et al. 2004). Thus, the main objective of this study was to elucidate evolutionary history and population genetic structure and to determine the genetic health of four of Alabama's imperiled spring inhabiting darters (*E. boschungi, E. ditrema, E. phytophilum,* and *E. tuscumbia*).

For each of the four target species, we conducted extensive, range-wide sampling and used a combination of mitochondrial (mt) DNA and nuclear microsatellite (m) DNA data to address the following objectives:

1. Determine population genetic structure within each species.

   Conservation relevance- Identify appropriate management units for conservation planning and better understand connectivity and migration between populations.

2. Determine levels of genetic variation for distinct populations within each species.

   Conservation relevance- Populations with low genetic variation have a higher extinction risk and should have higher conservation priority than populations with higher genetic variation.

3. Determine how demographic factors (e.g. recent versus historic bottlenecks) are reflected in the current patterns of genetic variation for each species.

   Conservation relevance- Management strategies may differ if reductions in genetic variability are recent due to anthropogenic activities versus historic, naturally low levels.

We conclude chapter three with recommendations that will be useful for conservation planning of all spring endemic fishes of the southeastern United States.

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EVOLUTIONARY HISTORY AND CONSERVATION GENETICS OF THE SLACKWATER DARTER (*ETHEOSTOMA BOSCHUNGI*) AND TUSCUMBIA DARTER (*ETHEOSTOMA TUSCUMBIA*)

Introduction

The Slackwater Darter, *Etheostoma boschungi*, is known from four tributaries to the lower bend of the Tennessee River (Cypress Creek, Shoal Creek, Swan Creek, and Flint River) and from tributaries to the Buffalo River system in Lawrence County, Tennessee (Wall and Williams 1974; Boschung and Nieland 1986; McGregor and Shepard 1995; Boschung and Mayden 2004). Based on shared male breeding coloration and breeding habitat preference, *E. boschungi* was placed in the subgenus *Ozarka* with four other darter species (*E. cragini*, *E. pallididorsum*, *E. punctulatum*, and *E. trisella*; Williams and Robison 1980). Males of *Ozarka* share exquisite breeding coloration (red-orange, orange, and blue combinations) and tuberculation patterns (Williams and Robison 1980). Of particular interest is the breeding habitat preference of *Ozarka*, which is unique to darters. During non-spawning times, members of *Ozarka* inhabit gentle riffles and slackwater areas of small to medium sized upland streams. However, for relatively brief periods during winter and early spring, they enter tiny spring-fed rivulets or ephemeral seeps, spawn, and subsequently return to the stream (Hambrick and Robison 1979; Williams and Robison 1980; Boschung and Nieland 1986; Bailey and Etnier 1988). Because of its limited distribution, small population sizes, and loss of habitat throughout its range, the United States Fish and Wildlife Service (USFWS) listed *E. boschungi* as federally threatened in 1977 (USFWS 1977). More recently, loss and alteration of breeding habitat for *E. boschungi* has coincided with decreased abundance at these sites, and has raised concerns about the species' future persistence (Boschung and Nieland 1986; Boschung and Mayden 2004; Johnston 2004).

The Tuscumbia Darter, *E. tuscumbia*, is restricted to limestone springs and spring runs of the lower bend of the Tennessee River (Armstrong and Williams 1971; Page 1983; Boschung and Mayden 2004; Kuhajda 2004). *Etheostoma tuscumbia* was once more widely distributed in springs of the lower bend of the Tennessee River, but impoundments of the river have inundated at least 10 of the historic localities (Etnier and Starnes 1993; Boschung and Mayden 2004). Thus, *E. tuscumbia* is now limited to 14 spring localities (Boschung and Mayden 2004) and a newly discovered locality in Limestone Creek (Bruce W. Stallsmith, pers. comm.; personal observation) where habitat degradation threatens the species' persistence (Jones et al. 1995; Kuhajda 2004). Although *E. tuscumbia* does not receive federal protection, the species is protected under Alabama non-game regulations and is considered to be of high conservation concern (Boschung and Mayden 2004; Kuhajda 2004).

Only recently have molecular phylogenetic studies identified *E. tuscumbia* as sister to *E. boschungi* (Mayden et al. 2006; Lang and Mayden 2007, Near et al. 2011), supporting previous hypotheses of its inclusion in *Ozarka* (Page 2000; Sloss et al. 2004). Although *E. tuscumbia* shares a sister relationship and geographic distribution with *E. boschungi* in the lower bend of the Tennessee River (Figs. 1 and 2), it has a number of uniquely derived characteristics compared to other members of *Ozarka*. In contrast to *E. boschungi* and other *Ozarka*, *E. tuscumbia* lacks male breeding coloration and does not participate in the brief annual breeding migrations into ephemeral seep and spring habitats. Rather, *E. tuscumbia* prefers isolated limestone spring pools throughout its range where it is rarely found in streams or beyond groundwater influence (Armstrong and Williams 1971; Page 1983; Boschung and Mayden 2004). Because of its thermally stable spring habitat, most populations of *E. tuscumbia* have protracted spawning periods, or spawn continuously throughout the year (Koch 1978; Boyce

1997). Remarkably, *E. tuscumbia* populations exhibit flexibility in egg laying strategies and activity patterns that are typically invariable within many darter clades and are rarely variable within species (Koch 1978; Page 1985; Boyce 1997).

In this study, we use comparative phylogeographic and population genetic methods to evaluate the evolutionary history of *E. boschungi* and *E. tuscumbia* and identify appropriate management units within each species. Further, we use several analytical methods to evaluate the relevance of historic versus contemporary events that have given rise to the endangered status of each species. We conclude by discussing how our results should guide conservation planning for *E. boschungi* and *E. tuscumbia*.

Materials and methods

Sample collection and DNA extraction

We obtained tissue samples from across the entire range of *E. boschungi* (*N* = 149) and *E. tuscumbia* (*N* = 295) in tributaries to the lower bend of the Tennessee River (Table 1; Figs. 1 and 2). Samples were collected by seine, or obtained from the University of Alabama Ichthyology Collection (UAIC) frozen tissue collection (Table 2). Due to the difficulty of collecting *E. boschungi* outside of breeding sites (McGregor and Shepard 1995; Boschung and Nieland 1986) and putative decrease in abundance at several extant breeding sites (Hartup 2005; Johnston and Henderson 2007; Rakes and Shute 2008), two of the eight known extant breeding sites yielded only five individuals each despite multiple attempts over a four-year period. Individuals of *E. tuscumbia* were obtained from 12 of the 14 extant spring localities and a newly discovered site in Limestone Creek (Bruce W. Stallsmith pers. comm.). Tissues and voucher specimens taken in the field were preserved in 95% ethanol and 10% formalin, respectively, and deposited into UAIC (Table 2). Genomic DNA was extracted from tissues using the DNeasy kit (Qiagen).

DNA sequencing and microsatellite genotyping

The material for phylogenetic analyses was amplified for the complete mitochondrial (mt) DNA NADH dehydrogenase subunit 2 (ND2) gene (Table 1). A subset of these individuals was amplified for the first intron of the nuclear ribosomal protein S7 (Table 1). Polymerase chain reaction (PCR) amplifications for both loci were conducted using primers and conditions listed in Lang and Mayden (2007). The resulting products were purified using the QIAquick PCR purification kit (Qiagen), cycle sequenced using BigDye® Terminator v3.1 chemistry and read on an ABI PRISM® 3100 Genetic Analyzer (Steven Johnson Molecular Systematics Laboratory, University of Alabama).

All individuals for both species (Table 1) were genotyped for nine microsatellite (m) DNA loci using primers designed for *E. caeruleum* (Eca10EPA, Eca46EPA, Eca48EPA; Tonnis 2006), *E. osburni* (EosC3, EosC6, EosD11, EosD107; Switzer et al. 2008), and *E. scotti* (Esc26b, Esc120; Gabel et al. 2008). PCR amplifications were performed using the Failsafe™ PCR system (Epicentre Biotechnologies) or GoTaq* Flexi DNA polymerase (Promega) under conditions used in Fluker et al. (2010). Fluorescently labeled PCR fragments (HEX and 6-FAM) were mixed with GeneScan™ 500 ROX™ Size Standard (Applied Biosystems) and read on an ABI 3730 DNA analyzer (University of Maine DNA sequencing facility). Prior to analyses, alleles were binned using the program FLEXIBIN 2.0 (Amos et al. 2007) and the dataset was screened for genotyping errors with MICRO-CHECKER (van Oosterhout et al. 2004).

DNA sequence alignment and model selection

Two sets of DNA sequence alignments were constructed; one for phylogeographic reconstruction and one for divergence time estimates. The first alignment consisted of ND2 sequences to reconstruct the phylogeographic history of *E. boschungi* and *E. tuscumbia*. Unique haplotypes were determined for both species using the program DnaSP v5.10 (Librado and Rozas 2009) and the resulting haplotypes were aligned with darter outgroups, including all members of *Ozarka* (Table 2). Best-fit models of nucleotide substitution were evaluated for individual codon positions of ND2 using Akaike information criterion in the program MrModeltest v2.3 (Nylander 2004).

The second alignment consisted of concatenated ND2-S7 sequences to estimate divergence times among *E. boschungi* and *E. tuscumbia*. Because fossil evidence for darters is poor and fossils of close relatives such as *Perca* have been difficult to assess (Cavender 1986), we employed external fossil calibration methods of Hollingsworth and Near (2009), which

utilized DNA sequence data and five fossil calibration points for the closely related family Centrarchidae (Near et al. 2005). Preliminary phylogenetic analysis revealed four highly divergent, well supported clades within *E. boschungi* and shallow divergence within *E. tuscumbia*. Thus, the final alignment consisted of one concatenated sequence from each of the four *E. boschungi* clades, the two most divergent *E. tuscumbia* sequences, 47 Centrarchid taxa (GenBank accession nos. listed in Near et al. 2005), and the same darter outgroups as the phylogeographic analysis (Table 2). The S7 data set was aligned using the program MUSCLE v3.8 (Edgar 2004), ND2 sequences were easily aligned by eye, and the partitions were concatenated in Geneious v5.1.7 (Biomatters Ltd).

Phylogeographic reconstruction and divergence time estimates

To reconstruct phylogeographic histories of both species, Bayesian phylogenetic inference was performed using MrBayes v3.12 (Ronquist and Huelsenbeck 2003) on the ND2 data set. Codon positions were partitioned and the appropriate substitution models were used to set unlinked priors for each (first pos. = HKY + Γ; second pos. = GTR + I; third pos. = GTR + I + Γ). Four separate runs were conducted for 20 million generations with sampling every 1000 generations. Convergence was assessed by monitoring the standard deviation of the split frequencies and all runs prior to convergence (> 0.01) were discarded as burnin to construct the 50% majority-rule consensus phylogram. Nodes with ≥ 0.95 posterior probability were considered to have significant support. We also used the program Tracer v1.5 to determine whether MCMC chains mixed well (effective sample size > 200) during MrBayes and BEAST runs. To visualize genealogical relationships among mtDNA sequences, haplotype networks were constructed using statistical parsimony in TCS v1.21 (Templeton et al. 1992; Clement et al. 2000). TCS constructs haplotype relationships by calculating the maximum number of

substitutions to connect two haplotypes parsimoniously with 95% confidence. TCS also estimates outgroup probabilities, identifying probable roots in the network and revealing the most ancient haplotypes (Castelloe and Templeton 1994).

To estimate divergence times between clades of the two species, the program BEAST v1.6.1 (Drummond and Rambaut 2007) was implemented with priors for minimum bound lognormal age estimates on Centrarchid fossil calibration points used in Hollingsworth and Near (2009). These methods have been used in several other analyses, all of which generated consistent estimates of time of most common recent ancestor (TMRCA) for Centrarchidae and major darter groups (Near and Benard 2004; Near and Keck 2005; Keck and Near 2010). Substitution models were unlinked among ND2 (TrN + I + Γ) and S7 partitions (GTR + Γ). To allow for uncertainty in substitutions rates on adjacent branches, an uncorrelated lognormal clock model was employed with a Yule process speciation tree prior. Four independent runs of 40 million generations were conducted and tree and log files were combined with 40% of the generations discarded as burnin using LogCombiner v1.6.1.

Estimates of genetic diversity, population structure, and gene flow

Departures from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were assessed in GENEPOP v4.0.10 (Rousset 2008) using a Markov Chain algorithm with 10,000 dememorizations, 200 batches and 10,000 iterations per batch. Measures of genetic diversity among STRUCTURE defined populations (see results) were estimated from mDNA as mean number of alleles per locus (A), heterozygosity observed (H_o), and heterozygosity expected (H_e) using GENEPOP. To provide an estimate of the number of alleles and private alleles per locus with equal sample sizes, allelic richness (AR) and private allelic richness (PA) were estimated using the program HP-RARE (Kalinowski 2005).

Genetic differentiation was assessed using mtDNA and mDNA for differing hierarchical groupings using analysis of molecular variance (AMOVA, Excoffier et al. 1992) and Φ_{st}, an analogue of F_{st} that incorporates molecular distance, in ARLEQUIN v3.5 (Excoffier and Lischer 2010) with 10,000 permutations. First, we tested the null hypothesis that individuals from each locality represent an arbitrary subsample relative to the entire range of the species. Second, we asked whether genetic variation was more proportionally distributed among vs. within tributaries to the Tennessee River. In the latter test, spring sites were grouped within tributaries that shared a single connection to the Tennessee River. For *E. boschungi*, groups were as follows: 1) Cypress Creek (CB, DD); 2) Shoal Creek (SH, CH, NF, SF); 3) Swan Creek (SW); and 4) Flint River (FL) (Table 1; Fig. 2). Samples from the Buffalo River (CH, NF, SF) were grouped with Shoal Creek (SH) because preliminary analyses suggested that Buffalo River populations were recently founded from Shoal Creek. For *E. tuscumbia*, groups were as follows: 1) Spring Creek west (TS); 2) Cypress Creek (BF); 3) Spring Creek east (WH); 4) Pryor Branch (PY); 5) Limestone Creek (LM, TH, PK, BD); 6) Indian Creek (KL, BR, BY); and 7) Flint River (MV, WC) (Table 1; Fig. 1).

The program STRUCTURE 2.3 (Pritchard et al. 2000) was used with mDNA data to determine the number of genetically differentiated clusters (K) within both species without *a priori* designations. For both species, we implemented models allowing admixture of genotypes and correlated allele frequencies (Falush et al. 2003). Ten replicate STRUCTURE runs of 1,000,000 iterations (300,000 burnin) were conducted for K values ranging from 2 to 10 and 2 to 15 for *E. boschungi* and *E. tuscumbia*, respectively. Best estimates of K for each species were determined using the ad hoc summary statistic ΔK (Evanno et al. 2005) as implemented in

STRUCTURE Harvester (Earl 2011). Bar plots of the best estimates of *K* were constructed using *Distruct* v1.1 (Rosenberg 2004).

We performed coalescent analyses to estimate the parameter Θ (mtDNA = $N_e\mu$, mDNA = $4N_e\mu$) and migration rates (*M*) for mtDNA sequences and mDNA loci using the Bayesian method in LAMARC v. 2.1.6 (Kuhner 2006). We were specifically interested in comparing levels of gene flow among and within tributaries to the Tennessee River for both species. To avoid prohibitively large gene genealogies and number of parameters, which can lead to poor estimates (see LAMARC documentation), we constructed reduced data sets for LAMARC analyses. Estimates of among tributary migration for *E. boschungi* were conducted using the following groups: (1) Cypress Creek (CB); (2) Shoal Creek (SH/CH); (3) Swan Creek (SW); and (4) Flint River (FL) (Table 1; Fig. 2). Two independent tests were conducted to evaluate within tributary migration for *E. boschungi*. The first compared populations within Cypress Creek (CB and DD) and the second compared populations within the Buffalo River (NF and SF) (Table 1; Fig. 2). We used the following groupings to estimate among tributary migration for *E. tuscumbia*: (1) Cypress Creek (BF); (2) Spring Creek west (WH); (3) Limestone Creek (TH/PK/BD); and (4) Flint River (MV) (Table 1; Fig. 1). Three independent analyses were conducted to estimate within tributary migration for *E. tuscumbia*: (1) populations within the Limestone Creek drainage (LM and TH/PK/BD); (2) populations within Indian Creek (KL, BR, and BY); and (3) populations within Flint River (MV and WC) (Table 1; Fig. 1). All mtDNA sequences for each population were used in estimations, while mDNA data sets consisted of 15 randomly sampled individuals from their respective STRUCTURE group. Each run was conducted with three replicates of four initial chains of 20,000 steps (1,000 trees sampled every 20 reps, burn-in = 2000) and one final chain of 600,000 steps (30,000 trees sampled every 20 reps, burn-in =

30,000) using an adaptive heating scheme with 4 settings (1.0, 1.1, 1.2, 1.3). Priors for Θ ranged from 1.0×10^{-5} to 10.0 and priors for *M* ranged from 1.0×10^{-2} to 1000. The number of effective immigrants per generation ($4Nm$) for each population pair was estimated by multiplying *M* by its estimate of Θ for the recipient population (Kuhner 2006; Kuhner and Smith 2007). Differences in among tributary and within tributary migration rates between the two species were evaluated using Mann-Whitney U tests in SPSS® (IBM®).

To test for isolation-by-distance (IBD), matrices of pairwise F_{st} values of mtDNA and mDNA were compared to geographic distance using the Mantel permutation test (Mantel 1967) as implemented in ARLEQUIN. We performed four Mantel tests that followed groupings in AMOVA analyses. The first two tests were conducted independently for both species to evaluate the possibility IBD between all localities range-wide. The next two tests were conducted independently for both species to evaluate the possibility of IBD among major tributaries to the Tennessee River. Geographical distances were measured as the shortest distance (by water) in kilometers.

Demographic history

A battery of statistics including F_s (Fu 1997), R_2 (Ramos-Onsins and Rozas 2002), and Tajima's *D* (Tajima 1989) was employed with our mtDNA data to test for departures from constant population size (or neutrality) in the five clades defined by our phylogeographic analysis (Fig. 3). Of these statistics, Ramos-Onsins and Rozas (2002) showed that F_s and R_2 have the greatest power to detect population growth, where growth is indicated by significantly large negative and small positive values of each, respectively. Specific combinations of the estimated value and significance of Tajima's *D* are also useful to distinguish between differing evolutionary processes. For example, significantly negative values of Tajima's *D* suggest growth

or selective sweep, whereas the reverse may indicate selection, population subdivision, or recent population bottleneck (Tajima 1989). Estimates and corresponding significance of F_s, R_2, and Tajima's D were determined with 10,000 coalescent simulations in DnaSP.

To evaluate whether populations have experienced recent change in effective population size (N_e) based on mDNA, we used M_P_Val.exe and Critical_M.exe (Garza and Williamson 2001) to compare the ratio of the number of alleles to the range in allele size (M) to population specific critical M values (M_c). Briefly, M is expected to decrease with reduced N_e. For example, declining populations are expected to have M-ratios $< \sim 0.7$ due to the rapid loss of alleles when compared to the range in allele size, whereas more stable population sizes should have M-ratios closer to one (Garza and Williamson 2001). The M-ratio was estimated using a two-phase model (Di Rienzo et al. 1994) with 95% single-step mutations, average size of non one-step mutations = 3.5, and pre-bottleneck Θ values of 10. The probability that a smaller M-ratio would be expected at equilibrium was assessed with 10,000 simulations.

Results

Phylogeographical structure and divergence time estimates

Of the 106 darters sequenced, 42 unique mtDNA haplotypes were identified (*E. boschungi* = 17, *E. tuscumbia* = 25; Table 1). Standard deviation of split frequencies in MrBayes runs fell below 0.01 in 2.5 x 10^6 generations and parameter values were highly convergent among runs. Thus, the 50% majority rule consensus phylogram was constructed from four runs of 20,000 trees each (15,000 trees used, 5,000 discarded; Fig. 3). The Bayesian analysis recovered *E. boschungi* and *E. tuscumbia* as sister with substantial mtDNA divergence among species (11.6%; Fig. 3). Four highly divergent clades were detected within *E. boschungi* (6.1% mean among clade sequence divergence), all but one of which was geographically structured according to distinct tributaries to the Tennessee River (Figs. 2, 3). Clade 2 (Figs. 2, 3) was the only exception, containing individuals from Shoal Creek and Buffalo River localities. The analysis revealed shallow divergence among all *E. tuscumbia* haplotypes (0.43% mean sequence divergence), with no apparent geographic structuring based on locality or tributary (Fig. 3). The statistical parsimony analysis recovered four disconnected clades for *E. boschungi* (not shown) that corresponded to clades defined in the MrBayes analysis (Figs. 2, 3). The analysis was able to connect all *E. tuscumbia* haplotypes with 95% confidence and revealed that most low frequency or singleton haplotypes derive from the ancestral haplotype 26 or the highly abundant haplotype 25 (Fig. 3). Both haplotypes 25 and 26 are widespread and abundant in the central portion of the range of *E. tuscumbia* (Pryor Branch [PY], Limestone Creek [LM, TH, PK, BD], and Indian Creek [KL, BY, BR]; Table 1; Figs. 1, 3). However, each of these central localities had one or two uniquely derived haplotypes (Table 1; Figs. 1, 3). All localities at the margin of the range of *E. tuscumbia*, i.e. Cypress Creek, Spring Creek (west and east), and Flint River were

characterized by unique, derived haplotypes (Table 1; Figs. 1, 3). In particular, haplotypes 34 (PK) and 42 (MV) showed the highest degree of divergence from all other haplotypes, with six and seven mutational differences from the ancestral haplotype 26, respectively (Fig. 3).

The BEAST analysis of ND2-S7 data yielded TMRCA for Centrarchidae (33.9 million years ago [Mya], 95% highest posterior density (HPD): [27.2, 41.9]) that was highly consistent with previous estimates using this calibration method (Hollingsworth and Near 2009; Near et al. 2011). BEAST runs with no data (empty) resulted in older divergence time estimates, suggesting that calibration priors did not overpower information in our data set. Figure 4 shows the chronogram of all darter taxa, pruned from the larger analysis with centrarchids. The analysis indicated deep divergence between *E. boschungi* and *E. tuscumbia* (8.4 Mya [5.9, 11.3]) and among the four clades of *E. boschungi* (4.9 Mya [3.3, 6.8]; Fig. 4). The analysis indicated TMRCA for *E. tuscumbia* in the late Pleistocene (0.6 Mya [0.2, 1.1]), which was much younger than estimates for clades within *E. boschungi* (Fig. 4).

Population genetic diversity, structure, and gene flow

Of the nine mDNA loci amplified for *E. boschungi*, EosC3 was monomorphic and EosD11 failed to amplify for all populations, thus both were removed from the data set. MICRO-CHECKER suggested the possibility of null alleles for some populations at locus Esc120, but subsequent analyses with and without this locus were highly consistent. Thus Esc120 was used in all final analyses of mDNA data. Following Bonferroni correction, only three of the 39 population-locus comparisons for *E. boschungi* deviated from HWE and resulted from a deficiency of heterozygotes. Seven of the 9 loci were successfully amplified for *E. tuscumbia*. Loci Eca48 and Esc120 failed to amplify across all populations and were removed from the data set. Seven of the 80 tests for HWE showed significant deviations following Bonferroni

correction, all of which resulted from deficiency of heterozygotes. However, as with *E. boschungi*, there was no clear pattern of deviation from HWE within a single population and analyses with and without potentially problematic loci were highly congruent. No loci showed evidence of linkage.

Both *E. boschungi* and *E. tuscumbia* were similar for number of mDNA alleles per locus (2−15 and 2−19, respectively). Measures of allelic variation (*A* and AR) varied widely among populations within both species, with *E. tuscumbia* showing slightly lower average values (Table 3). Average values of H_o and H_e were slightly lower in *E. tuscumbia* relative to *E. boschungi*, and *E. tuscumbia* exhibited a greater range of heterozygosity values compared to *E. boschungi* (Table 3).

The STRUCTURE analysis for *E. boschungi* revealed a high degree of population structure, with clusters corresponding to individual breeding sites or geographically proximate breeding sites ($K = 6$; Fig. 2). Analysis of *E. tuscumbia* also revealed high levels of genetic structure across the study area ($K = 8$; Fig. 1). However, clusters were more structured at the eastern and western margins of the range, and there was a high degree of admixture among localities within the central portion of the species' range (Fig. 1). Plots of the 'estimated ln probability of the data' versus K and ΔK were unambiguous for $K = 6$ and $K = 8$ for *E. boschungi* and *E. tuscumbia*, respectively.

The AMOVAs were largely consistent with results of the phylogeographic and STRUCTURE analyses, in which *E. boschungi* (F_{st} [mtDNA] = 0.98; F_{st} [mDNA] = 0.29) showed a higher degree of among locality differentiation compared to *E. tuscumbia* (F_{st} [mtDNA] = 0.63; F_{st} [mDNA] = 0.25; Table 4). When pair-wise F_{st} values were compared to geographic distance among localities, there were significant IBD patterns in *E. tuscumbia* for

both mtDNA (r = 0.53, P = 0.006) and mDNA (r = 0.58, P = 0.001). Comparisons in *E. boschungi* showed significant IBD patterns for mtDNA (r = 0.65, P < 0.001), but not for mDNA (r = 0.10, P = 0.338). In AMOVAs that grouped populations among tributaries, differentiation was significant and similar to among locality values for *E. boschungi* (F_{st} [mtDNA] = 0.95, P < 0.001; F_{st} [mDNA] = 0.10, P < 0.05; Table 4). However, among tributary differentiation for *E. tuscumbia* was not significantly different from zero (F_{st} [mtDNA] = 0.11, P = 0.323; F_{st} [mDNA] = 0.07, P = 0.134; Table 4). Patterns of IBD among tributaries for *E. boschungi* were significant for mtDNA (r = 0.58, P < 0.001), but not for mDNA (r = -0.53, P = 0.973). Tests of IBD among tributaries for *E. tuscumbia* were not carried out due to non-significant F_{st} values.

Coalescent estimations of effective migrants per generation among tributaries were low for both *E. boschungi* ($\bar{x}$ = 0.24 [mDNA], $\bar{x}$ = 0.02 [mtDNA]) and *E. tuscumbia* ($\bar{x}$ = 0.31 [mDNA], $\bar{x}$ = 0.11 [mtDNA]), with only one comparison > 1.0 for *E. tuscumbia* (Tables 5 and 6). Migration within tributaries was higher for both *E. boschungi* ($\bar{x}$ = 1.78 [mDNA], $\bar{x}$ = 0.51 [mtDNA]) and *E. tuscumbia* ($\bar{x}$ = 2.19 [mDNA], $\bar{x}$ = 0.95 [mtDNA]), with several comparisons among localities > 1.0 for both species (Tables 5 and 6). MtDNA based migration rates among tributaries were significantly lower in *E. boschungi* when compared to *E. tuscumbia* (P = 0.014). However, migration rates between species for all other comparisons were not significantly different.

Demographic history

Coalescent estimations of F_s, R_2, and Tajima's D for the four clades of *E. boschungi* (Fig. 3) were not significant, indicating no evidence of departure from constant population size. Only F_s (-9.39, P < 0.001) was significant for *E. tuscumbia* (clade 5; Fig. 3), indicating a signature of population growth. Microsatellite based M-ratios of all *E. boschungi* populations were at or

above critical values, indicating no evidence for recent reductions in N_e (Table 3). In contrast, *M*-ratios of five of the eight populations of *E. tuscumbia* were significantly below critical *M* values, indicating recent or prolonged reductions in N_e (Table 3). Four of the five *E. tuscumbia* populations with evidence for reduced N_e were at localities on the eastern and western margins of the species range (Table 3; Fig. 1).

Discussion

Habitat preference and dispersal ability

Etheostoma boschungi is clearly structured among tributaries to the Tennessee River and our BEAST analysis suggested a lack of gene flow among these tributaries since mid-early Pleistocene times (Figs. 2 and 4). In contrast, the shallow divergence within *E. tuscumbia* suggests very recent divergence (mid-late Pleistocene) within this species (Figs. 1 and 4). The patterns observed in *E. tuscumbia* were unexpected based on patterns seen in other spring inhabiting darters of the region. For example, the Watercress Darter (*E. nuchale*) and the Rush Darter (*E. phytophilum*) both exhibit high degrees of phylogeographic structure among populations distributed in small tributaries to the Black Warrior River (Fluker et al. 2010; Fluker unpubl. data).

Large streams and rivers are thought to pose strong barriers to dispersal for spring inhabiting fishes of the southeastern United States (Starnes and Etnier 1986). In addition, recent studies suggest that darters with highly specific habitat requirements, particularly in headwaters, can experience micro-allopatric divergence or reduced gene flow among populations separated by major river courses (Turner and Robison 2006; Hollingsworth and Near 2009). Thus, we predicted that both *E. boschungi* and *E. tuscumbia* would show greater population differentiation among tributaries to the Tennessee River, versus within. Given the strict breeding habitat requirements of *E. boschungi*, we further predicted greater differentiation both among and within tributaries compared to *E. tuscumbia*. The AMOVA and migration estimates indicated that both species were highly structured across their ranges and *E. boschungi* exhibited higher levels of differentiation compared to *E. tuscumbia* (Table 4). However, when compared among tributaries to the Tennessee River, significant differentiation was only detected for *E. boschungi* (Table 4).

Interestingly, IBD was detected for *E. boschungi* based on mtDNA, but not for mDNA. Hutchison and Templeton (1999) suggested that IBD patterns become difficult to interpret when populations experience long-term isolation and are separated by considerable distance. Thus, we suspect significant IBD patterns in mtDNA for *E. boschungi* reflect a strong correlation between barriers to gene flow (i.e. the Tennessee River) and geographic distance rather than an explicit IBD effect. Significant patterns of IBD were detected in both data sets across the range of *E. tuscumbia*, suggesting populations are in migration-drift equilibrium. Both species exhibited moderate levels of differentiation within tributaries, but values varied little among species and migration rates were not significantly different. For example, *E. tuscumbia* showed moderate levels of mtDNA structure, and both species showed approximately equal levels of mDNA structure among localities within tributaries (Table 4).

The odd occurrence of *E. boschungi* in tributaries to the Buffalo River provides a unique insight into the dispersal mechanisms for this species. The distribution of suitable breeding habitats for *E. boschungi* is highly discontinuous, and determined by stream order and local geological conditions. For example, breeding habitats are ephemeral tributaries of small order streams within the Fort Payne Chert formation of the Interior Low Plateaus Physiographic Province (Osborne et al. 1988; Szabo et al. 1988; Boschung and Nieland 1986; McGregor and Shepard 1995). The propensity of *E. boschungi* to migrate into these seeps is great, and breeding adults have been found at the most upstream reaches where water depths may be as little as 4 cm (Boschung and Nieland 1986). These ephemeral breeding tributaries closely interdigitate among the low divides separating the Buffalo River and Shoal Creek and it is likely that rare chance events such as stream capture or flooding allow brief periods of dispersal between drainage basins. Our genetic data indicate recent and possibly ongoing gene flow between the Buffalo

River and Shoal Creek populations. For example, NF and SF are differentiated from SH and CH but all fall within clade 2 (Fig. 2). Further evidence from both data sets suggest very recent or ongoing gene flow between SH and CH (Fig. 2). Thus, inter-tributary dispersal in *E. boschungi* is likely determined by proximity of favorable breeding sites that interdigitate among drainage divides, rather than dispersal through larger order streams and rivers.

Dispersal mechanisms are much different for *E. tuscumbia*. That the Tennessee River was not a significant barrier to dispersal was surprising for this spring specialist and evidence that all extant populations of *E. tuscumbia* recently stemmed from the more diverse, centrally located populations supports the extraordinary dispersal ability of this spring endemic. With the exception of locality BD, which falls within the Fort Payne Chert Formation, the location of suitable spring habitats for *E. tuscumbia* lies within the Tuscumbia Limestone formation, which adheres closely to the valley floor of the Tennessee River (Osborne et al. 1988; Szabo et al. 1988; Etnier and Starnes 1993). Numerous known, and probably many unknown, spring localities for *E. tuscumbia* were inundated with the construction of Pickwick Reservoir in 1938 (Fig. 1), indicating a formerly more widespread distribution of this species and its habitats (Etnier and Starnes 1993; Mettee et al. 1996; Boschung and Mayden 2004). Thus historic spring to spring dispersal may have involved brief forays along the margins of the Tennessee River when springs were much more abundant along the valley floor.

Combined with the phylogeographic analysis, our population genetic analyses show that the Tennessee River poses a barrier to gene flow for the more specialized *E. boschungi*, but not for *E. tuscumbia*. However, both species can be highly isolated to habitats within the same tributary, which is not uncommon for spring specialists (Fluker et al. 2010). The strikingly different patterns of population structure between *E. boschungi* and *E. tuscumbia* indicate that

habitat preference influences dispersal ability and genetic differentiation within these species. However, their differing demographic histories point to possible differences in habitat stability, and ignoring factors such as drainage history may confound the ability to understand the current patterns of genetic diversity (Burridge et al. 2008). Thus, differences in genetic structure and demographic history between these two species must be understood in the context of the complex paleohydrologic history of the lower bend of the Tennessee River.

Habitat stability and genetic differentiation

We found no evidence for population expansion or prolonged bottlenecks for *E. boschungi*, suggesting relatively constant long-term population sizes for this species. Based on the BEAST analysis (Fig. 4), this would suggest long-term stability, at least since early-mid Pleistocene, of suitable habitat for *E. boschungi*. The star-like patterns observed in the mtDNA haplotype network and the significantly negative F_s values for *E. tuscumbia* are indicative of recent population growth (Fu 1997; Avise 2000; Fig. 3). However, four of the marginal populations and one central population of *E. tuscumbia* have passed through recent or prolonged bottlenecks based on mDNA data (Table 3). Additionally, marginal populations exhibited higher mDNA genetic differentiation, generally lower levels of mDNA variation, and reduced mtDNA haplotype diversity (Table 3; Figs. 1 and 3). Collectively, these patterns are indicative of a history of frequent extinction and colonization events (Pannell 2003), suggesting that springs inhabited by *E. tuscumbia* were less stable throughout the Pleistocene. Habitat instability of springs inhabited by *E. tuscumbia* could have occurred during the Pleistocene in two main ways. Climate fluctuations during the Pleistocene had a dramatic effect on species range dynamics in North America (Hewitt 2011). During climate fluctuations, dryer periods could have resulted in local extinction of springs or range contraction, whereas wetter periods could have caused local

reconfigurations of spring out flows or range expansions (Williams 1968; Fluker unpubl. data). An alternative explanation can be drawn from hypothesized course reconfigurations of lower portions of the Tennessee River during the Pleistocene (Thornbury 1965; Braasch and Mayden 1985; Starnes and Etnier 1986). These reconfigurations could have drastically altered the flow regime of the Tennessee River, resulting in periods of incision and aggradation, and aquifers associated with the river system would have been heavily impacted by such events (Creuzé des Châtelliers et al. 1994). Drill cores from the lower Tennessee River show Pleistocene valleys buried at least 21 meters below the present valley depth, and groundwater levels were equally depressed during this time (Moneymaker 1941). Thus, groundwater habitats closely aligned with the Tennessee valley (i.e. the Tuscumbia Limestone formation) may have been more heavily impacted during the Pleistocene, and those at higher elevations and further distance from the valley (i.e. the Fort Payne Chert formation) may have served as refugia during times of instability. For example, the two most upstream localities in Indian Creek (KL) and Flint River (WC) were resolved as a homogeneous cluster in the structure analysis and WC is monomorphic for an mtDNA allele present in KL (Fig. 1), suggesting a recent connection between these two disjucnt upstream localities or relicts of a shared distribution. The two divergent haplotypes recovered in the analysis (34 and 42; Table 1; Fig. 3) were found at the central locality PK and an upstream locality MV, possibly reflecting relict haplotypes retained in refugia. Interestingly, BD is one of the largest spring wetlands in the region and the only spring inhabited by *E. tuscumbia* that falls outside of the Tuscumbia Limestone formation. Accordingly, central positioned localities near the unique BD show the highest levels of genetic variation and extensive mtDNA haplotype sharing and mDNA admixture (Figs. 1 and 3). The co-occurrence of two other spring endemic fishes provides further evidence for long-term stability of spring

habitats within the range center of *E. tuscumbia*. The Spring Pygmy Sunfish (*Elassoma alabamae*) was once known from two tributaries to the Tennessee River, but now is only known from the Beaverdam Creek system (Mayden 1993; Localities BD and TH, Fig. 1). The Whiteline Topminnow (*Fundulus albolineatus*) was known only from Big Spring (Indian Creek; Fig. 1), but is now extinct due to recent anthropogenic disturbance (Williams and Etnier 1982). Although we can only speculate on the causes of instability and the effects on spring habitats, our results clearly indicate recent patterns of extinction and recolonization within *E. tuscumbia*. There is further evidence that areas within its range center were more stable during the Pleistocene and may have served as refugia during instability.

It is likely that the impoundment of the Tennessee River has also acted to recently isolate *E. tuscumbia* populations on the range margins. For example, our data indicate that most, if not all, populations of *E. tuscumbia* were intermittently connected throughout the species history. However, the presence of recent bottlenecks and low genetic diversity in marginal populations indicates the possibility that migration routes between central and marginal populations may now be blocked by inundated waters.

Conservation implications and recommendations

Slackwater Darter, *Etheostoma boschungi*

Taxonomic status

Phylogenetic analyses conducted in this study showed that *E. boschungi* consists of four highly divergent clades (Figs. 3 and 4), suggesting that the species, as currently described, is harboring cryptic diversity that may warrant species recognition. The degree of genetic divergence observed among these four clades approaches or exceeds levels of divergence between currently recognized darter species. Accordingly, a thorough morphological analysis of *E. boschungi* is needed to determine the extent of cryptic diversity within this species. Unfortunately, there are few museum specimens of *E. boschungi* for morphological analysis and collection of additional breeding adults may further threaten the rarer breeding populations. Thus, modern approaches of species delimitation using DNA sequence data from multiple nuclear markers may be a viable approach in the future to resolve species boundaries within *E. boschungi*.

Genetically distinct groups within E. boschungi

Based on analysis of mitochondrial DNA sequence data and multiple nuclear microsatellite DNA markers, we identify four groups within *E. boschungi* that warrant independent conservation consideration, and each group may very well represent distinct species. Based on mtDNA, these four groups are reciprocally monophyletic and based on both mtDNA and mDNA, these groups are significantly genetically differentiated from one another. Together, these results indicate that the four groups have been historically isolated from one another, share no ongoing or recent gene flow, and represent major elements of diversity for conservation (analogous to the 'evolutionary significant unit' [ESU] of Moritz [1994, 1999]). The analysis of

mDNA allowed us to further identify groups analogous to 'management units' (MU; *sensu* Moritz 1994, 1999) within two of the four major groups. Below, we define the four groups within *E. boschungi* and provide explanations of genetic structure within each group:

1. Cypress Creek group

Cypress Creek was once considered the 'stronghold' for *E. boschungi* because of its relatively widespread presence throughout the system (Bruton Branch, Burcham Creek, Lindsey Creek, Cypress Creek mainstem, North Fork, Dulin Branch, Greenbrier Branch, and Middle Cypress Creek; Boschung and Nieland 1986). However, surveys by McGregor and Shepard (1995) revealed an absence of *E. boschungi* at several historic sites within the Cypress Creek system. This study and other recent surveys (Johnston and Hartup 2001, 2002; Johnston and Henderson 2007, 2008) have only detected *E. boschungi* at or in the vicinity of two breeding sites: (1) Dodd Site #1 (DD), Middle Cypress Creek (35.06060°N, -87.77250°W); and (2) Unnamed tributary to Cooper Branch (CB) on Natchez Trace Parkway (35.01589°N, -87.82322°W). Our genetic analysis of mDNA indicated that *E. boschungi* from these two breeding sites are genetically distinct from one another (Fig. 2) and should be treated as distinct management units within the Cypress Creek group. Although census sizes of these two breeding populations are relatively low (Boschung and Nieland 1986), our genetic analysis showed that neither of these breeding populations have suffered from recent declines in effective population size (i.e. recent genetic bottlenecks; Table 3). Thus, there is limited evidence that either breeding population has suffered recent ill genetic effects from isolation or inbreeding depression.

2. Shoal Creek-Buffalo River group

As mentioned above (see discussion), the occurrence of *E. boschungi* in the Buffalo River system is likely the result of recent, natural interdrainage transfers from the Shoal Creek

system. In this study, the Shoal-Buffalo group is comprised of samples from four breeding sites: (1) Little Shoal Creek (SH) headwaters (35.32694°N, -87.27278°W); (2) Chief Creek (CH) at Barnett Road (Buffalo River system, 35.35972°N, -87.41917°W); (3) Gum Springs Branch (North Fork Buffalo River [NF], 35.42472°N, -87.28139°W); and (4) South Fork Buffalo River (SF) at Jap Lane (35.36194°N, -87.25528°W). Our genetic analysis of mDNA indicated that *E. boschungi* from Shoal and Chief creeks share recent or ongoing gene flow and are genetically indistinguishable. Similarly, *E. boschungi* from North and South forks of the Buffalo River share recent or ongoing gene flow and were not genetically distinct from each other. However, samples from Shoal and Chief creeks are genetically distinct from those in the North and South forks of the Buffalo River (Fig. 2). Thus, we recognize Shoal + Chief creek samples and North + South Fork Buffalo River samples as two distinct breeding populations that should be treated as distinct management units within the Shoal-Buffalo group. Although census sizes of these two breeding populations are relatively low and recent repeated attempts by us failed to collect any specimens from Shoal or Chief creeks, our genetic analysis showed that neither of these breeding populations have suffered from recent declines in effective population size (i.e. recent genetic bottlenecks; Table 3). Thus, there is limited evidence that either breeding population has suffered recent ill genetic effects from isolation or inbreeding depression.

3. Swan Creek group

We made several attempts to collect *E. boschungi* from historic localities in the Swan Creek system (see McGregor and Shepard 1995) throughout the study period. Despite multiple attempts, we were only able to obtain samples of *E. boschungi* from the mainstem of Swan Creek at Elkton Road (34.8319°N, -86.95156° W) and from an unnamed tributary to Swan Creek approximately 100 meters downstream of the Elkton Road Bridge (34.8333°N, -86.9475°W).

Although our *E. boschungi* samples from Swan Creek represent a single breeding population, genetic variability (*A*, AR, H_o, H_e; Table 3) of this population is relatively high compared to breeding populations in the Cypress Creek and Shoal-Buffalo groups. Additionally, we found no evidence for recent genetic bottlenecks within the Swan Creek group (Table 3). The unnamed tributary represents a newly discovered breeding site for *E. boschungi* in the Swan Creek system and land owners (Mr. and Mrs. Broadwater) were very cooperative during our collection efforts. This breeding site would likely benefit from habitat restoration projects that aimed to replace piped road culverts with box culverts or span bridges to improve accessibility, prevent channel incision and increase instream habitat for *E. boschungi* (i.e. aquatic vegetation).

4. Flint River group

We obtained samples of *E. boschungi* from two breeding sites within the Brier Fork of the Flint River: (1) Unnamed tributary to Brier Fork at Scott Orchard Road (34.99897°N, -86.67643° W); and (2) Brier Fork at Fowler Road (35.01518°N, -86.65516° W). These two breeding sites are separated by approximately 3 km and, based on mDNA, represent a single breeding population. Despite poor breeding habitat conditions at these two sites throughout the study period, we found no evidence for recent genetic bottlenecks and levels of genetic variability (Table 3) were higher in the Flint River group compared to all other groups of *E. boschungi*.

Priorities for conserving E. boschungi

The unique breeding strategy of *E. boschungi* makes it a very elusive fish to monitor by standard sampling and survey techniques. For example, detection of *E. boschungi* at breeding sites may depend heavily on the annual physical condition of the breeding site (e.g. volume output of seepage water, inundation by beaver dams) or simply sampling the breeding site during

the small window of time when *E. boschungi* are present and spawning (Boschung 1976; McGregor and Shepard 1995). Thus, knowledge of the levels of population genetic diversity for imperiled species is a viable supplement to standard sampling for prioritization of populations for conservation. For example, populations with lower genetic diversity are more susceptible to stochastic events and are less likely to adapt to environmental change (Frankel 1974; Spielman et al 2004; Frankham 2005). Based on genetic variability (Table 3), we prioritize distinct groups of *E. boschungi* for conservation action in the following order: (1) Cypress Creek group; (2) Shoal Creek-Buffalo River group; (3) Swan Creek group; (4) Flint River group. However, all four distinct groups of *E. boschungi* suffer equally from extensive habitat alteration and loss of breeding habitat (Boschung and Nieland 1986; Johnston and Hartup 2001, 2002; Boschung and Mayden 2004; Johnston and Henderson 2007, 2008). Thus, habitat restoration projects that restore connectivity between non-breeding and breeding habitat and that maintain the integrity of breeding sites are highest priority for all distinct groups of *E. boschungi* and should be carried out for lower priority populations when funding is available.

Recommendations for captive propagation programs

Captive propagation techniques have been established for *E. boschungi* (Rakes and Shute 2008). If future conservation actions for *E. boschungi* involve propagation, translocation, reintroduction, and/or augmentation (PTRA; George et al. 2009), we recommend the following protocols.

1. Any and all activities involving PTRA of *E. boschungi* should follow the protocols set forth in George et al. (2009). To our knowledge, George et al. (2009) is the most current and comprehensive guide for PTRA of freshwater fishes and would provide the greatest chances of success for such programs involving *E. boschungi*.

2. Under NO circumstances should stocks from the four genetically distinct groups of *E. boschungi* (Cypress Creek group, Shoal Creek-Buffalo River group, Swan Creek group, and Flint River group) be mixed. Each of these groups represents a distinct and significant component of diversity that has a unique history in its respective geographic setting. Even in the event that one of these major groups goes extinct, it is unadvisable to reintroduce *E. boschungi* from one of the other distinct groups. Reintroduction practices of this nature often do more harm than good and can threaten other species native to the area (George et al. 2009).
3. Distinct management units within the Cypress Creek and Shoal-Buffalo groups should be treated separately in any PTRA activities. If, at some time in the future, it is determined that a management unit is suffering ill genetic effects from isolation, population collapse, inbreeding depression, or have gone extinct, it would be advisable to reintroduce *E. boschungi* from another management unit within its major group. However, this action would only be advisable under the following circumstances: (1) future genetic studies reveal that isolation of management units is causing substantial loss of genetic variation or high levels of inbreeding depression; (2) extensive, long-term surveys (10–20 years) using multiple collection techniques (i.e. dipnets, seines, backpack electro-shocker) reveal an absence of *E. boschungi* at all known breeding sites for a given management unit; (3) appropriate breeding and non-breeding habitat are sufficient to support reintroduced *E. boschungi*; and (4) protocols follow those of George et al. (2009).

Tuscumbia Darter, *Etheostoma boschungi*

Taxonomic status

Phylogenetic analyses conducted in this study showed that *E. tuscumbia* forms a single clade comprised of closely related spring populations that share a very recent history (Figs. 3 and 4). Thus, we consider *E. tuscumbia* as a single species throughout its range.

Genetic structure and priority units for conservation within E. tuscumbia

Although our analysis of mtDNA revealed that spring populations share a recent history of connectivity, fine-scale analyses of mDNA revealed some alarming trends for several spring populations of *E. tuscumbia*. Our analyses indicate that the Tennessee River (historically) was not a significant barrier to dispersal for *E. tuscumbia*, suggesting that spring populations were connected via gene flow in the recent past (late Pleistocene times). However, most of the springs on the margins of the range of *E. tuscumbia* are highly structured, have low genetic variability, and have experienced recent genetic bottlenecks (Table 3). These results suggest that connectivity between central and marginal springs has been recently disrupted. Given the spatially isolated nature of springs inhabited by *E. tuscumbia*, the observed genetic structure and low genetic diversity in marginal populations may be somewhat natural. However, it is likely that the impoundment of the Tennessee River created a barrier to long-distance dispersal for *E. tuscumbia*. Below, we identify the following groups analogous to 'management units' (MU; *sensu* Moritz 1994, 1999) within *E. tuscumbia* and rank them highest to lowest conservation concern based on genetic factors. However, we recommend that future conservation plans incorporate status of habitat and relative abundance at each spring (see Jones et al. 1995; Boyce 1997) in conjunction with our rankings based on genetic factors.

1. Buffler Spring (BF)

The Buffler (or King) Spring population (BF, 34.8333°N, -86.9475°W) forms a highly distinct group in the mDNA structure analysis (Fig. 1). Genetic variability is extremely low at this site and we found evidence for a recent decline in effective population size (Table 3). Additionally, BF should receive special conservation concern because the reproductive behavior of *E. tuscumbia* is unique here compared to other spring populations. For example, *E. tuscumbia* at BF use gravel and sand (as opposed to aquatic vegetation) as the primary substrate for egg deposition (Koch 1978; Boyce 1997).

2. Meridianville Spring (MV)

The Meridianville Spring population (MV, 34.84530°N, -86.56830°W) forms a highly distinct group in the mDNA structure analysis (Fig. 1). Genetic variability is extremely low at this site and we found evidence for a recent decline in effective population size (Table 3).

3. Beaverdam Creek-Byrd Spring group

Individuals of *E. tuscumbia* from Pryor Spring (PY, 34.67560°N, -86.95000°W), Thorsen Spring (TH, 34.64000°N, -86.80920°W), Pickens Spring (PK, 34.66690°N, -86.81280°W), Beaverdam Spring (BD, 34.70280°N, -86.82940°W), and Byrd Spring (BY, 34.66420°N, -86.58250°W) were highly genetically admixed in the mDNA structure analysis (Fig. 1). Our analyses indicated a high degree of recent connectivity among springs within this group, the highest levels of genetic variability, and no evidence for recent declines in effective population size. Thus, we treat these springs as a part of the same management unit. Although this group should be ranked lower in conservation priority based on genetic characteristics, we consider springs from this group to constitute the 'stronghold' for *E. tuscumbia*. Focusing conservation efforts on the Beaverdam-Byrd group is high priority because it would ensure conservation of a substantial proportion of the genetic variability housed within *E. tuscumbia*.

4. Kelly Spring (KL)-Unnamed Spring, Flint River (WC)

The mDNA structure analysis revealed that Kelly Spring (KL, 34.81560°N, -86.71250°W) and the unnamed spring, Flint River (WC, 34.9275°N, -86.39420°W) were genetically indistinguishable (Fig. 1). Additionally, *E. tuscumbia* from WC shares mtDNA sequences with some individuals from KL. Patterns in the mtDNA suggest that WC was likely founded by individuals from KL. Together, these results indicate recent connectivity between these two springs, which was unexpected given that KL (Indian Creek) and WC (Flint River) are in independent tributaries to the Tennessee River. Thus, KL and WC would be best treated separately in management practices unless some form of PTRA is needed in the future. If it is determined that one of these springs requires PTRA to recover the population, KL should serve as a source for WC, but not vice versa. Based on the mtDNA composition of KL, Beaverdam Spring (BD) *E. tuscumbia* would likely be the best source for PTRA should *E. tuscumbia* go extinct at KL. Genetic variability of this group approaches the average of all populations, but we found evidence for recent decline in effective population size (Table 3).

5. Tuscumbia Spring (TS)

The Tuscumbia (or Big) Spring population (TS, 34.72970°N, -87.70330°W) forms a highly distinct group in the mDNA structure analysis (Fig. 1). Although genetic variability is relatively high within Tuscumbia Spring, we found evidence for a recent decline in effective population size (Table 3).

6. Wheeler Spring (WH)

The Wheeler Spring population (WH, 34.65220°N, -87.25220°W) forms a highly distinct group in the mDNA structure analysis (Fig. 1). Genetic variability approaches the average of all populations and we found no evidence for recent decline in effective population size (Table 3),

suggesting the WH population has remained relatively stable throughout recent history. However, we place higher conservation priority on this population compared to those below because of its genetic distinctiveness and the isolated location of the spring (south side of the Tennessee River).

7. Braham Spring (BR)

Although *E. tuscumbia* from Braham Spring (BR, 34.70670°N, -86.60050°W) form a moderately structured group (Fig. 1), there is some genetic admixture with the larger Beaverdam-Byrd group (defined above). Further, we found no evidence for recent declines in effective population size, indicating relative stability for the BR population.

8. Limestone Creek (LM)

Samples of *E. tuscumbia* from Limestone Creek (LM) used in this study were collected from a newly discovered site (Unnamed tributary to Limestone Creek at County Road 71, 34.6842°N, -86.8783° W) identified by Bruce W. Stallsmith (UA Huntsville). Although *E. tuscumbia* from LM show a moderate degree of genetic admixture with the larger Beaverdam-Byrd group (defined above), individuals from LM form a distinct group that has experienced recent decline in effective population sized (Fig. 1, Table 3).

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Table 1 Details of *Etheostoma boschungi* and *E. tuscumbia* specimens used in this study showing locality ID (see Figs. 1 and 2), number of individuals genotyped for microsatellite loci (*N* m), sequenced for the mitochondrial ND2 gene (*N* mt) and nuclear intron S7 (*N* S7), and mtDNA haplotype code

Species/drainage/locality	ID	*N* m	*N* mt	*N* S7	haplotype
Etheostoma boschungi					
Cypress Creek					
Cooper Branch	CB	31	5	–	1, 2, 3, 4
Dodd Site	DD	27	5	1	1, 5
Shoal Creek	SH	5	5	1	6, 7
Buffalo River					
Chief Creek	CH	5	5	–	6
North Fork	NF	18	5	–	8, 9
South Fork	SF	11	5	–	8, 10, 11
Swan Creek	SW	21	5	1	12, 13, 14
Flint River					
Brier Fork	FL	31	6	1	15, 16, 17
E. tuscumbia					
Spring Creek (west)					
Tuscumbia Spring	TS	24	5	–	18, 19, 20, 21
Cypress Creek					
Buffler Spring	BF	22	5	1	22, 23
Spring Creek (east)					
Wheeler Spring	WH	24	5	–	24
Pryor Branch					
Pryor Spring	PY	24	5	–	25, 26, 27, 28
Limestone Creek					
Limestone Creek	LM	20	5	–	25, 26, 29, 30, 31
Thorsen Spring	TH	22	5	–	25, 32
Pickens Spring	PK	24	5	–	25, 33, 34
Beaverdam Spring	BD	23	5	–	26, 29, 35, 36
Indian Creek					
Kelly Spring	KL	24	5	–	26, 37, 38
Byrd Spring	BY	16	5	–	25, 26, 39, 40
Braham Spring	BR	24	5	–	25, 41
Flint River					
Meridianville Spring	MV	24	5	1	42
Unnamed Spring	WC	24	5	–	37

Table 2 GenBank accession numbers for the mitochondrial ND2 gene and the nuclear ribosomal S7 intron 1 for outgroup taxa used in this study, followed by University of Alabama Ichthyological Collection (UAIC) catalog numbers for tissue and voucher specimens

Taxon/Locality	ND2	S7	UAIC catalog No.
Percina caprodes	EF027178	EF035498	–
Etheostoma boschungi			
Cypress Creek			
Cooper Branch	–	–	15128.01, 15496.02
Dodd Site	–	–	15129.01, 15232.01
Shoal Creek	–	–	15230.01
Buffalo River			
Chief Creek	–	–	15228.01
North Fork	–	–	15282.01,15231.01, 15544.01
South Fork	–	–	15229.01, 15281.01
Swan Creek	–	–	15133.01, 15134.01, 15546.01, 15563.01
Flint River			
Brier Fork	–	–	15225.01, 15226.02, 15130.01, 15131.01, 15561.01, 15562.01
E. cragini	EF027191	EF035511	
E. edwini	EF027193	EF035513	
E. histrio	EF027199	EF035519	
E. pallididorsum	EF027211	EF035531	
E. parvipinne	EF027212	EF035532	
E. punctulatum	EF027217	EF035537	
E. tuscumbia			
Spring Creek (west)			
Tuscumbia Spring	–	–	10772.03, 11081.01, 11100.01
Cypress Creek			
Buffler Spring	–	–	10697.03
Spring Creek (east)			
Wheeler Spring	–	–	10691.04
Pryor Branch			
Pryor Spring	–	–	11080.02
Limestone Creek			
Limestone Creek	–	–	15560.01
Thorsen Spring	–	–	11150.01
Pickens Spring	–	–	10685.05
Beaverdam Spring	–	–	10773.01
Indian Creek			
Kelly Spring	–	–	10690.01
Byrd Spring	–	–	11301.01
Braham Spring	–	–	11101.01
Flint River			
Meridianville Spring	–	–	10688.06
Unnamed Spring	–	–	10687.02
E. trisella	EF027226	EF035546	–

Table 3 Genetic diversity estimates (averaged over seven microsatellite loci) for *Etheostoma boschungi* and *E. tuscumbia*. Genetic diversity estimates are followed by critical *M* values (M_c), *M*-ratios, and associated significance as performed using the methods of Garza and Williamson (2001). Significant values ($P < 0.05$) are shown in bold. See Fig. 3 for clade membership. Populations described and mapped in Table 1 and Figs. 1 and 2

Species/Structure group	mtDNA Clade	*N*	*A*	AR	PA	H_o	H_e	M_c	*M*-ratio	*P*-value *M*-ratio
E. boschungi										
CB	1	31	3.43	3.34	0.38	0.48	0.46	0.72	0.79	0.266
DD	1	27	4.71	4.66	0.22	0.54	0.55	0.71	0.74	0.099
SH, CH	2	10	4.00	4.00	0.31	0.52	0.59	0.63	0.77	0.535
NF, SF	2	29	6.57	6.13	1.68	0.67	0.64	0.71	0.71	0.059
SW	3	21	8.43	8.43	1.99	0.73	0.74	0.69	0.75	0.188
FL	4	31	10.00	9.28	3.31	0.80	0.85	0.72	0.86	0.680
Population mean	–	–	6.74	6.50	1.50	0.65	0.67	–	–	–
E. tuscumbia										
TS	5	24	7.29	6.93	1.05	0.71	0.64	0.70	0.63	**0.007**
BF	5	22	1.86	1.82	0.05	0.25	0.16	0.66	0.63	**0.025**
WH	5	24	4.86	4.69	0.27	0.60	0.61	0.70	0.75	0.157
PY, TH, PK, BD, BY	5	109	11.29	7.93	0.81	0.63	0.68	0.77	0.84	0.333
LM	5	20	5.43	5.43	0.28	0.61	0.65	0.69	0.66	**0.019**
BR	5	24	5.29	5.14	0.48	0.67	0.63	0.70	0.76	0.205
MV	5	24	2.29	2.26	0.21	0.40	0.28	0.67	0.43	**<0.001**
KL, WC	5	48	4.86	4.68	0.66	0.47	0.53	0.74	0.69	**0.007**
Population mean	–	–	5.39	4.86	0.48	0.54	0.52	–	–	–

Number of individuals (*N*); mean number of alleles per locus (*A*); allelic richness (AR); private allelic richness (PA); heterozygosity observed (H_o); heterozygosity expected (H_e)

Table 4 Analysis of molecular variance (AMOVA) for different hierarchical groupings of *Etheostoma boschungi* and *E. tuscumbia* based on the mitochondrial (mt) ND2 gene and seven microsatellite loci. Non-significant results (> 0.05) are shown in bold

Species/Data type	Source of variation	d.f.	SS	% of variance	Fixation index	P-value
E. boschungi						
mtDNA	Among localities	7	831.47	98.04	F_{st} = 0.98	<0.00001
	Within localities	33	15.27	1.96	–	
Microsatellites	Among localities	7	243.10	29.20	F_{st} = 0.29	<0.00001
	Within localities	141	327.06	3.79	F_{is} = 0.05	<0.01
	Within individuals	149	310.50	67.01	F_{it} = 0.33	<0.00001
mtDNA	Among tributaries	3	807.87	94.91	F_{ct} = 0.95	<0.00001
	Among localities within tributaries	4	23.60	3.57	F_{sc} = 0.70	<0.00001
	within localities	33	15.27	1.52	F_{st} = 0.98	<0.01
Microsatellites	Among tributaries	3	162.50	9.66	F_{ct} = 0.10	<0.05
	Among localities within tributaries	4	80.61	20.63	F_{sc} = 0.23	<0.00001
	within localities	290	637.56	69.70	F_{st} = 0.30	<0.00001
E. tuscumbia						
mtDNA	Among localities	12	99.88	63.17	F_{st} = 0.63	<0.00001
	Within localities	52	45.20	36.83	–	
Microsatellites	Among localities	12	382.65	25.21	F_{st} = 0.25	<0.00001
	Within localities	282	583.32	4.53	F_{is} = 0.06	<0.00001
	Within individuals	295	540.50	70.26	F_{it} = 0.30	<0.00001
mtDNA	Among tributaries	6	56.53	10.53	F_{ct} = 0.11	**0.32327**
	Among localities within tributaries	6	43.35	53.14	F_{sc} = 0.59	<0.00001
	within localities	52	45.20	36.33	F_{st} = 0.64	<0.00001
Microsatellites	Among tributaries	6	238.94	7.05	F_{ct} = 0.07	**0.13366**
	Among localities within tributaries	6	143.62	18.91	F_{sc} = 0.20	<0.00001
	within localities	577	1123.82	74.05	F_{st} = 0.26	<0.00001

Degrees of freedom (d.f.); sum of squares (SS)

Table 5 Rates of migration (*M*) for *Etheostoma boschungi* as estimated in LAMARC. Number of effective migrants per generation (4*Nm*) is shown for microsatellite (m) DNA and number of effective females migrating per generation (*N*m) is shown for mitochondrial (mt) DNA. Locality codes are described and mapped in Table 1 and Fig. 2

	mDNA		mtDNA	
Comparison	*M*	4*N*m	*M*	*N*m
Among tributaries				
CB -> SH/CH	0.088	0.186	29.320	0.014
CB -> SW	0.038	0.324	0.043	0.000
CB -> FL	0.019	0.140	1.415	0.002
SH/CH -> CB	0.119	0.170	26.910	0.053
SH/CH -> SW	0.044	0.374	1.090	0.003
SH/CH -> FL	0.017	0.131	7.890	0.009
SW -> CB	0.080	0.114	5.513	0.011
SW -> SH/CH	0.085	0.179	9.897	0.005
SW -> FL	0.057	0.434	26.111	0.030
FL -> CB	0.054	0.077	2.330	0.005
FL -> SH/CH	0.042	0.089	7.738	0.004
FL -> SW	0.071	0.611	21.827	0.062
Within tributaries				
NF -> SF	0.372	1.478	568.899	0.307
SF -> NF	1.421	4.973	780.948	0.772
CB -> DD	0.157	0.312	692.741	0.515
DD -> CB	0.272	0.359	419.806	0.461

Table 6 Rates of migration (*M*) for *Etheostoma tuscumbia* as estimated in LAMARC. Number of effective migrants per generation (4*Nm*) is shown for microsatellite (m) DNA and number of effective females migrating per generation (*N*m) is shown for mitochondrial (mt) DNA. Locality codes are described and mapped in Table 1 and Fig. 1

	mDNA		mtDNA	
Comparison	*M*	4*N*m	*M*	*N*m
Among tributaries				
BF -> WH	0.048	0.096	146.917	0.012
BF -> BD	0.034	0.243	85.785	0.376
BF -> MV	0.115	0.073	15.221	0.005
WH -> BF	0.141	0.061	15.539	0.005
WH -> BD	0.181	1.293	98.860	0.434
WH -> MV	0.266	0.169	15.984	0.005
BD -> BF	0.190	0.083	462.550	0.159
BD -> WH	0.246	0.491	793.380	0.063
BD -> MV	0.423	0.269	137.900	0.044
MV -> BF	0.070	0.030	140.558	0.048
MV -> WH	0.124	0.247	48.830	0.004
MV -> BD	0.095	0.676	46.343	0.203
Within tributaries				
LM -> BD	0.282	1.647	910.780	2.806
BD -> LM	0.359	1.206	906.924	3.848
MV -> WC	0.061	0.139	102.920	0.026
WC -> MV	0.128	0.115	127.409	0.024
KL -> BR	0.174	0.475	279.673	0.217
KL -> BY	0.685	3.889	246.730	0.373
BR -> KL	0.189	0.734	337.010	0.299
BR -> BY	1.630	9.261	627.070	0.947
BY -> KL	0.854	3.311	435.514	0.386
BY -> BR	0.398	1.086	729.434	0.566

Fig. 1 (a) Map of the lower bend of the Tennessee River showing sample locations for *Etheostoma tuscumbia*. Locality codes correspond to Table 1. Pie charts represent mitochondrial DNA haplotype endemicity, where white represents the proportion of haplotypes unique to the locality and colors represent the proportion shared with other localities. Red X indicates extirpated localities. (b) Results from the microsatellite DNA based STRUCTURE analysis of *E. tuscumbia*, showing the most likely genetic structure ($K = 8$). Bars correspond to multilocus genotypes of individuals and colors represent the probability of ancestry to each cluster (K)

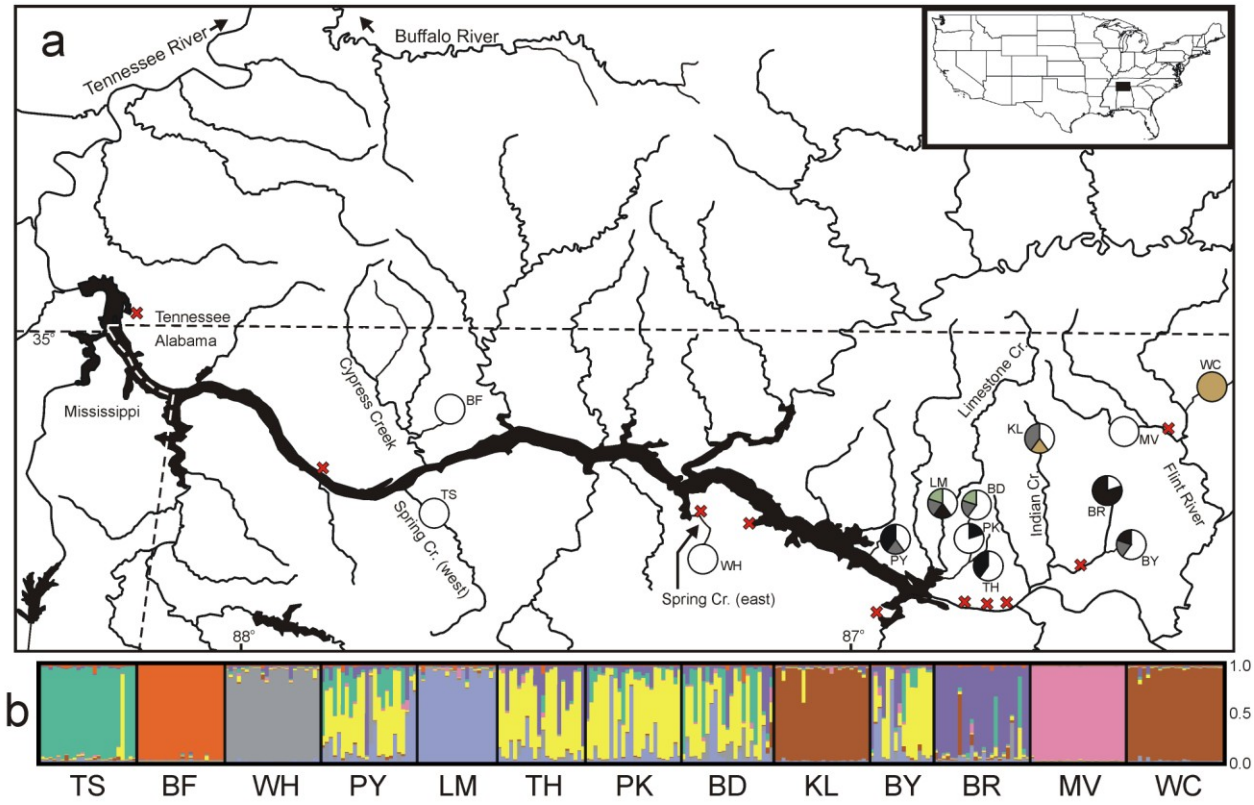


Fig. 2 (a) Map of the lower bend of the Tennessee River showing sample locations for *Etheostoma boschungi*. Locality codes correspond to Table 1. Pie charts represent mitochondrial DNA haplotype endemicity, where white represents the proportion of haplotypes unique to the locality and colors represent the proportion shared with other localities. (b) Results from the microsatellite DNA based STRUCTURE analysis of *E. boschungi*, showing the most likely genetic structure ($K = 6$). Bars correspond to multilocus genotypes of individuals and colors represent the probability of ancestry to each cluster (K). Clades correspond to Fig. 3

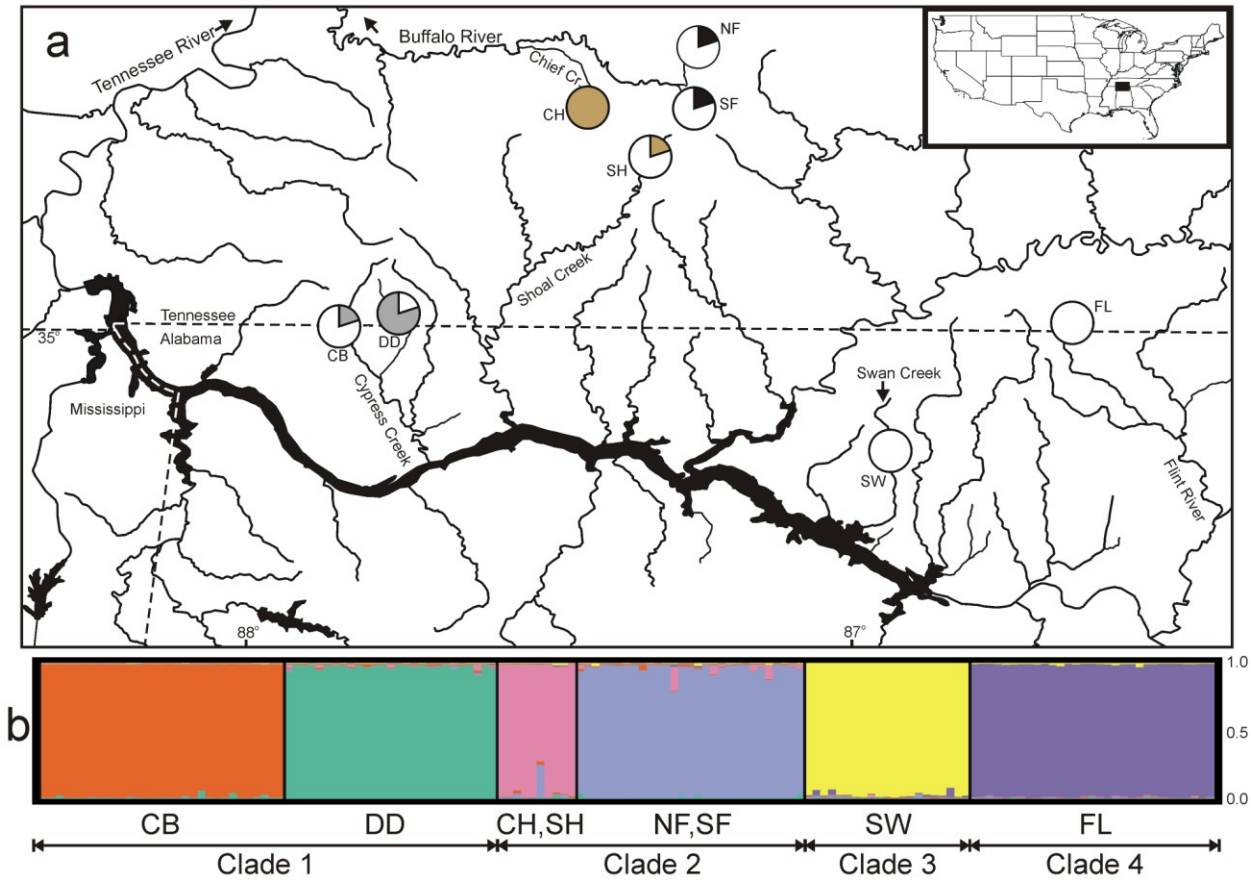


Fig. 3 (a) Fifty percentage majority-rule consensus phylogram for Bayesian analyses of the mitochondrial (mt) ND2 data set. Mean posterior probabilities shown at nodes with significant support and haplotype codes at terminals follow Table 1. Outgroup taxa not shown for simplicity. (b) mtDNA haplotype network from the statistical parsimony analysis of *Etheostoma tuscumbia*. Circles (haplotypes) are proportional to sample size and lines indicate one mutational step between haplotypes. Haplotype and locality codes correspond to Table 1 and Fig. 1

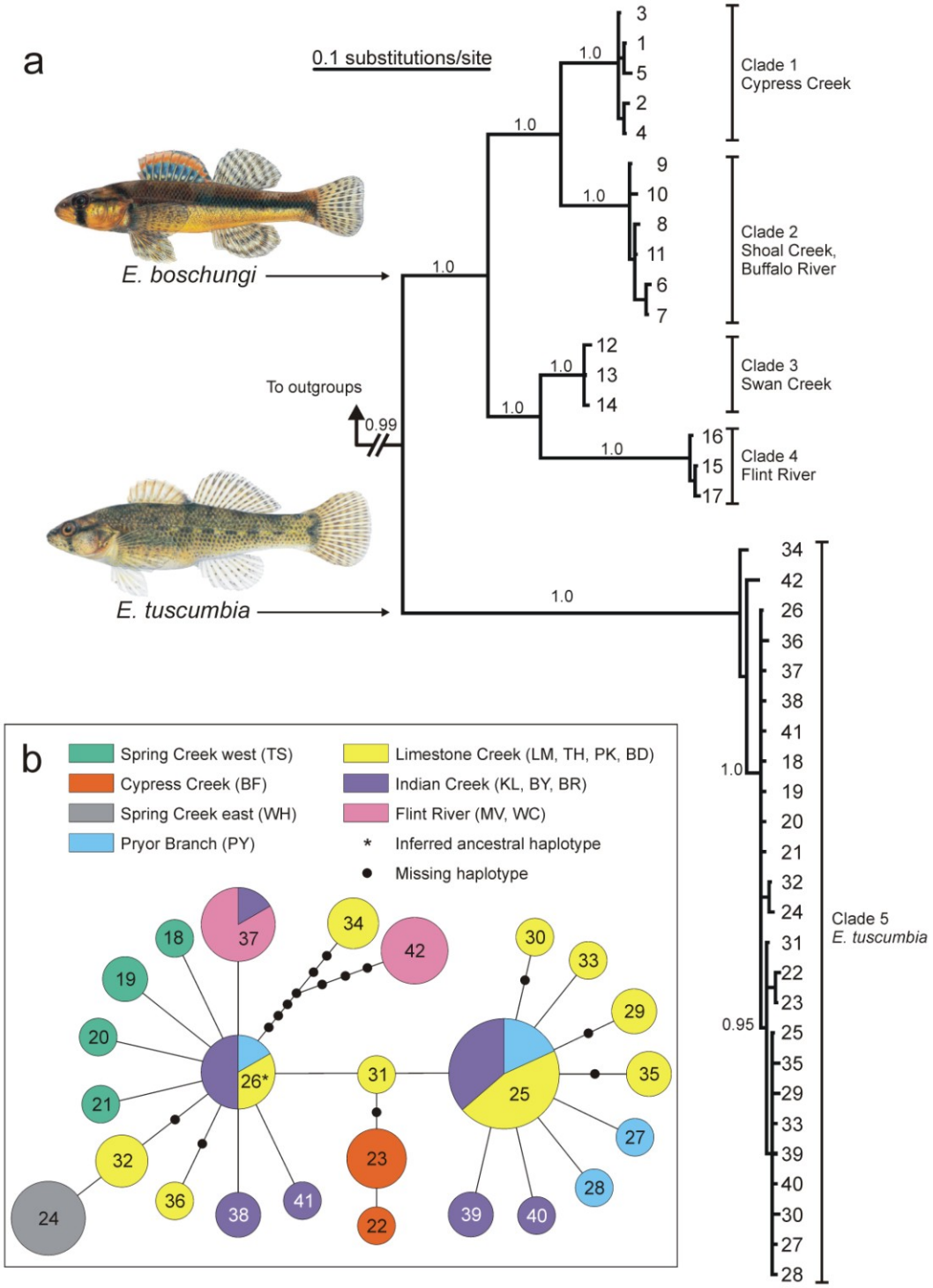
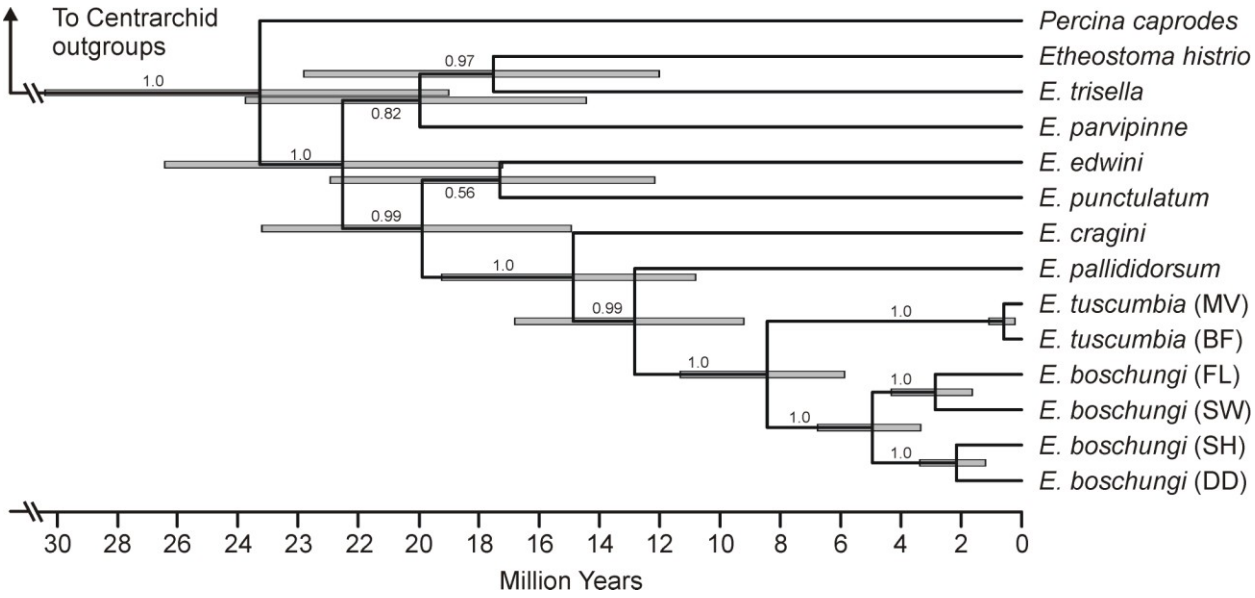


Fig. 4 Chronogram for darter species (pruned from the BEAST analysis with centrarchid outgroups) based on the combined analysis of mitochondrial ND2 and nuclear S7 gene sequences. Posterior probabilities are shown at nodes. Gray bars at nodes represent the 95% highest posterior density of age estimates. Taxon names are followed by codes corresponding to Table 1 and Figs. 1 and 2



EVOLUTIONARY HISTORY OF THE *ETHEOSTOMA SWAINI* SPECIES COMPLEX IN THE MOBILE BASIN, WITH EMPHASIS ON CONSERVATION GENETICS OF THE COLDWATER DARTER (*E. DITREMA*)

Introduction

Within the Mobile Basin (MOB), the *Etheostoma swaini* species complex consists of three described species and one undescribed form spanning the lowland Eastern Gulf Coastal Plain physiographic province (GCP) and the upland Appalachian Plateau (APP), Piedmont (PIE), and Valley and Ridge (VRP) physiographic provinces (Mayden et al. 2005). *Etheostoma swaini* is a widespread species, inhabiting small streams to large rivers from the Lake Pontchartrain drainage to the Apalachicola drainage of the GCP (Fig. 1; Boschung and Mayden 2004). *Etheostoma ditrema* has a narrow distribution, where it is confined to coldwater springs in the Coosa River Drainage within the VRP (Fig. 1; Ramsey and Suttkus 1965; Mayden et al. 2005). An undescribed stream inhabitant (*E.* sp. cf. *ditrema*) occurs in soft-water streams of the central Coosa River drainage in upland portions of the PIE and at the geologically complex junction of the PIE and VRP (MIX; Fig. 1) (Mayden et al. 2005). The third described species (*E. nuchale*) has an extremely narrow distribution, confined to just four coldwater springs in the upper Black Warrior River Drainage of the VRP (Fig.1) (Howell and Caldwell 1965; Fluker et al. 2010).

Phylogenetic relationships of *E. swaini* throughout its entire range have been notoriously difficult to assess and, as currently descried, is likely an unnatural grouping of multiple species and highly distinct lineages (Lang and Mayden 2007; Nicholas J. Lang, pers. comm.). However, Mayden et al. (2005) and Fluker et al. (2010) showed that the spring endemic *E. nuchale* was a distinct species, most closely related to populations of *E. swaini* from the Black Warrior River. Mayden et al. (2005) revealed that the spring endemic *E. ditrema* was most closely related to, but genetically distinct from central Coosa River samples of *E*. sp cf. *ditrema* and *E. swaini* from the

Cahaba river (CR; Fig. 1). Mayden et al. (2005) further revealed significant genetic structure within *E. ditrema*, but their study only included four sample sites for this species.

Using mitochondrial (mt) and microsatellite (m) DNA data, Fluker et al. (2010) found that spring populations of *E. nuchale* were highly structured and warranted independent conservation consideration. Genetic analyses further showed that effective population sizes of *E. nuchale* were small, making them more susceptible to extinction risks (Fluker et al. 2010). However, evolutionary relationships and genetic structure are not well understood throughout the entire ranges of the spring endemic *E. ditrema* and closely related stream counterparts (*E*. sp. cf. *ditrema* and *E. swaini* [CR]).

In this study, we first use phylogenetic analyses of DNA sequence data to reevaluate relationships and divergence time estimates among all members of the *E. swaini* complex from the MOB. Second, we employ a spatial phylogeographical approach to reconstruct the pattern and timing of divergence between all known populations of *E. ditrema* and closely related stream relatives (*E*. sp. cf. *ditrema* and *E. swaini* [CR]). Third, we use mDNA data to determine levels of population genetic structure and genetic variability for *E. ditrema* and closely related stream relatives (*E*. sp. cf. *ditrema* and *E. swaini* [CR]). Finally, we use several analytical methods to determine whether populations have experienced recent declines in effective population size or have remained stable throughout recent history. We conclude by discussing how our results should guide conservation planning for *E. ditrema*.

Materials and methods

Samples and DNA extraction

A total of 508 individuals were collected by seine including 309 *E. ditrema* from 13 localities, 166 *E.* sp. cf. *ditrema* from 8 localities, and 33 *E. swaini* from three localities in the CR (Table 1; Fig. 1B). Fin clips were obtained from all individuals, fins were promptly placed in 95% ethanol, and fishes were either released live or preserved in formalin. Tissues and voucher specimens were cataloged into The University of Alabama Ichthyological Collection (UAIC). Total genomic DNA was extracted from fin tissue using the DNEasy kit (Qiagen, Valencia, CA).

DNA sequencing and microsatellite genotyping

The complete mtDNA NADH dehydrogenase subunit 2 gene (ND2) was amplified and sequenced for109 samples and at least one individual from each population (Table 1) was amplified and sequenced for the nuclear ribosomal protein S7 using previously published primers and conditions (Lang and Mayden 2007; Fluker et al. 2010). A total of 505 individuals (Table 1) were genotyped for nine mDNA loci (Eca10EPA, Eca11EPA, Eca22EPA, Eca36EPA, Eca37EPA, Eca46EPA, Eca48EPA, Eca49EPA, and Eca71EPA; Tonnis 2006) following detailed procedures listed in Fluker et al. (2010). PCR fragments were tagged with HEX or 6-FAM fluorescent labels and read on an ABI 3730 DNA analyzer (University of Maine DNA sequencing facility) using GeneScan™ 500 or 1000 ROX™ Size Standard (Applied Biosystems). Alleles were binned using the program FLEXIBIN 2.0 (Amos et al. 2007) and the dataset was screened for genotyping errors using the program MICRO-CHECKER (van Oosterhout et al. 2004).

Divergence time estimates

BEAST v1.6.1 (Drummond and Rambaut 2007) was used with concatenated ND2-S7 sequences to estimate the timing of the colonization of the VRP and subsequent spring colonization by *E. ditrema* and *E. nuchale* within the MOB. Because darters are poorly represented in the fossil record (Cavender 1986), we employed external fossil calibration methods of Hollingsworth and Near (2009), which utilized DNA sequence data and five fossil calibration points for the closely related family Centrarchidae (Near et al. 2005). Studies using these methods for darters have generated consistent estimates of time of most recent common ancestor (TMRCA) for Centrarchidae and major darter groups (Near and Benard 2004; Near and Keck 2005; Near et al. 2011; Keck and Near 2010). Based on the phylogeographic analysis of Fluker et al. (2010) and preliminary runs (not shown), a single individual was chosen from each distinct clade of *E. swaini* (4, 5, 6, 7), *E. ditrema* (1, 13), *E. nuchale* (1, 2), and *E.* sp cf. *ditrema* (8) (Table 1). Thus, the final alignment consisted of concatenated ND2-S7 sequences from the individuals listed above, 47 Centrarchid taxa (GenBank accession nos. listed in Near et al. 2005), and various darter outgroups (Table 2). ND2 sequences were easily aligned by eye, the S7 data set was aligned using MUSCLE v3.8 (Edgar 2004), and heterozygous positions in S7 were coded as ambiguous. Best-fit substitution models were determined using Akaike information criterion in MrModeltest v2.3 (Nylander 2004). Minimum bound lognormal age estimates on Centrarchid fossil calibration points (Hollingsworth and Near 2009) were used and substitution models for the ND2 (TrN + I + Γ) and S7 partitions (GTR+ Γ) were unlinked. To permit uncertainty in substitutions rates on adjacent branches, an uncorrelated lognormal clock model (UCLN, Drummond et al. 2006) was employed with a Yule process speciation tree prior. Four replicate runs were conducted for 40 million generations and tree and log files were combined with 30%

of the generations discarded as burnin using LogCombiner v1.6.1 (Drummond and Rambaut 2007). To evaluate the influence of calibration priors on divergence time estimates, the BEAST analysis was replicated with an empty alignment (sampling from the prior only). Tracer v1.5 (Rambaut and Drummond 2007) was used to assess convergence and to confirm mixing efficiency of MCMC chains (effective sample size > 200).

To reconstruct the colonization history of spring habitats of the VRP by *E. ditrema* through time, the full mtDNA dataset for *E. ditrema* and closely related populations of *E.* sp. cf. *ditrema* and *E. swaini* (CR) was analyzed using a relaxed Bayesian phylogeographic approach as implemented in BEAST (Lemey et al. 2009). We were specifically interested to evaluate the diffusion process across physiographic boundaries (GCP, PIE, VRP; Fig. 1B) in the CR and Coosa river drainages that may have been important in the isolation of *E. ditrema*. Populations of *E. ditrema*, *E.* sp. cf. *ditrema* (5, 6, 7, 8) and *E. swaini* (CR) were assigned to VRP, PIE, and GCP, respectively (Table 1; Fig. 1). However, streams with the remaining populations of *E.* sp. cf. *ditrema* (1, 2, 3, 4; Table 1; Fig. 1) meander on the highly geologically complex boundary of PIE and VRP and were assigned to a fourth location (MIX). We employed a discrete geospatial model to infer the probability distributions of the geographic locations of each node of the tree, thus allowing the reconstruction of historical movements of or between populations (Bloomquist et al. 2010). In brief, each sequence is assigned to a specified geographic location and rates of diffusion are estimated among locations using a continuous-time Markov chain. Under this model, the ancestral location at the root of the tree derives from a uniform distribution over all locations and dispersal then proceeds conditionally independently along each branch, ultimately giving rise to the observed locations at the tips (Lemey et al. 2009; Campos et al. 2010). BEAST runs were conducted with the HKY substitution model (as determined in MrModeltest) unlinked

among ND2 codon positions under a relaxed molecular clock (UCLD), and a coalescent constant size tree prior. The UCLD was modeled as a normal distribution using the ND2 substitution rate of 9.29×10^{-3} (7.65×10^{-3}, 1.09×10^{-2}) substitutions/site/million years derived from the Centrarchid only data set of Near et al. (2005). This method of secondary calibration using externally calibrated rates from the Centrarchid data has been shown to provide consistent divergence time estimates for darter groups when compared to analyses using fossil calibrations (Near et al. 2011). The Bayesian stochastic search variable selection (BSSVS; see Lemey et al. 2009) was implemented to construct a Bayes factor (BF) test to identify parsimonious descriptions of the colonization process. SPREAD (Bielejec et al. 2011) was used to calculate BF, in which rates with BF > 3 were considered well supported. Three runs were conducted for 80 million generations and sampled every 8,000 generations. Convergence diagnostics and log and tree file concatenation followed methods in the BEAST analysis above.

Population genetic structure and diversity: mDNA

Departures from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were assessed in GENEPOP v4.0.10 (Rousset 2008) using a Markov Chain algorithm with 10,000 dememorizations, 200 batches and 10,000 iterations per batch. Bonferroni corrections were performed for multiple comparisons to control for type-I error. The mean number of alleles per locus (A), observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated in ARLEQUIN v3.5 (Excoffier and Lischer 2010). Allelic richness (AR) and private allelic richness (PA), measures of allelic variation that control for differences in sample sizes, were calculated in HP-RARE (Kalinowski 2005). FSTAT v2.9.3.2 (Goudet 2001) was used to test the hypothesis that genetic diversity (AR, H_o, and H_e) was lower in *E. ditrema* compared to *E.* sp. cf. *ditrema* and *E. swaini* (CR).

The Bayesian clustering method of Pritchard et al. (2000) was implemented in STRUCTURE v2.3.3 to determine the number of genetically differentiated clusters (*K*) from combined *E. ditrema*, *E.* sp. cf. *ditrema*, and *E. swaini* (CR) mDNA samples (Table 1). The analysis was run without *a priori* population designations and a model allowing admixture of genotypes and correlated allele frequencies (Falush et al. 2003). Ten replicates of 400,000 iterations (100,000 burnin) were conducted with *K* values ranging from 2 to 23. Best estimates of *K* were determined by evaluating plots of the 'mean ln probability of the data' (LnPD) versus *K* and using the ad hoc summary statistic ΔK (Evanno et al. 2005) as implemented in STRUCTURE harvester v0.6.7 (Earl 2011). Bar plots were constructed using *Distruct* v1.1 (Rosenberg 2004). Populations groupings resolved in STRUCTURE analyses were used in estimates of genetic diversity and population genetic analyses listed below.

Population genetic differentiation (F_{st}) and associated significance was estimated by AMOVA as implemented in ARLEQUIN with 10000 permutations. Three hierarchical groupings were analyzed separately to test the null hypothesis that populations represent an arbitrary subsample relative to the total sample: 1) All *E. ditrema*, *E.* sp. cf. *ditrema*, and *E. swaini* (CR) as a single group; 2) All *E. ditrema* as a single group; and 3) all *E. sp. cf. ditrema* and *E. swaini* (CR) as a single group. The first AMOVA was conducted to evaluate levels of genetic differentiation among all samples. The second and third AMOVAs were conducted to evaluate potential differences in genetic differentiation among spring populations versus among stream populations. The significance of differences in F_{st} between tests two and three was assessed in FSTAT.

N_e estimates, migration, and demographic history

To test the prediction of little to no gene flow between spring populations of *E. ditrema* and stream populations of *E. sp. cf. ditrema* and *E. swaini* (CR), we used the program LAMARC v2.1.6 (Kuhner 2006) with mDNA to jointly estimate the parameter Θ ($4N_e\mu$) and immigration rates ($M = m/\mu$), where m equals the chance of immigration/individual/generation, and μ is the chance of mutation/site/generation (Kuhner 2006; Kuhner and Smith 2007). Multi-population LAMARC analyses comparing more than five populations can lead to poor estimates due to over parameterization (see program documentation). Thus, we restricted our analysis to five populations: 1) the most downstream population of *E. ditrema* on the eastern side of the Coosa River (13; Table 1; Fig. 1); 2) the most downstream population of *E. ditrema* on the western side of the Coosa River (9; Table 1; Fig. 1); 3) *E.* sp. cf. *ditrema* from MIX (1, 2, 3, 4; Table 1; Fig. 1); 4) *E.* sp. cf. *ditrema* from PIE (5, 6, 7, 8; Table 1; Fig. 1); and 5) *E. swaini* from CR (Table 1; Fig. 1). For each group, 15 individuals were selected at random from STRUCTURE defined populations. Initial runs were conducted with final chains of 200,000–400,000 steps to evaluate convergence of parameter values and to obtain starting values for subsequent runs. Three final runs employed three initial chains of 20,000 steps (1,000 trees sampled every 20 reps, burn-in = 2,000) and one final chain of 1,000,000 steps (50,000 trees sampled every 20 reps, burn-in = 20,000) using an adaptive heating scheme with 4 temperatures (1.0, 1.1, 1.2, 1.3). Priors for Θ and M ranged from 1.0×10^{-2} to 12.0 and 1.0×10^{-2} to 1000, respectively. For each population, Θ was converted to long-term N_e using the mDNA mutation rate of 5×10^{-4} (Goldstein and Schlötterer 1999; Yue et al. 2007). Immigration rates were converted to the number of effective immigrants per generation ($4Nm$) by multiplying M by its estimate of Θ for the recipient population (Kuhner 2006; Kuhner and Smith 2007).

We used mDNA with two complementary bottleneck methods that detect reductions in N_e due to different demographic processes. The H_e excess test was implemented in the program BOTTLENECK 1.2.02 (Cornuet and Luikart 1996; Piry et al. 1999), which identifies reductions in N_e that are very recent or less severe (Luikart et al. 1998; Garza and Williamson 2001; Williamson-Natesan 2005). We conducted 10,000 replicates using a two-phase model (TPM), 95% single-step mutations, and 12% variance of multi-step mutations (Piry et al. 1999). The second method (*M*-ratio; Garza and Williamson 2001) is more powerful at detecting prolonged, severe, or older reductions in N_e (Williamson-Natesan 2005). This method compares the ratio of number of alleles to the range in allele size (M) to population specific critical M values (M_c) using M_P_Val.exe and Critical_M.exe (Garza and Williamson 2001). We considered several parameter sets to estimate *M*-ratios for populations of both species: pre-bottleneck Θ values of 10, 15, and 20 (N_e = 5,000, N_e = 7,500, and N_e = 10,000, respectively); average size of non one-step mutations = 3.5; and TPM with 90% single-step mutations. The probability that a smaller *M*-ratio would be expected under equilibrium conditions was tested with 10,000 simulations.

Results

Phylogeographic analyses and divergence time estimates

The BEAST analysis recovered Centrarchids and darters as reciprocally monophyletic (not shown) and TMRCA for the former (33.6 [26.3, 41.8] million years ago [Mya]) was highly consistent with previous published analyses using this calibration method (Hollingsworth and Near 2009; Keck and Near 2010; Near et al. 2011). Figure 2 shows the chronogram (pruned from the complete analysis) for the *E. swaini* species complex within the MOB plus the closely related *E. asprigene*. Within the MOB, *E. swaini* from the Alabama River formed a well-supported sister relationship to a trichotomus clade (node B; Fig. 2) of *E. swaini* from the Tombigbee River, *E. swaini* + *E. nuchale* from the Black Warrior River, and *E. ditrema* + *E.* sp. cf. *ditrema* + *E. swaini* (CR), all of which shared a MRCA 7.3 [5.2, 9.8] Mya (node A; Fig. 2). Within the Black Warrior River (node C; Fig. 2), *E. nuchale* and closely related populations of *E. swaini* shared a MRCA 4.1 [2.7, 5.7] Mya and distinct populations of *E. nuchale* began diverging from one another 1.3 [0.6, 2.1] Mya (node D; Fig 2). Members of the focal group (*E. ditrema*, *E.* sp. cf. *ditrema*, and *E. swaini* [CR]) shared a MRCA 1.9 [1.1, 2.8] Mya (node E; Fig. 2). The stream inhabiting *E.* sp. cf. *ditrema* shared a MRCA with *E. swaini* (CR) 0.6 Mya [0.2, 1.1] Mya (node F; Fig. 2) and *E. ditrema* began diversifying in the Coosa River Drainage 0.7 [0.3, 1.1] Mya (node G; Fig. 2).

Spatial phylogeographic reconstruction

A total of 36 mtDNA haplotypes were identified among focal taxa (*E. ditrema* = 14, *E.* sp. cf. *ditrema* = 18, *E. swaini* = 4; Table. 1), none of which were shared between taxa. Figure 3 shows the location-annotated maximum clade credibility (MCC) tree for all individuals of the focal group (also see clade E; Fig. 2). With the exception of a slightly younger age estimate for

clade F (Fig. 3), node age estimates were largely consistent with the reduced BEAST analysis (Fig. 2), indicating a root node age of 1.8 [1.0, 2.6] Mya. The analysis recovered two divergent haplotype sets within *E.* sp. cf. *ditrema* from MIX (Fig. 3) that varied little within major clades G (0.19 % mean sequence divergence) and F (0.34 % mean sequence divergence), but substantially between clades G and F (2.77% mean sequence divergence). The discrete phylogeographic analysis revealed a high uncertainty on the root state posterior probabilities for all locations (0.12−0.36) with MIX and VRP having the highest probabilities (0.31 and 0.36, respectively). The posterior probability of the ancestral distribution of clade F (*E. swaini* [CR] and *E.* sp. cf. *ditrema*; Fig. 3) was highest for PIE (0.64) and substantially lower for other locations (0.01−0.21). The MCC tree shows strong support for VRP as the ancestral distribution of clade G (0.91), with a TMRCA of 0.6 (0.3, 0.9) Mya. This clade is composed of all spring inhabiting populations of *E. ditrema* and divergent haplotypes of *E.* sp. cf. *ditrema* from MIX that coalesce with distinct populations of *E. ditrema* in different parts of the clade (Fig. 3). Haplotypes of *E. ditrema* were further characterized by strong signal of clustering to spring localities (Fig. 3). Despite uncertainty of the ancestral distribution of the root node and members of clade F (Fig. 3), BF tests under the BSSVS identified support for three migration routes: GCP-PIE (BF = 3.7); PIE-MIX (BF = 18.3); and VRP-MIX (BF = infinity).

Population genetic diversity and structure

Eight of the nine mDNA loci were successfully amplified across samples. Locus Eca46EPA proved problematic with respect to amplification and scoring, thus was removed from all analyses due to the presence of null alleles. Following Bonferroni correction, 15 of the 104 locus/population comparisons deviated from HWE. All deviations resulted from a deficiency of heterozygotes with no clear pattern of multi-locus deviations in any one population. Linkage was

not detected among the eight mDNA loci. Genetic diversity measures AR ($P < 0.05$), H_o ($P < 0.05$), and H_e ($P < 0.01$) were significantly lower in spring populations of *E. ditrema* when compared to stream relatives *E.* sp. cf. *ditrema* and *E. swaini* (CR) (Table 3).

The STRUCTURE analysis including all 505 individuals indicated $K = 14$ as the most likely configuration of genetic subdivision (Fig. 4A). However, the ΔK distribution revealed the possibility of lower levels of genetic subdivision ($K = 6$ and $K = 4$; Fig. 4B and C). Examination of the lower levels of structure showed that *E. ditrema* exhibited higher levels of structure, while *E.* sp. cf. *ditrema* and *E. swaini* (CR) were less structured and were resolved as a single homogeneous cluster in the $K = 4$ scenario (Fig. 4C). Thus, we conducted two additional independent STRUCTURE analyses; one with only *E. ditrema* samples and one with only *E.* sp. cf. *ditrema* plus *E. swaini* (CR) samples. For the independent runs, LnPD and ΔK plots revealed clearer resolution of K values and similar results compared to the combined analysis. These analyses indicated $K = 10$ for *E. ditrema* and $K = 3$ for *E.* sp. cf. *ditrema* plus *E. swaini* (CR) (Fig. 3B) and were used as population groupings for all mDNA analyses. The genetic partitioning within *E. ditrema* suggested a high degree of structuring to individual springs and spring groups within the same creek basin (Fig. 3B). Members of the stream group showed a strong pattern of genetic partitioning with respect to physiography. For example, samples of *E.* sp. cf. *ditrema* were subdivided between PIE (5, 6, 7, 8) and MIX (1, 2, 3, 4) and samples of *E. swaini* (CR) from the GCP was assigned to its own cluster (Table 1; Fig. 3B).

The AMOVA including all taxa suggested that most of the mDNA variation (70.9%) was within individuals ($F_{it} = 0.29$, $P < 0.01$). However, 19.8% of the variation was partitioned among populations ($F_{st} = 0.20$, $P < 0.01$) and all pair-wise population F_{st} values were highly significant ($P < 0.00001$; Table 4). The AMOVA including only spring populations of *E. ditrema* revealed a

similar value for F_{it} (0.34, 65.2%, $P < 0.01$), but a much higher degree of among population variation ($F_{st} = 0.26$, $P < 0.01$). The AMOVA including only stream populations (*E.* sp. cf. *ditrema* plus *E. swaini* [CR]) revealed 81.8% of the variation was within individuals ($F_{it} = 0.18$, $P < 0.01$), yet lower levels of F_{st} (0.08, $P < 0.01$). All three analyses yielded similar levels of individual variation at the population level ($F_{is} = 0.12$, $P < 0.01$ for all). Results from the FSTAT analysis indicated that F_{st} differed significantly between *E. ditrema* (0.26) and stream populations (0.08, $P < 0.01$).

N_e estimates and demographic history

Coalescent-based estimates of Θ varied widely among the subset of five populations included in the LAMARC analysis (Table 5). The corresponding estimates of long-term N_e for *E. ditrema* and *E. swaini* (CR) were 13−46% of the values observed for *E.* sp. cf. *ditrema* (Table 5). With the exception of three comparisons, estimated values of effective immigrants per generation ($4Nm$) were below one (Table 6). The analysis revealed somewhat symmetrical migration between *E.* sp. cf. *ditrema* (PIE and VRP), and evidence for low levels of migration (1.52) into *E.* sp. cf. *ditrema* (PIE) from *E. swaini* (CR) (Table 6).

The analysis of mDNA using bottleneck revealed no significant deviations between H_e and H_e based on the number of alleles and sample size (not shown). Results from the *M*-ratio tests showed that eight of the 10 populations of *E. ditrema* had *M*-ratios significantly lower than their respective critical values (Table 3). However, populations of *E.* sp. cf. *ditrema* and *E. swaini* (CR) showed no significant reductions (Table 3).

Discussion

Our phylogeographic analysis indicated that *E. ditrema* arose *in situ* in the VRP, diverging from stream-inhabiting relatives of the PIE and GCP 1.8 [1.0, 2.6] Mya (Fig. 3A). The analysis further suggested that spring populations of *E. ditrema* began diversifying in the mid-late Pleistocene (Figs. 2 and 3A). However, mtDNA provided evidence of recent secondary contact between *E. ditrema* and *E.* sp. cf. *ditrema* that occupy the geologically complex boundary between the PIE and VRP. Analysis of mDNA indicated a lack of long-term migration and a clear break between *E. ditrema* and stream relatives. Populations of *E. ditrema* exhibited lower levels of genetic variation, small long-term N_e, and evidence for prolonged bottlenecks compared to stream relatives.

Colonization of the VRP and spring habitats

The phylogeographic reconstruction showed a deep division between two lineages that corresponds to the break between the VRP and PIE + GCP (clades F and G, respectively; Fig. 3A). However, the analysis identified divergent haplotypes within *E.* sp. cf. *ditrema* from MIX that were recovered in each major lineage indicating the possibility of retention of ancestral '*E. ditrema* like' mtDNA alleles or hybridization and introgression of *E. ditrema* alleles into *E.* sp. cf. *ditrema* (MIX) and subsequent divergence. Retention of ancestral alleles by *E.* sp. cf. *ditrema* (MIX) cannot be ruled out completely, but seems unlikely in this case. Given the deep divergence between lineages F and G (Fig. 3A), ancestral alleles retained within *E.* sp. cf. *ditrema* (MIX) would be expected to have deeper or basal positions within clade G (Funk and Omland 2003). Further, these divergent alleles would also be expected to be present in *E.* sp. cf. *ditrema* (PIE). This is not the case, as they are only present in the most geographically proximate populations with respect *E. ditrema* in the VRP. The BSSVS also provide significant BF results

supporting that haplotypes in clade G arose in the VRP and subsequently spread to MIX (Fig. 3A). Although LAMARC analyses revealed evidence for low levels of long-term migration between *E.* sp. cf. *ditrema* from PIE and MIX, STRUCTURE analysis clearly indicated genetic subdivision between the two (Table 6; Fig. 3B), which may explain why divergent '*E. ditrema* like' alleles were not present within the PIE. We propose that these divergent mtDNA alleles within *E.* sp. cf. *ditrema* (MIX) are the result of multiple recent, but temporally intermittent, hybridization and mtDNA introgression events between *E. ditrema* and *E.* sp. cf. *ditrema* (MIX). Fluker et al. (2010) found the same pattern of recent (approx. 0.3 Mya) directional mtDNA introgression of *E. nuchale* haplotypes into closely related populations of *E. swaini* within the Black Warrior River. Despite strong support for long-term geographical isolation of both *E. ditrema* and *E. nuchale* from their respective progenitors, these boundaries appear to be temporally 'leaky' for mtDNA. Our S7 data were mostly uninformative among members of the *E. swaini* complex in the MOB, thus additional nuclear DNA sequence data are needed to further understand patterns of incomplete sorting versus introgression based on mtDNA markers. However, both spring-adapted species and their closest stream inhabiting relatives clearly maintain species identity based on mDNA and morphological data.

There is strong support that *E. ditrema* was isolated in the VRP in the early-mid Pleistocene (node E; Figs. 2 and 3A). This is much younger than the putative isolation of *E. nuchale* in the VRP (4.1 [2.7, 5.7] Mya) based on the TMRCA of *E. nuchale* and close relatives in the Black Warrior River (Fig. 2). However, diversification of spring populations of both species within the VRP fall completely within the Pleistocene (*E. nuchale* 1.3 [0.6, 2.1] Mya and *E. ditrema* 0.7 [0.3, 1.2] Mya; Fig. 2). Interestingly, this is largely overlapping with diversification of *E. phytophilum* populations (1.2 [0.6, 1.8] Mya), another spring-adapted darter

that is co-distributed with *E. nuchale* in the Black Warrior River in the VRP (B. L. Fluker unpubl. data). This replicated pattern of population diversification within three spring-adapted darters from the VRP coincides closely with the transition from relatively shorter Pleistocene glacial cycles (41,000 years) to relatively longer, more dramatic cycles (100,000 years) approximately 0.9 Mya (Hewitt 2000; Hewitt 2011). This is consistent with the hypothesis of Pleistocene spring specialization of *Cottus paulus* in the Coosa River drainage (Williams 1968). However, Williams (1968) hypothesized that contraction of a formerly more wide spread ancestral species (*C. bairdii*) during Pleistocene warming periods gave rise to *C. paulus*. For spring-adapted darters in the VRP, it seems equally likely that extended periods of colder, dryer climate following the onset of 100,000 year glacial cycles may have facilitated adaptation in isolated groundwater outflows.

Geographical isolation versus ecology in spring endemics

Geographic isolation within the MOB has no doubt played an important role in lineage diversification with the *E. swaini* complex. This is clearly shown in the high degree of distinctiveness among *E. swaini* populations inhabiting different physiographies and drainage basins. For example, those populations that have surmounted the Fall Line and inhabit upland streams in the Black Warrior River (APP) and Coosa River (PIE and MIX) form highly distinct lineages compared to lowland GCP populations in the Alabama and Upper Tombigbee rivers (Figs. 1 and 2; also see Mayden et al. 2005; Fluker et al. 2010). One exception is the close relationship of *E. swaini* (CR) and *E.* sp. cf. *ditrema*. However, this relationship is not unexpected given evidence of former connections between Cahaba and Coosa rivers based on distributional patterns of several other fishes (Swift et al. 1986). Isolation of *E. ditrema* and *E.*

nuchale within the geologically stable VRP also supports the strong association of lineage divergence and geographic isolation.

The prediction of no or little gene flow between *E. ditrema* and stream relatives was supported by our mDNA data. However, the analysis revealed additional insights as to how ecological attributes of each species affect gene flow. Analysis of mDNA showed that stream inhabitants (*E.* sp. cf. *ditrema* and *E. swaini* [CR]) were structured among physiographic provinces, but clearly showed high genetic diversity, low levels of structure among localities, and moderate levels of long-term migration. Conversely, mtDNA and mDNA revealed high levels of population structure among spring localities (or spring groups within the same tributary) for *E. ditrema*. That spring populations are highly structured and have reduced dispersal capability has been shown for a number of fishes (Duvernell and Turner 1998; Martin and Wilcox 2004; Bernardi et al. 2007). However, differences in dispersal ability between spring populations and closely related stream inhabitants have received less attention. Similar to this study, spring populations of *E. nuchale* (F_{st} = 0.24) and *E. phytophilum* (F_{st} = 0.40) in the Upper Black Warrior River show reduced dispersal ability and high levels of population structure compared to populations of stream inhabiting relatives *E. swaini* (F_{st} = 0.07) and *E. parvipinne* (F_{st} = 0.14), respectively (Fluker et al. 2010; B. L. Fluker unpubl. data). These findings suggest that colonization and adaptation to spring environments has facilitated the evolution of reduced dispersal ability in spring endemics of the MOB, which likely played a key role in their divergence from stream relatives.

Conservation implications and recommendations

Taxonomic status

Phylogenetic analyses conducted in this study further revealed the difficulties of resolving species boundaries within the *E. swaini* group. These difficulties are due to the propensity of historic hybridization events between distinct forms within the group (Lang and Mayden 2007; Near et al. 2011). Despite these difficulties, our results allow us to better define species boundaries for *E. ditrema* and better understand genetic structure among its spring populations. Although we identified recent (but not ongoing) hybridization between *E. ditrema* and *E*. sp. cf. *ditrema*, *E. ditrema* clearly maintains its species identity based on mtDNA, nuclear mDNA data and morphological characteristics (Utter 1984). Our results indicate that true 'spring form' *E. ditrema* are distributed exclusively in the VRP, from Poorhouse Branch and upstream in the Coosa River drainage (Fig. 1). We confirmed the results of Mayden et al. (2005) which show a close relationship between *E*. sp. cf. *ditrema* and *E. swaini* from the Cahaba River. However, a better understanding of relationships among the undescribed stream form (*E*. sp. cf. *ditrema*) and *E. swaini* will require an intensive analysis of *E. swaini* throughout its entire range. Until these relationships are better understood, we recommend that *E*. sp. cf. *ditrema* be treated independently from *E. ditrema* and *E. swaini* (CR) in future conservation practices.

Genetic structure and priority units for conservation

Coldwater Darter, *Etheostoma ditrema* (spring form)

Samples of *E. ditrema* from the 13 localities in this study are structured into 10 genetically distinct groups (Fig. 3B, Tables 3 and 4) that can be considered management units. Because nearly all management units of *E. ditrema* show reductions in effective population size based on genetic characteristics (Table 3), we do not prioritize the conservation concern for the

10 management units on the basis of genetic characteristics alone. Rather, we recommend that data on relative abundance and threats to habitat (see Kuhajda and Mayden 2002) be considered to best prioritize conservation planning for each of the 10 management units defined herein. Below we provide descriptions of the 10 management units (listed from upstream to downstream in the Coosa River system) and list the source of samples used in our genetic analyses. Source localities are followed by University of Alabama Ichthyological Collection (UAIC) catalog numbers.

1. Deverell Spring-Colvard Spring

Both of these springs are in close proximity to one another in the upper Conasauga River. However, these are the only two springs in which *E. ditrema* from distinct creek drainages share connectivity and gene flow. During this study, a habitat restoration project was being conducted to Colvard Spring and ongoing surveys were being conducted to monitor the status of *E. ditrema* at this spring (Anna George, pers. comm.). Genetic variability of this group was above the average of all *E. ditrema*, however, we detected evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- Deverell Spring along Union Road, 0.7 mi WNW of Union Church (35°1'3"N, 84°49'21"W) (Felker quad.), Bradley Co., Tennessee. UAIC 15670.02.
- Colvard Spring at GA Hwy 225, 0.2 mi N of jct. with GA Hwy 2, 2.5 mi W of Cisco (34°56'41"N, 84°46'49"W) (Beaverdale quad.), Murray Co., Georgia. UAIC 15668.01.

2. Cohutta Spring

Cohutta Spring is within the Coahulla Creek drainage of the upper Conasauga River. This spring receives some protection because it is located on the property of the former Cohutta National Fish Hatchery. Genetic variability of this group was near average values of all *E.*

ditrema, however, we detected evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- Cohutta Spring and run at Cohutta National Fish Hatchery, 1.0 mi N of Cohutta (34°58'25"N, 84°57'4"W) (Cohutta quad.), Whitfield Co., Georgia. UAIC 15667.01.

3. Moseley Spring

Moseley Spring is the type locality for *E. ditrema* (Ramsey and Suttkus 1965). Genetic variability of this group was slightly below average values of all *E. ditrema*. However, we detected no evidence for recent decline in effective population size, indicating that *E. ditrema* has been relatively stable at Moseley Spring (Table 3) even though the spring has been impounded within the last decade (B. R. Kuhajda, personal observation). Source of individuals for genetic analysis:

- Moseley (Moses) Spring, 4.3 mi W of Lyerly, 0.2 mi ENE of AL State line (34°23'47"N, 85°28'54"W) (Lyerly quad.), Chattooga Co., Georgia. UAIC 15672.01.

4. Terrapin Creek group- Smart Spring and Todd Spring

Smart and Todd springs are situated < 1 km from one another on the western and eastern side of Nances Creek in the Terrapin Creek system. Thus, it is no surprise that these springs share extensive gene flow and constitute a single breeding population. Genetic variability of this group was the lowest observed for any group of *E. ditrema* and we detected evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- Smart Spring [unmarked] on E side of bend in Steinburg Rd., 0.75 mi. W of Ladiga (33°56'53"N, 85°35'37"W) (T12S, R10E, S33) (Piedmont quad.), Calhoun Co., Alabama. UAIC 15665.02.

- Todd Spring [unmarked] at end of gravel road, 0.5 mi. W of Ladiga, just W of abandoned house (33°56'49"N, 85°35'26"W) (T12S, R10E, S33) (Piedmont quad.). Calhoun Co., Alabama. UAIC 15666.01.

5. Ballplay Creek

Our collection site for *E. ditrema* in Ballplay Creek was more stream-like than other collection sites throughout the range of *E. ditrema*. However, the discovery of breeding Trispot Darters (*E. trisella*) at this site suggests heavy spring influence (Johnson et al. 2011). Similarly, *E. ditrema* has been collected in stream-like habitat at another site in the Ballplay Creek system where spring influence is present (unnamed tributary to Ballplay Creek downstream of Dripping Rock Road, 2.1 mi SSW of Knightens Crossroads [33°55'42"N, 85°43'23"W]; Kuhajda and Mayden [2002]). Genetic variability of this group was the highest observed for any group of *E. ditrema* and we detected no evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- Unnamed tributary to Ballplay Creek at County Road 24 (Rocky Ford Road), 1.0 mi. W of Ball Flat (34°00'55"N, 85°45'03"W). Cherokee Co., Alabama. UAIC 15709.01, 15710.01.

6. Glencoe Spring

Glencoe Spring is in the Cove Creek system and property is owned by the City of Glencoe. Genetic variability of this group was slightly above average values of all *E. ditrema,* but we detected evidence for recent decline in effective population size (Table 3). This spring is considered to have some of the highest abundances of *E. ditrema* compared to other springs throughout its range (Kuhajda and Mayden 2002). Source of individuals for genetic analysis:

- Glencoe (Jeffers) Spring [unmarked] and adjacent stream, just W of US Hwy 431, 0.5 mi SSE of Glencoe (33°56'59"N, 85°55'45"W) (T12S, R7E, S29/32) (Glencoe quad.), Etowah Co., Alabama. UAIC 15615.01.

7. Big Canoe Creek group

Based on collections made in this study and by Johnson et al. (2011), *Etheostoma ditrema* is relatively widespread throughout Little Canoe Creek, but we only obtained one individual from a direct tributary to Big Canoe Creek (North Fork Dry Creek). *Etheostoma ditrema* was never collected in large numbers from sites used in this study. We collected *E. ditrema* from springs and spring-fed stream habitat within Little Canoe Creek, where it co-occurred with *E. trisella* in some collections. Genetic variability of this group was slightly below average values of all *E. ditrema* and we detected evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- North Fork Dry Creek (Big Canoe Creek) at Hwy 231, approx. 3 mi. S of Ashville (33°48'03"N, 86°16'14"W), St. Clair Co., Alabama. UAIC 15490.01.
- Little Canoe Creek at Beulah Circle Rd., approx. 2 mi. SW of Cool Springs (33°46'50"N, 86°21'47"W), St. Clair Co., Alabama. UAIC 15491.01, 15552.01.
- Little Canoe Creek just east of Beulah Circle Rd. (33°47'05"N, 86°21'38"W), St. Clair Co., Alabama. UAIC 15551.01.
- Unnamed tributary to Little Canoe Creek just below confluence with St. Clair Springs run (33°46'05"N, 86°24'14"W), St. Clair Co., Alabama. UAIC 15549.01, 15550.01.
- St. Clair Springs head and run (33°45'49"N, 86°24'19"W), St. Clair Co., Alabama. UAIC 15547.01, 15548.01, 15734.02.

8. Ohatchee Creek group

Etheostoma ditrema is historically known from three spring localities within the Ohatchee Creek system (Kuhajda and Mayden 2002). We sampled two of those springs (Edwards Spring and an unnamed spring 3 mi NW of Angel [33°52'10"N, 85°52'21"W]), but only successfully collected *E. ditrema* from Edwards Spring. If *E. ditrema* are collected at the other springs in Ohatchee Creek (Kuhajda and Mayden 2002), they should be considered a part of the Ohatchee Creek group. Genetic variability of this group was slightly below average values of all *E. ditrema* and we detected evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- Edwards Spring [unmarked], 1.5 mi NNW of Jacksonville (33°50'11"N, 85°46'33"W) (T14S, R8E, S2 SW 1/4) (Jacksonville West quad.), Calhoun Co., Alabama. UAIC 15733.02.

9. Choccolocco Creek group

Etheostoma ditrema is historically known from three spring localities within the Choccolocco Creek system (Coldwater Spring, Blue Spring, and Murray Spring run; Kuhajda and Mayden 2002). We obtained samples from Coldwater and Blue springs and found no genetic distinctiveness between the two. Genetic variability of this group was slightly above average values of all *E. ditrema* and we detected evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- Coldwater Spring run, just off Co. Rd. 109, 2.4 mi WNW of Oxford (33°36'13"N, 85°55'34"W) (T16S, R7E, S29 S 1/2) (Munford quad.), Calhoun Co., Alabama. UAIC 15732.01.

- Blue Spring [unmarked] run, just W of AL Hwy 21 & just S of I-20 in Oxford (33°36'11"N, 85°50'7"W) (T16S, R8E, S30 SE 1/4) (Oxford quad.), Calhoun Co., Alabama. UAIC 15616.01.

10. Poorhouse Branch

Poorhouse Branch is the downstream most creek in the Coosa River drainage where true *E. ditrema* (those considered the spring form) is found. Habitat at this site is stream-like with several nearby springs. During our collection efforts at Poorhouse Branch on 19 Sept. 2009, we collected several juvenile and adult individuals of the non-native oriental weatherfish (*Misgurnus anguillicaudatus*). Subsequent sampling trips revealed that *M. anguillicaudatus* is established throughout Poorhouse Branch and in tributaries to Logan Martin Reservoir. The threats to *E. ditrema* from the non-native *M. anguillicaudatus* are not well understood. Thus, additional surveys in Poorhouse Branch and nearby tributaries may provide some insight into any negative impacts to *E. ditrema*. Genetic variability of this group was near average values of all *E. ditrema*, however, we detected evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- Poorhouse Branch at New Lincoln Road (33°29'19"N, 86°07'59"W), Talladega County, Alabama. UAIC 15617.02.

Undescribed stream form, *Etheostoma* sp. cf. *ditrema*

Prior to this study, samples from Tallaseehatchee Creek were considered to be *E. ditrema* (spring form; Kuhajda and Mayden 2002). However, our genetic analyses show that individuals from Tallaseehatchee Creek actually represent *E*. sp. cf. *ditrema* (stream form). Thus, Tallaseehatchee Creek is now considered the upstream most creek in the Coosa River drainage to contain *E*. sp. cf. *ditrema*. Samples of *E.* sp. cf. *ditrema* from the eight creek systems in this

study are structured into two genetically distinct groups (Fig. 3B, Table 4). Although the two groups are weakly differentiated (Table 4), we treat them as distinct management units for conservation because of the unique mtDNA alleles found in streams that meander on the complex boundary between the Valley and Ridge and Piedmont physiographic provinces (Tallaseehatchee, Kahatchee, Fourmile, and Beeswax creeks). See Kuhajda and Mayden (2002) for a detailed assessment of relative abundance of and threats to habitat for *E*. sp. cf. *ditrema*. Below we provide descriptions of the two management units and list the source of samples used in our genetic analyses. Source localities are followed by University of Alabama Ichthyological Collection (UAIC) catalog numbers.

1. Valley and Ridge group

As currently known, this group is found in Tallaseehatchee, Kahatchee, Fourmile, and Beeswax creeks. We consider this group as a distinct management unit from the Piedmont group (see below) because some individual *E*. sp. cf. *ditrema* from these sites have unique "*E. ditrema* like" mtDNA sequences. As mentioned in the discussion, this is likely the result of past (but not ongoing) hybridization events with *E. ditrema*. These unique mtDNA sequences are not found in the Piedmont group. Genetic variability of this group was high compared to *E. ditrema* and we detected no evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- Unnamed spring tributary to Tallaseehatchee Creek, 4.5 mi E of Bon Air (33°15'18"N, 86°15'33"W) (T20S, R4E, S29/31/32) (Childersburg quad.), Talladega Co., Alabama. UAIC 12172.03, 15737.01.
- Waters Branch (Kahatchee Creek) at County Road 008 (33°13'57"N, 86°24'23"W), Talladega Co., Alabama. UAIC 15738.01, 15729.01.

- Kahatchee Creek at County Road 008 (33°13’18”N, 86°24’20”W), Talladega Co., Alabama. UAIC 15730.01.
- Fourmile Creek at Co. Rd. 55, next to Co. Rd. 61, just S of Fourmile (33°14’50”N, 86°33’7”W) (T20S, R1E, S33 W 1/2) (Columbiana quad.), Shelby Co., Alabama. UAIC 15724.01.
- Unnamed tributary to Fourmile Creek at Co. Rd. 61, 1.3 mi NE of Fourmile (33°15’43”N, 86°32’19”W) (T20S, R1E, S28 SE 1/4) (Westover quad.), Shelby Co., Alabama. UAIC 15728.01.
- Unnamed tributary to Beeswax Creek at AL Hwy 25, 0.5 mi ENE of Nelson (33°13’32”N, 86°34’4”W) (T21S, R1E, S8 NW 1/4) (Columbiana quad.), Shelby Co., Alabama. UAIC 15723.01.

2. Piedmont group

As currently known, this group is found in the Waxahatchee Creek system (Camp Branch, Buxahatchee Creek, Mill Creek) and Blue Gut Creek. Genetic variability of this group was high compared to *E. ditrema* and we detected no evidence for recent decline in effective population size (Table 3). Source of individuals for genetic analysis:

- Unnamed tributary to Camp Branch, approx. 100 ft. S of Camp Branch bridge on AL Hwy 25, 0.7 mi NE of Shelby Springs (33°8’34”N, 86°40’46”W) (T22S, R1W, S6) (Bounds Lake quad.), Shelby Co., Alabama. UAIC 15719.01.
- Buxahatchee Creek at I-65, 0.8 mi SSE of Calera (33°05’37”N, 86°44’06”W) (T24N, R13E, S1 NW 1/4) (Ozan quad.), Shelby Co., Alabama. UAIC 15718.02.
- Mill Creek at or upstream of Co. Rd. 311, 4.1 mi S of Shelby (33°3’23”N, 86 °34’37”W) (T24N, R15E, S21 NW 1/4) (Shelby quad.), Shelby Co., Alabama. UAIC 15722.02.

- Blue Gut Creek at County Road 61 (32°57’47”N, 86°35’16”W), Chilton Co., Alabama. UAIC 15720.01.
- Blue Gut Creek at AL Hwy 145 (32°59’22”N, 86°36’56”W), Chilton Co., Alabama. UAIC 15721.01.

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Table 1 Location of taxa used in this study, showing locality identifier (ID; see Fig. 1), number of individuals genotyped for microsatellite DNA loci (*N* mDNA), sequenced for the mitochondrial ND2 gene (*N* mtDNA), and sequenced for nuclear intron 1 of S7 protein (*N* S7). The mtDNA haplotypes are indicated for focal taxa in the Cahaba and Coosa rivers

Species/Locality	ID	*N* mDNA	*N* mtDNA	haplotype	*N* S7
Etheostoma ditrema					
Deverell Spring	1	24	5	1	1
Colvard Spring	2	24	5	1, 3	–
Cohutta Spring	3	24	5	2	–
Moseley Spring	4	24	5	4	–
Todd Spring	5	24	5	4, 5	–
Smart Spring	6	24	5	5	–
Ballplay Creek	7	24	5	4	–
Glencoe Spring	8	24	5	6	–
Little Canoe Creek	9	21	5	7	–
Edwards Spring	10	24	5	8	–
Blue Spring	11	24	5	9	–
Coldwater Spring	12	24	5	10, 11,	–
Poorhouse Branch	13	24	5	12, 13, 14	1
E. sp. cf. *ditrema*					
Tallaseehatchee Creek	1	9	4	15, 16, 17	–
Kahatchee Creek	2	16	5	18, 19, 20, 21	–
Fourmile Creek	3	24	5	18, 19, 22	–
Beeswax Creek	4	24	5	18, 23	–
Camp Branch	5	24	5	24, 25, 26	–
Buxahatchee Creek	6	24	5	27, 28, 29	–
Mill Creek	7	24	5	24, 25, 30	–
Blue Gut Creek	8	21	5	31, 32	1
E. swaini					
Cahaba River (CR)					
Lightsey's Pond	1	30	2	34	–
Horse Branch	2	–	2	34, 35	–
Lick Branch	3	–	1	36	–
Sprott island	4	–	1*	33	1*
Alabama River					
Little Mulberry Creek	5	–	1*	–	1*
Tombigbee River					
Luxapalilla Creek		–	1*	–	1*
Black Warrior River	6				
Wolf Creek	7	–	1*	–	1*

*DNA sequences obtained from GenBank (see Table 2); Cahaba River (CR)

Table 2 Additional taxa used in estimates of divergence times and all taxa used in the morphological analysis. GenBank accession numbers for outgroups are listed for the mitochondrial ND2 gene and the nuclear ribosomal S7 intron 1.

Taxon/Locality	ND2	S7
Percina caprodes	EF027178	EF035498
Nothonotus jordani	EF027175	EF035495
Etheostoma asprigene	EF027180	EF035500
E. nuchale		
Glenn Spring	HM856125	HM856115
Roebuck Spring	HM856127	HM856115
E. proeliare	EF027214	EF035534
E. swaini		
Cahaba River		
Sprott island	HM856134	HM856121
Alabama River		
Little Mulberry Creek	HM856132	HM856119
Tombigbee River		
Luxapalilla Creek	HM856124	HM856137
Black Warrior River		
Wolf Creek	HM856131	HM856118
E. trisella	EF027226	EF035546

Table 3 Sample sizes and genetic diversity estimates (averaged over eight microsatellite DNA loci) for STRUCTURE based populations of *Etheostoma ditrema*, *E*. sp. cf. *ditrema*, and *E. swaini*. Population numbers described and mapped in Table 1 and Fig. 1. Genetic diversity estimates are followed by critical *M* values (M_c), *M*-ratios, and associated significance as performed using the methods of Garza and Williamson (2001). Significant values ($P < 0.05$) shown in bold

Species/population	*N*	*NA*	*A*	AR	PA	H_o	H_e	M_c	*M*	*P*-value
E. ditrema										
1, 2	48	74	9.25	7.04	0.87	0.49	0.64	0.69	0.66	**0.0204**
3	24	58	7.25	6.93	0.83	0.55	0.69	0.65	0.48	**0.0000**
4	24	52	6.50	6.02	0.26	0.58	0.65	0.66	0.67	0.0736
5, 6	48	45	5.63	4.70	0.26	0.59	0.61	0.69	0.48	**0.0000**
7	24	88	11.00	10.18	0.49	0.76	0.83	0.65	0.65	0.0552
8	24	65	8.13	7.44	0.14	0.78	0.76	0.65	0.44	**0.0000**
9	21	52	6.50	6.24	0.28	0.54	0.71	0.65	0.47	**0.0000**
10	24	57	7.13	6.63	0.26	0.56	0.64	0.65	0.63	**0.0217**
11, 12	48	91	11.38	7.94	1.09	0.59	0.71	0.69	0.65	**0.0090**
13	24	58	7.25	6.66	0.83	0.59	0.69	0.65	0.39	**0.0000**
population mean		64	8.00	6.98	0.53	0.60	0.69	–	–	–
E. sp. cf. *ditrema*										
1, 2, 3, 4	73	184	23.00	14.08	1.97	0.79	0.90	0.70	0.76	0.2725
5, 6, 7, 8	93	152	19.00	12.73	2.47	0.77	0.88	0.71	0.76	0.2216
population mean		168	21.00	13.40	2.22	0.78	0.89	–	–	–
E. swaini (CR)										
1	30	70	8.75	7.59	2.09	0.71	0.78	0.67	0.69	0.0991

Number of individuals (*N*); total number of alleles (*NA*); mean number of alleles per locus (*A*); allelic richness (AR); private allelic richness (PA); heterozygosity observed (H_o); heterozygosity expected (H_e); Cahaba River (CR)

Table 4 Microsatellite DNA based pair-wise population fixation indices (F_{st}) among STRUCTURE based populations of *Etheostoma ditrema*, *E.* sp. cf. *ditrema*, and *E. swaini*. Population numbers described and mapped in Table 1 and Fig. 1

	Species (locality)	1	2	3	4	5	6	7	8	9	10	11	12	13
1	*E. ditrema* (1,2)	–												
2	*E. ditrema* (3)	0.24	–											
3	*E. ditrema* (4)	0.34	0.31	–										
4	*E. ditrema* (5,6)	0.34	0.29	0.34	–									
5	*E. ditrema* (7)	0.24	0.18	0.18	0.18	–								
6	*E. ditrema* (8)	0.28	0.23	0.21	0.21	0.09	–							
7	*E. ditrema* (9)	0.25	0.23	0.29	0.28	0.16	0.22	–						
8	*E. ditrema* (10)	0.33	0.30	0.29	0.33	0.20	0.24	0.29	–					
9	*E. ditrema* (11,12)	0.28	0.26	0.22	0.31	0.19	0.19	0.25	0.23	–				
10	*E. ditrema* (13)	0.32	0.29	0.25	0.32	0.18	0.23	0.23	0.27	0.23	–			
11	*E.* sp. cf. *ditrema* (1,2,3,4)	0.18	0.16	0.17	0.20	0.09	0.12	0.15	0.15	0.12	0.13	–		
12	*E.* sp. cf. *ditrema* (5,6,7,8)	0.17	0.16	0.19	0.22	0.11	0.14	0.15	0.18	0.15	0.16	0.04	–	
13	*E. swaini* (1)	0.28	0.26	0.28	0.29	0.18	0.22	0.25	0.24	0.25	0.24	0.12	0.13	–

All values significant ($P < 0.0001$)

Table 5 Microsatellite based estimates of theta (Θ) and 95% credibility intervals calculated in LAMARC, followed by the corresponding estimates of long-term effective population size (N_e). Population numbers described and mapped in Table 1 and Fig. 1

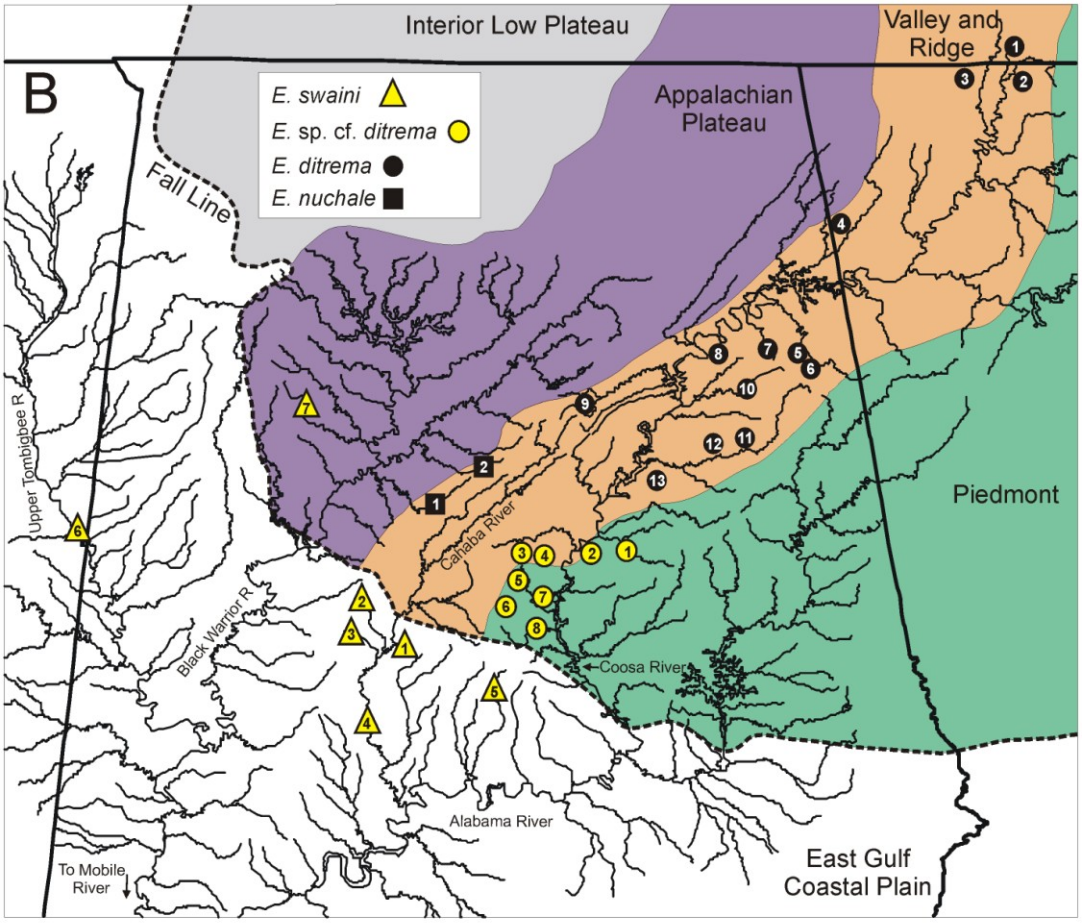
Species/Population	Θ ($4Ne\mu$)	0.05	0.95	Long-term *N*e	0.05	0.95
E. ditrema						
9	2.660	2.053	3.649	1330	1026	1824
13	1.468	1.078	2.076	734	539	1038
E. sp. cf. *ditrema*						
1, 2, 3, 4	10.957	9.944	11.619	5479	4972	5810
5, 6, 7, 8	11.128	10.079	11.716	5564	5039	5858
E. swaini						
1	5.054	4.056	7.117	2527	2028	3559

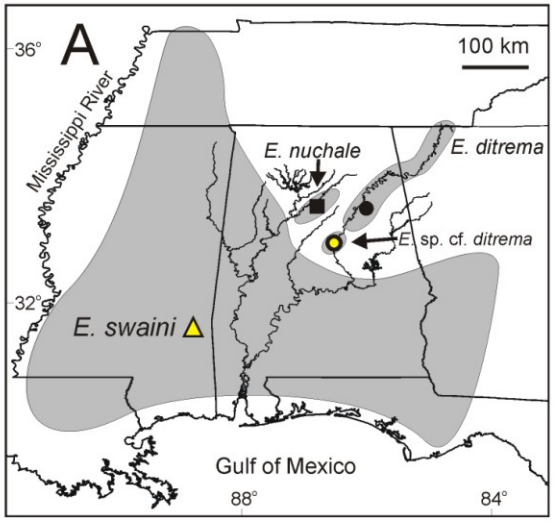
μ = mutation rate/site/generation

Table 6 Rates of migration (*M*), 0.05, and 0.95 credibility intervals inferred using Lamarc. Number of effective immigrants per generation (4*Nm*), 0.05, and 0.95 credibility intervals were obtained by multiplying each *M* times theta (Θ; Table 5) of the recipient population. Population numbers described and mapped in Table 1 and Fig. 1

Population/comparison	*M*	0.05	0.95	4*Nm*	0.05	0.95
E. ditrema (9)						
from *E. ditrema* (13)	0.087	0.053	0.194	0.232	0.140	0.515
from *E.* sp. cf. *ditrema* (1,2,3,4)	0.154	0.052	0.219	0.410	0.138	0.583
from *E.* sp. cf. *ditrema* (5,6,7,8)	0.074	0.032	0.167	0.197	0.084	0.444
from *E. swaini* (1)	0.037	0.015	0.095	0.097	0.039	0.252
E. ditrema (13)						
from *E. ditrema* (9)	0.157	0.066	0.254	0.230	0.097	0.373
from *E.* sp. cf. *ditrema* (1,2,3,4)	0.180	0.081	0.277	0.264	0.119	0.407
from *E.* sp. cf. *ditrema* (5,6,7,8)	0.171	0.073	0.295	0.251	0.108	0.433
from *E. swaini* (1)	0.042	0.017	0.139	0.062	0.025	0.205
E. sp. cf. *ditrema* (1,2,3,4)						
from *E. ditrema* (9)	0.033	0.019	0.071	0.363	0.210	0.779
from *E. ditrema* (13)	0.053	0.028	0.094	0.579	0.306	1.032
from *E.* sp. cf. *ditrema* (5,6,7,8)	0.558	0.406	0.758	6.112	4.449	8.303
from *E. swaini* (1)	0.029	0.014	0.076	0.320	0.158	0.834
E. sp. cf. *ditrema* (5,6,7,8)						
from *E. ditrema* (9)	0.032	0.014	0.066	0.354	0.160	0.740
from *E. ditrema* (13)	0.060	0.025	0.101	0.672	0.281	1.124
from *E.* sp. cf. *ditrema* (1,2,3,4)	0.720	0.629	0.808	8.012	6.998	8.990
from *E. swaini* (1)	0.137	0.065	0.185	1.522	0.723	2.056
E. swaini (1)						
from *E. ditrema* (9)	0.028	0.013	0.065	0.140	0.068	0.326
from *E. ditrema* (13)	0.027	0.013	0.061	0.135	0.066	0.310
from *E.* sp. cf. *ditrema* (1,2,3,4)	0.071	0.021	0.123	0.360	0.105	0.621
from *E.* sp. cf. *ditrema* (5,6,7,8)	0.157	0.146	0.301	0.796	0.740	1.521

Fig. 1 (a) Map of the Mobile Basin and species ranges (gray shading) of *Etheostoma ditrema*, (black circle), *E*. sp. cf. *ditrema* (yellow circle), *E. nuchale* (square), and *E. swaini* (yellow triangle). (b) Sample locations for taxa used in this study. Physiographic provinces are indicated by color. (c) Hypothesis of relationships of the *E. swaini* complex based on allozyme data (redrawn from Mayden et al. 2005). Taxon labels are followed by river drainage or specific localities in Fig. 1B





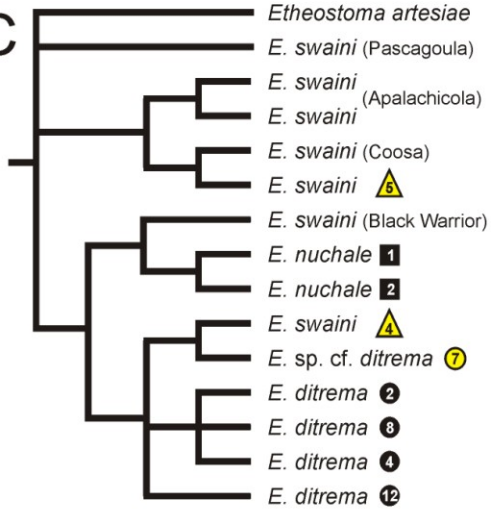


Fig. 2 Chronogram for the *Etheostoma swaini* complex of the Mobile Basin (pruned from the BEAST analysis with centrarchid and darter outgroups) based on the combined analysis of mitochondrial (ND2) and nuclear (S7) gene sequences. Letters at nodes correspond with age estimates in text and nodes with posterior probability < 0.95 are indicated with ns. Gray bars at nodes represent the 95% highest posterior density of age estimates. Taxon names are followed by codes corresponding to Table 1 and Fig. 1

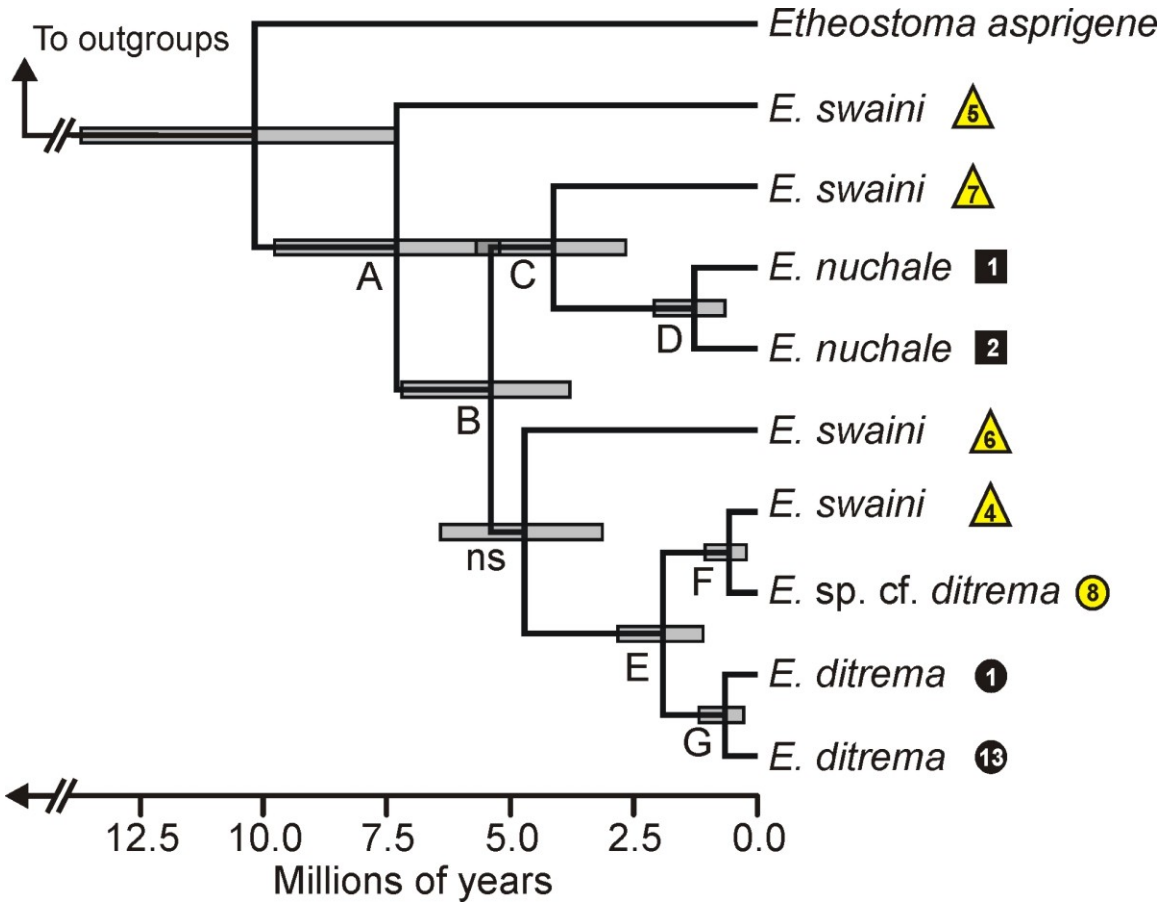


Fig. 3 (a) Maximum clade credibility tree from the discrete phylogeographic analysis of the complete mtDNA data set for *Etheostoma ditrema*, *E*. sp. cf. *ditrema*, and *E. swaini* (CR). Branches are colored according to the most probable location of their descendant nodes. Pie charts represent posterior probability distributions of ancestral locations. Nodes with > 0.95 posterior probability are shown with an asterisk and 95% highest posterior density of node ages are shown below focal clades E, F, and G. Taxon labels correspond to Table 1 and Fig. 1. (b) STRUCTURE bar plots showing the most likely genetic structure for *E. ditrema* ($K = 10$) and *E.* sp. cf. *ditrema* plus *E. swaini* (CR) ($K = 3$) from independent analyses of microsatellite data. Bars correspond to multilocus genotypes of individuals and colors represent the probability of ancestry to each cluster (K). Colors do not correspond to Fig. 3A

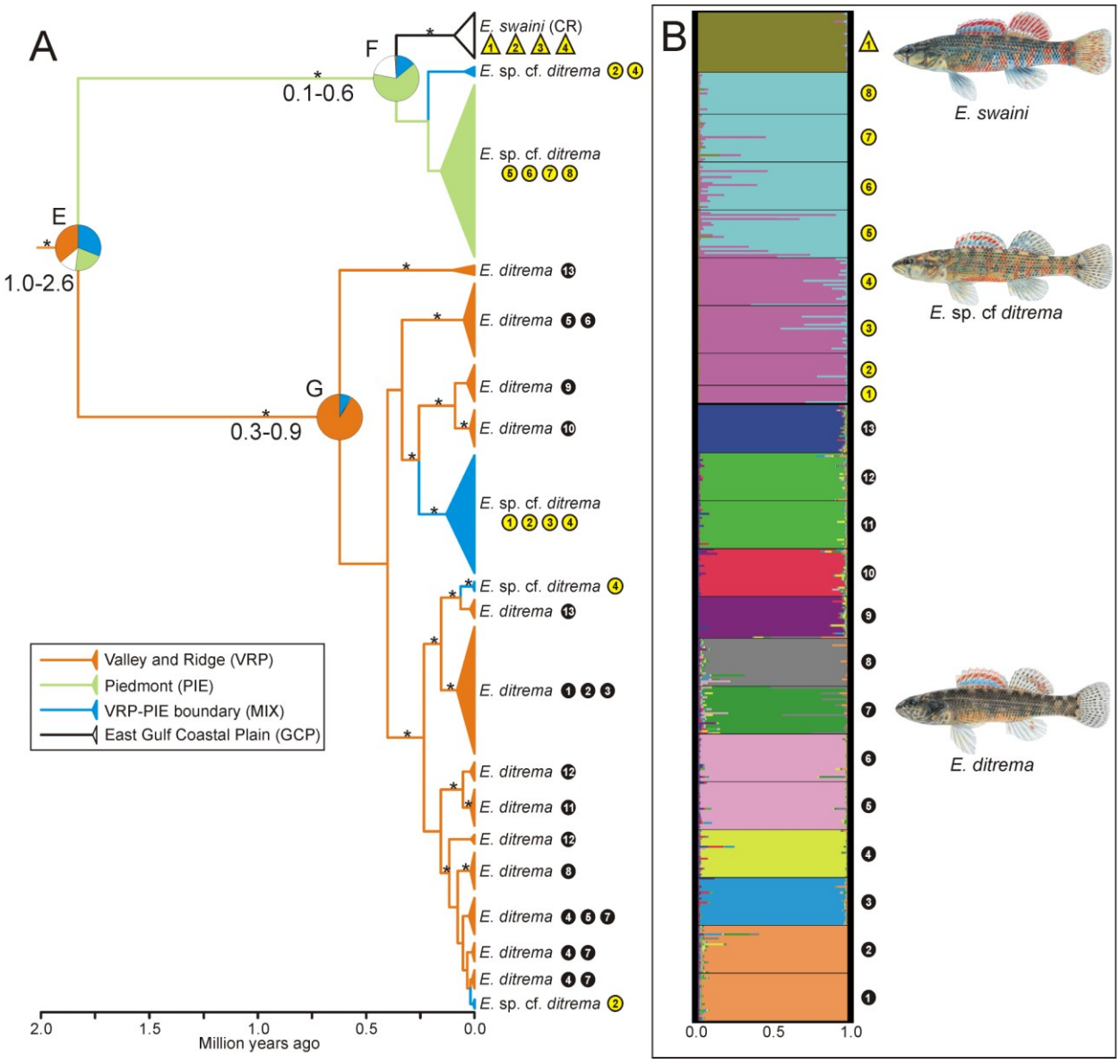
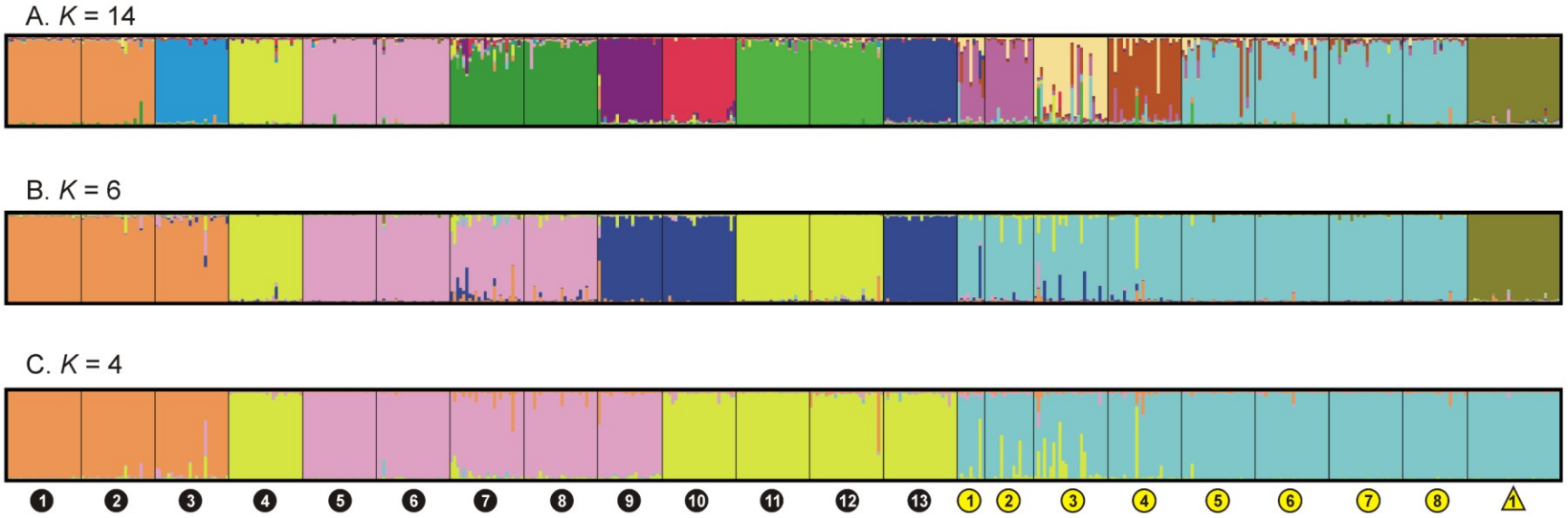


Fig. 4 Bar plots from the STRUCTURE analysis of microsatellite data from all 505 individuals of *Etheostoma ditrema*, *E*. sp. cf. *ditrema*, and *E. swaini*. (a) the most likely genetic structure for all samples (*K* = 14). Possible lower levels of genetic structure for all samples (b, *K* = 6; c, *K* = 4). Bars correspond to multilocus genotypes of individuals and colors represent the probability of ancestry to each cluster (*K*). Taxon labels correspond to Table 1 and Fig. 1



CONSERVATION GENETICS OF THE RUSH DARTER (*ETHEOSTOMA PHYTOPHILUM*): IMPLICATIONS FOR CONSERVATION OF SPRING ENDEMIC FISHES IN THE SOUTHEASTERN UNITED STATES

Introduction

Organisms inhabiting freshwater springs present unique challenges to conservationists. The naturally fragmented distribution of spring species render them extremely vulnerable to fine-scale disturbance and springs are among the most anthropogenically exploited freshwater habitats (Meffe and Vrijenhoek 1988; Hubbs 1995; Etnier 1997; Timpe et al. 2009; Fluker et al. 2010; Martin 2010). Spring endemic species are often confined to spring pools and short stretches of spring runs, with interconnecting streams and rivers acting as major barriers to dispersal (Starnes and Etnier 1986). Consequently, spring endemic species should share several characteristics with island endemic species, i.e. small genetically structured populations with low genetic diversity, making them more susceptible to local extinction compared to their mainland relatives (Frankham 1997; Wilson et al. 2009). Recent studies of spring taxa support this hypothesis in terms of genetic structure (Martin and Wilcox 2004; Hurt and Hedrick 2004; Wilmer and Wilcox 2007) and small population sizes with low genetic diversity (Duvernell and Turner 1999; Fluker et al. 2010). Most genetic studies of North American spring endemic species have focused on taxa from arid lands (Vrijenhoek et al. 1985; Meffe and Vrijenhoek 1988; Echelle et al. 1989; Thompson et al. 2002; Martin and Wilcox 2004; Hurt and Hedrick 2004; Bernardi et al. 2007) where demands from municipal and agricultural users for groundwater have long conflicted with biodiversity conservation (Deacon et al. 2007). Relatively few genetic studies have been conducted in the southeastern United States, however, where recent

groundwater demands due to rapid human population growth threaten the rich diversity of coldwater spring endemics (Hubbs 1995; Etnier 1997; Mirarchi et al. 2004).

The southern Appalachian Highlands (SAH) provides an intriguing landscape to better understand the origin and population genetic characteristics of coldwater spring-adapted species endemic to the southeastern United States. This region is dissected by diverse physiographic characteristics (Fig. 1), providing an abundance of soluble substrates that support at least 10 coldwater spring-dependent fishes (Boschung and Mayden 2004). Because of their endangered status, or extinction (i.e. *Fundulus albolineatus*, Williams and Etnier 1982), many aspects of the origin and genetic characteristics of spring-adapted fishes from the SAH are poorly understood (Warren et al. 2000; Boschung and Mayden 2004; Mirarchi et al. 2004). Previous studies indicate that the upland spring endemic *Etheostoma nuchale* exhibits highly structured populations and likely arose via peripheral isolation from the lowland stream-inhabiting *E. swaini* (Mayden et al. 2005; Fluker et al. 2010). Fluker et al. (2010) further demonstrated that *E. nuchale* exhibited small long-term population sizes and suggested that habitat stability promoted long-term persistence in highly isolated spring habitats despite low genetic diversity and prolonged bottlenecks. However, previous studies lacked temporal information to provide age estimates of the origin of *E. nuchale* and subsequent diversification among isolated spring populations. Additionally, genetic data is needed from additional spring endemic species to better understand patterns of spring endemism in the biodiversity rich SAH.

The Rush Darter, *Etheostoma phytophilum*, is known from three disjunct populations within upland portions of the Black Warrior River (Fig. 1B), where it is exclusive to springs and spring-fed reaches of streams (Bart and Taylor 1999). This species is sister to the Goldstripe Darter (*E. parvipinne*), which is widespread throughout the Gulf Coastal Plain (GCP) from

Texas to Georgia where it typically inhabits small first-order streams, seeps, and springs. Together, the two species comprise the darter subgenus *Fuscatelum* (Page 1981; Near et al. 2011). Two of the three known populations of *E. phytophilum* occupy the Valley and Ridge physiographic province (VRP) with *E. nuchale*, however, they do not naturally co-occur in the same creek systems. Within the VRP, *E. phytophilum* is found in a few spring tributaries of Turkey Creek (TC) of the Locust Fork and Little Cove Creek (LC) of the upper Locust Fork (Fig. 1B). The third population resides in the Clear Creek system of the Sipsey Fork (SF) in the Cumberland Plateau physiographic province (Fig. 1B). The TC population is jeopardized by rapid urbanization of the greater metropolitan Birmingham area, resulting in habitat loss due to channelization of spring runs and spring-fed creeks, modification and/or removal of spring habitat, and reduced recharge of spring aquifers due to increased impervious surfaces throughout the watershed (Bart 2004; Fluker et al. unpublished data). In the upper Locust Fork, *E. phytophilum* is extremely rare (only 53 collected individuals since 1975) and suffers from spring modifications and stream channelization. Although the SF population appears secure and some sites benefit from protection within the Bankhead National Forest (Bart and Taylor 1999), the population is threatened by increased sediment input putatively caused by poor timber harvest practices (Johnston and Kleiner 2002). The United States Fish and Wildlife Service listed *E. phytophilum* as federally endangered based on restricted distribution, sporadic occurrence, and imminent threats to water quality deterioration and habitat destruction (US Fish and Wildlife Service 2011). Currently, genetic and ecological data are lacking for *E. phytophilum* and there are no recovery strategies in place for this endangered species with the exception of a pilot captive propagation program (Rakes and Shute 2005; Petty and Rakes 2009).

Our goal in this study was to examine the origin and post-colonization evolution of *E. phytophilum*. First, we use data from three mitochondrial (mt) DNA regions to reconstruct the phylogeographic history of *E. phytophilum* and *E. parvipinne*. Second, we use nuclear (n) and mtDNA sequence data to estimate the temporal scale of colonization of spring habitats in the upper Black Warrior River by both *E. nuchale* and *E. phytophilum*. If both species have occupied this shared region over the same time scale, they have likely experienced similar historical and contemporary events that have shaped the current patterns of genetic variation. Third, we use microsatellite (m) DNA loci to evaluate post-colonization differentiation and gene flow among populations of *E. phytophilum* relative to widespread lowland populations of *E. parvipinne*. Based on prior results from *E. nuchale* (Fluker et al. 2010), we predict higher levels of genetic structure and lower levels of gene flow among populations of *E. phytophilum* compared to levels among populations of *E. parvipinne*. We also test the hypothesis that population genetic diversity is lower in *E. phytophilum* compared to populations of *E. parvipinne*. Finally, we use mDNA to estimate long-term and current effective population size (N_e) and evaluate how demographic history is reflected in genetic diversity and N_e of *E. phytophilum* since diverging from *E. parvipinne*. Our results are interpreted in the context of the evolution spring-adapted species in the SAH and how data from both *E. phytophilum* and *E. nuchale* can inform conservation decisions for other coldwater spring endemic species in the southeastern United States.

Materials and methods

Samples and DNA extraction

A total of 75 *E. phytophilum* were collected from seven localities representing the three known populations (Table 1; Fig. 1B). Tissues from 71 *E. parvipinne* were obtained from 22 localities throughout a portion of its range in the GCP, with extensive sampling near the upland-lowland boundary (Fall Line) in the Mobile Basin (Table 1; Fig. 1B). Fishes were fin clipped and either released live or retained as voucher specimens. All tissues and vouchers were preserved in 95% ethanol and formalin, respectively, and cataloged in various ichthyological collections (Table 1). Genomic DNA was extracted from fin clips using either standard CTAB-chloroform extraction procedures or the DNEasy kit (Qiagen, Valencia, CA).

DNA sequencing and mDNA genotyping

The complete mtDNA cytochrome *b* gene (cyt *b*), NADH dehydrogenase subunit 2 gene (ND2), and a portion of the control region (d-loop) was amplified for 45 darters (Table 1) using the following primers: cyt *b*, forward primer 5'-GTGACTTGAAAAACCACCGTTG-3' and reverse primer 5'-CTCCATCTCCGGTTTACAAGAC-3' (Song et al. 1998); ND2, 562L 5'-TAAGCTATCGGGCCCATACC-3' and 449H 5'-TGCTTAGGGCTTTGAAGGCTC-3'; and d-loop, Lpro 5'-AACTCTCACCCCTAGCTCCCAAAG-3'and TPhenR 5'-CTAGGGCCCATCTTAACATCTTCAG-3' (Porter et al. 2002). Amplifications were conducted in 25–50 µL reactions with the GoTaq* Flexi DNA polymerase kit (Promega) and cycled the ND2 thermal protocol of Lang and Mayden (2007). A subset of seven individuals recovered in distinct mtDNA clades (Table 1; Fig. 1A) was amplified for the first intron of the nuclear ribosomal protein S7 using primers and conditions in Lang and Mayden (2007). PCR products were purified with the QIAquick PCR purification kit (Qiagen), cycle sequenced using BigDye®

Terminator v3.1 chemistry and read on an ABI PRISM® 3100 Genetic Analyzer (Steven Johnson Molecular Systematics Laboratory, University of Alabama). Cycle sequence reactions for mtDNA regions utilized the amplification primers listed above and internal primers were designed for ND2: 292L, 5'-ATTAYTCTTGCYYTAGCACTAAA-3'; 292L_par, 5'-ATTACTCTTGCCTTAGCCCTAAA-3'; and 523L, 5'-CGTAAAATTCTTGCYTAYTC-3'.

A total of 128 individuals consisting of 73 *E. phytophilum* and 55 *E. parvipinne* from three populations of each species (Table 1) were genotyped for nine mDNA loci using primers designed for *E. caeruleum* (Eca10EPA, Eca11EPA, Eca48EPA, Eca71EPA; Tonnis 2006), *E. osburni* (EosC6, EosC112, EosD107; Switzer et al. 2008), and *E. scotti* (Esc26b, Esc132b; Gabel et al. 2008). Genotyped *E. parvipinne* were from populations with close relationships to disparate populations of *E. phytophilum* based on the mtDNA phylogeny (Fig. 1A). The relatively small sample sizes of *E. parvipinne* samples used in mDNA analyses represent our best efforts to obtain larger number of specimens from the three populations from 2007-2011; this species has a spotty occurrence despite its widespread distribution throughout the GCP (Robison 1977; Mettee et al. 1996; Boschung and Mayden 2004; McAllister et al. 2007). Amplification of mDNA loci followed protocols detailed in Fluker et al. (2010). Genotypes were read on an ABI 3730 DNA analyzer (University of Maine DNA sequencing facility) using HEX or 6-FAM fluorescent labels and GeneScan™ 500 or 1000 ROX™ Size Standard (Applied Biosystems). Allele binning was performed using FLEXIBIN v2.0 (Amos et al. 2007) and the dataset was screened for genotyping errors with MICRO-CHECKER (van Oosterhout et al. 2004).

Phylogeographic analysis: mtDNA

To reconstruct spring colonization by *E. phytophilum*, Bayesian phylogenetic inference was performed in MrBayes v3.12 (Ronquist and Huelsenbeck 2003) using a concatenated

alignment of three mtDNA regions derived from this species and from and *E. parvipinne* samples from the Mobile Basin and neighboring drainages (Table 1). Unique haplotypes were determined for both species using DnaSP v5.10 (Librado and Rozas 2009) and the resulting haplotypes were aligned with outgroups *Percina caprodes* and *E. edwini* (Table 2). Cyt *b* and ND2 genes were partitioned by codon position, d-loop was treated as a single non-coding partition, and best-fit substitution models were determined for each of the seven partitions (Table 3) using Akaike information criterion in the program Modeltest v3.7 (Posada and Crandall 1998; Posada and Buckley 2004). Four partitioned MrBayes runs were conducted for 10 million generations with sampling every 1,000 generations using unlinked variable Dirichlet priors (Table 3). Convergence was assessed via examination of the standard deviation of the split frequencies and runs prior to convergence (> 0.01) were discarded as burnin to construct the 50% majority-rule consensus phylogram. Nodes with ≥ 0.95 posterior probability were considered to have significant support.

Divergence time estimates: mtDNA and nDNA

To estimate the timing of colonization and subsequent diversification of spring endemic darters in the upper Black Warrior River, BEAST v1.6.1 (Drummond and Rambaut 2007) was used with concatenated ND2-S7 sequences of *E. phytophilum* and *E. parvipinne* (*Fuscatelum* group) and *E. nuchale* and its closest relatives (*E. asprigene* species group) (Table 2). The *Fuscatelum* group included a single individual from the three *E. phytophilum* populations, a single individual of *E. parvipinne* from each of the two distinct lineages containing *E. phytophilum*, and a single individual from a western population of *E. parvipinne* (Table 1; Figs. 1, 2). The *E. asprigene* group consisted of a single individual of *E. nuchale* from two distinct populations (Valley and Village creeks), a single individual of *E. swaini* from closely related

upland (Mulberry Fork) and lowland (Alabama River) populations, and a single individual of the western Mud Darter *E. asprigene* (see Fluker et al. 2010; Table 2; Figs. 1, 2). Darters are poorly represented in the fossil record and fossils of close relatives such as *Perca* have been difficult to assess (Cavender 1986). Thus, we employed external fossil calibration methods of Hollingsworth and Near (2009), which utilized DNA sequence data and five fossil calibration points for the closely related family Centrarchidae (Near et al. 2005). These methods have generated consistent estimates of time of most common recent ancestor (TMRCA) for Centrarchidae and major darter groups (Near and Benard 2004; Near and Keck 2005; Keck and Near 2010). Our final alignment consisted of concatenated ND2-S7 sequences from *Fuscatelum* and *E. asprigene* groups listed above, 47 Centrarchid taxa (GenBank accession nos. listed in Near et al. 2005), and representative darter outgroups (Table 2). The S7 data set was aligned using MUSCLE v3.8 (Edgar 2004) with heterozygous positions coded as ambiguous based on standard nomenclature of the IUPAC (Domingues et al. 2005; Ray et al. 2008). ND2 sequences were easily aligned by eye, and the partitions were concatenated in Geneious v5.1.7 (Biomatters Ltd). The BEAST analysis was implemented with priors for minimum bound lognormal age estimates on Centrarchid fossil calibration points used in Hollingsworth and Near (2009) and substitution models were unlinked among ND2 (TrN + I + Γ) and S7 partitions (GTR+ Γ). To allow for uncertainty in substitutions rates on adjacent branches, an uncorrelated lognormal clock model (UCLN, Drummond et al. 2006) was employed with a Yule process speciation tree prior. Four independent runs of 40 million generations were conducted and tree and log files were combined with 40% of the generations discarded as burnin using LogCombiner v1.6.1 (Drummond and Rambaut 2007). The BEAST analysis was replicated with an empty alignment (sampling from the prior only) to evaluate the influence of calibration priors on divergence time estimates.

Tracer v1.5 (Rambaut and Drummond 2007) was used to assess convergence and to determine whether chains mixed well (effective sample size > 200) during BEAST and MrBayes runs.

Genetic diversity and population structure: mDNA

Departures from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were assessed in GENEPOP v4.0.10 (Rousset 2008) under a Markov Chain algorithm with 10,000 dememorizations, 200 batches and 10,000 iterations per batch. Genetic diversity estimates, i.e. the mean number of alleles per locus (A), observed heterozygosity (H_o), and expected heterozygosity (H_e) were calculated in GENEPOP. We also calculated allelic richness (AR) and private allelic richness (PA), measures of allelic variation that control for differences in sample sizes and missing data, using HP-RARE (Kalinowski 2005). FSTAT v2.9.3.2 (Goudet 2001) was used to evaluate the significance of differences in genetic diversity (AR, H_o, and H_e) between *E. phytophilum* and *E. parvipinne*. Mann-Whitney U tests were performed to further evaluate the significance of differences in genetic diversity parameters among populations within species.

The Bayesian clustering method of STRUCTURE v2.3.3 (Pritchard et al. 2000) was used to determine the number of genetically differentiated clusters (K) from combined *E. phytophilum* and *E. parvipinne* mDNA samples (Table 1) without *a priori* designations. Given the close relationship of *E. phytophilum* and *E. parvipinne* populations based on our phylogenetic analyses (Figs. 1A, 2), we implemented models allowing admixture of genotypes and correlated allele frequencies (Falush et al. 2003). Ten replicates of 400,000 iterations (100,000 burnin) were conducted for K values ranging from 1 to 7. Best estimates of K were determined using the ad hoc summary statistic ΔK (Evanno et al. 2005) as implemented in STRUCTURE harvester v0.6.7 (Earl 2011) and bar plots were constructed using *Distruct* v1.1 (Rosenberg 2004).

Population genetic structure, differentiation (F_{st}), and associated significance were assessed by analysis of molecular variance (AMOVA) as implemented in ARLEQUIN v3.5 (Excoffier and Lischer 2010) with 10000 permutations. In the first AMOVA, we treated both species as a single group to test the null hypothesis that the six populations (*E. phytophilum* [SF, TC, LC] and *E. parvipinne* [Pascagoula River (PR), Tyro Creek (TY), Cahaba River (CR)]) represent an arbitrary subsample relative to the entire sample. Next, the two species were analyzed independently to test the null hypothesis that individuals from each population represent an arbitrary subsample relative to the respective species. Finally, FSTAT v2.9.3.2 (Goudet 2001) was used to assess whether F_{ST} differed significantly between *E. phytophilum* and *E. parvipinne*.

N_e estimates, migration, and demographic history: mDNA data

Bayesian coalescent analyses were implemented with mDNA in LAMARC v2.1.6 (Kuhner 2006) to estimate the parameter Θ ($4N_e\mu$) and immigration rates (M) for populations of both species. Multi-population analyses were conducted independently for each species. Initial runs were conducted with final chains of 600,000–1,000,000 steps to evaluate convergence of parameter values among runs. Final runs consisted of five initial chains of 100,000 steps (5000 trees sampled every 20 reps, burn-in = 20,000) and one final chain of 2,000,000 steps (100,000 trees sampled every 20 reps, burn-in = 50,000) using an adaptive heating scheme with 4 temperatures (1.0, 1.1, 1.2, 1.3). Priors for Θ and M ranged from 1.0×10^{-5} to 10.0 and 1.0×10^{-2} to 1000, respectively. Number of effective immigrants per generation ($4N$m) was estimated for each population pair by multiplying M by its estimate of Θ for the recipient population (Kuhner 2006; Kuhner and Smith 2007). LAMARC based estimates of Θ and the mDNA mutation rate (μ) of 5×10^{-4} (Goldstein and Schlötterer 1999; Yue et al. 2007) were substituted into the

equation $\Theta = 4N_e\mu$ to estimate long-term N_e. Because long-term N_e may not reflect recent population history, LDNe (Waples and Do 2008) was used to estimate current N_e based on the linkage disequilibrium method with a bias correction (Hill 1981; Waples 2006).

Two complementary bottleneck tests were conducted to evaluate whether populations have experienced recent declines in N_e. The first method, H_e excess test implemented in the program BOTTLENECK v1.2.02 (Cornuet and Luikart 1996; Piry et al. 1999), takes advantage of the observation that gene diversity (H_e) will be significantly greater than heterozygosity expected based on number of alleles and sample size (H_{eq}) in recently bottlenecked populations (Luikart and Cornuet 1998; Luikart et al. 1998; Piry et al. 1999). We conducted 10,000 replicates using a two-phase model (TPM), in which 95% of mutations were single-step with a 12% variance of multi-step mutations (Piry et al. 1999). Significance of deviations between H_e and H_{eq} were assessed with Wilcoxon signed-rank tests. The second method estimated the ratio of number of alleles to the range in allele size (M) and compared them to population specific critical M values (M_c) using M_P_Val.exe and Critical_M.exe (Garza and Williamson 2001). As suggested by Garza and Williamson (2001), a conservative parameter set was used to estimate M-ratios for populations of both species: pre-bottleneck Θ values of 10 (N_e = 5,000); average size of non one-step mutations = 3.5; and TPM with 90% single-step mutations. The probability that a smaller M-ratio would be expected under equilibrium conditions was tested with 10,000 simulations.

Results

Phylogenetic analyses and divergence time estimates

Amplification of the three mtDNA regions was successful for all *E. phytophilum* and *E. parvipinne* used in the phylogeographic analysis. The complete ND2 gene (1047 bp) was obtained for all individuals, whereas d-loop and cyt *b* sequences were truncated to 821 bp and 990 bp, respectively. The d-loop sequences had four alignment gaps and cyt *b* aligned with bases 58–1047 of the complete 1140 bp sequence of *E. parvipinne*. Of the 45 darters sequenced, 24 unique composite mtDNA haplotypes were identified (*E. phytophilum* = 5, *E. parvipinne* = 19; Table 1). When aligned with outgroup sequences, the composite mtDNA data set (2866 bp) exhibited 2,125 constant and 386 phylogenetically informative characters. The standard deviation of split frequencies in MrBayes runs fell below 0.01 in 6.5 x 10^6 generations and, when visualized in Tracer, parameter values were highly convergent among runs. Thus, the first 65% of trees of each run were discarded as burnin to construct the 50% majority rule consensus phylograms (3500 trees used, 6500 discarded; Fig. 1A). The Bayesian analysis recovered two major clades with substantial mtDNA divergence (6.74%) corresponding to samples of *Fuscatelum* west (clade 1) and east (clade 2) of the Mississippi River (Fig. 1A). Within clade 2, *E. parvipinne* from the Tennessee, Hatchie, and Obion river drainages showed moderate divergence (1.84%), but unresolved relationships, with respect to focal haplotypes in the well supported clade 3 comprised of *E. phytophilum* and *E. parvipinne* from the PR, Mobile, and Chattahoochee river drainages (Fig. 1A, B). *Etheostoma phytophilum* was polyphyletic with respect to *E. parvipinne* from clade 3. However, the SF and Locust Fork (TC, LC) populations were independently recovered in well-supported clades that correspond to samples west (clade 4) and east (clade 5) of the divide between the Tombigbee and Alabama river drainages (Fig. 1A,

B). Moderate mtDNA divergence was revealed between clades 4 and 5 ($\bar{x}$ = 1.70%) and comparable levels were found among haplotypes within clades 4 and 5 (0.04–1.5% and 0.04–2.0%, respectively). Although relationships were unresolved among *E. phytophilum* and *E. parvipinne* haplotypes in both clades 4 and 5, populations of *E. phytophilum* within these clades were well supported with moderate mtDNA divergence compared to other distinct *E. parvipinne* populations (Table 4; Fig. 1A).

The combined ND2-S7 data set for the BEAST analysis consisted of 1811 characters; 823 constant, 145 variable uninformative, and 843 phylogenetically informative characters. BEAST runs with empty data yielded substantially older divergence time estimates when compared to runs with data, suggesting that model priors did not overpower information in our data set. The analysis recovered darters and centrarchids in reciprocally monophyletic groups (not shown). The TMRCA of Centrarchidae (33.7 Mya, 95% highest posterior density (HPD): [27.2, 40.8]) was consistent with a previous estimate (32.6 Mya, 95% HPD: [25.3, 39.9]) using this calibration method (Hollingsworth and Near 2009). The *Fuscatelum* and *E. asprigene* species group chronograms pruned from the larger analysis are shown in Figure 2. Within *Fuscatelum*, the TMRCA for focal *E. phytophilum* and *E. parvipinne* haplotypes (see clade 3, Fig. 1A) was 2.2 Mya, 95% HPD: [1.5, 3.1] (node B; Table 5; Fig. 2). The analysis indicated a slightly younger split between SF *E. phytophilum* and *E. parvipinne* west of the Alabama River (node C, 1.2 Mya, 95% HPD: [0.8, 1.9]) when compared to the split between Locust Fork (TC, LC) *E. phytophilum* and *E. parvipinne* east of the Tombigbee River (node E, 1.9 Mya, 95% HPD: [1.2, 2.7]) (Table 5; Fig. 2). The former was comparable to age estimates of TMRCA for Locust Fork (TC, LC) populations of *E. phytophilum* (node F, 1.2 Mya, 95% HPD: [0.6, 1.8]) (Table 5; Fig. 2). Within the *E. asprigene* species group, the TMRCA for upland *E. swaini*

(Mulberry Fork) and *E. nuchale* was 4.6 Mya, 95% HPD: [2.9, 6.4] and *E. nuchale* populations shared a common ancestor 1.3 Mya, 95% HPD: [0.7, 2.2] (nodes I, J; Table 5; Fig. 2).

Characteristics of mDNA loci and genetic diversity

All nine mDNA loci amplified were polymorphic; however, loci EosC112 and EosD107 failed to amplify for *E. parvipinne* from PR and *E. phytophilum* from SF, respectively. Due to the possibility of null alleles in populations amplified for these loci, they were omitted and the remaining seven loci were used in subsequent mDNA analyses. Following Bonferroni correction, only one of 37 tests for HWE showed significant deviation (*E. phytophilum* [TC], Locus Eca71EPA). For locus Eca71EPA, the TC population showed a unique allele configuration (555−587 bp) compared to all other populations of both species (191−311 bp). Further examination by DNA sequencing revealed a 284 bp insert within the tetranucleotide repeat region. Analyses with and without locus Eca71EPA were highly concordant, thus it was used in all mDNA analyses. Pairwise comparisons of all loci revealed no evidence of linkage.

The number of alleles ranged from 3−22 and 2−22 per locus across samples of *E. phytophilum* and *E. parvipinne*, respectively. Genetic diversity measures (A, AR, PA, H_o, H_e) varied widely among populations for both species, with *E. phytophilum* showing slightly lower means of all but PA when compared to *E. parvipinne* (Table 6). However, the FSTAT analysis revealed no significant differences between species for the above measures. Pairwise population comparisons using Mann-Whitney U tests revealed significant differences in A, AR, and H_e between PR and LC, TC, TY, and CR ($P < 0.05$ for all), however, these were not significant following Bonferroni correction.

Population structure

The mean 'estimated ln probability of the data' in STRUCTURE runs rose to $K = 6$ and leveled off, corresponding to the best estimate of the ΔK method (not shown). The plot of ΔK also indicated a peak at $K = 4$, which corresponded to a lower level of structure grouping all *E. parvipinne* populations together (Fig. 1C). When species were analyzed independently, each had a best estimate of $K = 3$; thus, we interpret $K = 6$ to be the most appropriate level of population structure for the mDNA data set (Fig. 1D). Group assignments for $K = 6$ indicated high proportions of population membership for both *E. phytophilum* (0.97–0.98) and *E. parvipinne* (0.90–0.97) to STRUCTURE based clusters.

The AMOVA treating both species as a single group indicated that 65% of the genetic variation was distributed within individuals (Table 7). However, the analysis revealed a high degree of structure among populations (average $F_{st} = 0.34$, $P < 0.00001$; Table 7) and all pair-wise population comparisons of differentiation were highly significant ($P < 0.0001$ for all; Table 4). When species were analyzed separately, genetic structure between *E. phytophilum* populations (average $F_{st} = 0.40$; $P < 0.00001$) was more than double the value observed among *E. parvipinne* populations (average $F_{st} = 0.14$; $P < 0.00001$). The FSTAT analysis indicated that the mean F_{st} value for *E. phytophilum* was significantly greater than *E. parvipinne* ($P = 0.048$).

N_e estimates, migration, and demographic history

Bayesian estimates of Θ varied greatly for both species and estimates of long-term N_e ranged from 1,202−3,463 and 1,547−4,802 for *E. phytophilum* and *E. parvipinne* populations, respectively (Table 8). LDNe was unable to estimate current N_e for TC, but the remaining populations ranged from 45−infinity and 12−infinity for *E. phytophilum* and *E. parvipinne*, respectively (Table 8). Bayesian estimates of migration ($4Nm$) were below 0.27 among *E. phytophilum* populations and values ranged from 0.37−0.95 among *E. parvipinne* populations

(Table 9). Results from the H_e excess tests revealed a recent bottleneck in the PR population of *E. parvipinne*, but all other populations showed no evidence for H_e excess (Table 6). The *M* value for only one of the six populations (TY, *E. parvipinne*) was significantly below its respective M_c value (Table 6).

Discussion

Spring colonization history of E. phytophilum in the upper Black Warrior River

The Bayesian analysis recovered *E. phytophilum* in two major lineages that each included lowland populations of *E. parvipinne*, suggesting two independent upland colonization events for *E. phytophilum* (clades 4, 5; Fig. 1A). Reciprocal monophyly of clades 4 and 5 strongly corresponds to a split between eastern and western tributaries of the Mobile Basin (see exceptions for Cottondale Creek [CD], LC, and TC below), suggesting a lack of gene flow since their MRCA 2.2 Mya, HPD: [1.5, 3.1] (Table 5; Fig. 2). The timing of divergence between these two lineages (late Pliocene-early Pleistocene) corresponds closely with sea level fluctuations that often resulted in extended periods of isolation between the Alabama and Tombigbee rivers (Swift et al. 1986). Continued isolation of these lineages is likely facilitated by Quaternary deposits in the lower Mobile Basin that are avoided by *E. parvipinne* as evidenced by its distribution (Rohde 1980). Phylogenetic and divergence time estimates suggest that the earliest upland colonization event by *Fuscatelum* involved members of eastern Mobile tributaries, resulting in the extant Locust Fork populations of *E. phytophilum* (clade 5, Fig. 1A; node E, Table 5; Fig. 2). Two lines of evidence suggest that the current distribution of *E. phytophilum* in the Locust Fork is likely the result of historic intermittent inter-drainage connections between Cahaba and Black Warrior River or Locust Fork tributaries. First, at least two other fishes (*Cyprinella trichroistia* and *Notropis chrosomus*) share this peculiar distribution. For example, each species has the majority of its distribution in tributaries to the Alabama River, but each also has highly restricted populations in the upper Black Warrior River and Locust Fork (Boschung and Mayden 2004). Second, our phylogenetic analysis revealed a recent connection between a CR and a Black Warrior River population where their stream tributaries closely interdigitate

(CD; Fig. 1). Phylogenetic and divergence time estimates suggest that the extant SF population of *E. phytophilum* was the result of a more recent upland colonization by *Fuscatelum* from western Mobile tributaries (clade 4, Fig. 1A; node C, Table 5; Fig. 2). It seems likely that the SF was colonized via inter-tributary movements from tributaries of the Black Warrior River or upper Tombigbee rivers (Fig. 1A, B). In fact, the distributions of several fishes including an undescribed darter (*E*. sp. cf. *zonistium*) and two minnows (*Lythrurus bellus* and *Notropis baileyi*) reveal evidence for stream capture events in the area at the Fall Line where tributaries of the upper Tombigbee and Tennessee rivers interdigitate with the SF (Wall 1968; Boschung and Mayden 2004). Given that members of *Fuscatelum* are extreme headwater specialists (Moore and Cross 1950; Robison 1977; Boschung and Mayden 2004), this sort of inter-drainage dispersal is likely favorable where tributaries interdigitate and opportunistic events such as flooding or stream capture occur. The multiple, upland Pliocene-Pleistocene colonization events by *Fuscatelum* revealed in this study was in stark contrast to the single, older colonization of upland habitats by the ancestor of *E. nuchale* (Fluker et al. 2010). Our analyses suggest that the ancestor of upland *E. nuchale* and *E. swaini* (Mulberry Fork) shared a MRCA with lowland *E. swaini* in the late Miocene-early Pliocene (7.0 Mya, HPD: [4.9, 9.5]) and the subsequent split of upland *E. swaini* and *E. nuchale* predates upland colonization by *Fuscatelum* (Table 5; Fig. 2).

Post-colonization differentiation of spring populations in the upper Black Warrior River

We predicted that upland populations of *E. phytophilum* would exhibit greater levels of inter-population genetic structure, thus lower levels of migration, compared to its lowland relative *E. parvipinne*. Our prediction was based on the results of Fluker et al. (2010), in which populations of the spring endemic *E. nuchale* showed substantially higher levels of among population genetic structure (F_{st}, 0.05–0.29, mean = 0.21) compared to its stream-dwelling

relative *E. swaini* (F_{st}, 0.06–0.08, mean = 0.07). Results from STRUCTURE and AMOVA strongly supported our prediction, revealing significantly higher levels of genetic structure among populations of *E. phytophilum* (F_{st}, 0.33–0.50, mean = 0.40; Tables 4, 7), when compared among lowland populations of *E. parvipinne* (F_{st}, 0.09–0.21, mean = 0.14; Tables 4, 7). The distinctive, well supported clades of *E. phytophilum* in our phylogeographic analysis (Fig. 1A), in conjunction with relatively higher among population mtDNA divergence (*E. phytophilum* mean = 1.7%, *E. parvipinne* mean = 1.2%; Table 4), corroborate the findings based on mDNA.

Although *E. nuchale* has a much longer upland history compared to *E. phytophilum*, the timing of divergence between their respective populations in the SAH in is surprisingly similar and suggests that Pleistocene events played an important role in their isolation (nodes F, J; Table 5; Fig. 2). Features of the SAH within our study area have remained relatively stable since mid-Mesozoic times and were unaffected by Pleistocene glaciations (Swift et al. 1986). Thus, changes in climate during this time probably had a strong influence on colonization of spring habitats and subsequent diversification of spring populations in the SAH. The overlapping Pleistocene divergence of *E. phytophilum* and *E. nuchale* populations corresponds to the transition from relatively shorter Pleistocene glacial cycles (41,000 years) to relatively longer, more dramatic cycles (100,000 years) approximately 0.9 Mya (Hewitt 2000; Hewitt 2011). This is consistent with the hypothesis of Williams (1968) that Pleistocene warming periods facilitated spring specialization of *Cottus paulus* in the Coosa River drainage. Alternatively, extended periods of colder, dryer climate following the onset of 100,000 year glacial cycles may have facilitated adaptation in isolated groundwater outflows.

Genetic diversity, N_e, and demographic history

Relatively little is known about levels of genetic diversity and corresponding N_e of spring endemic species in the SAH. However, to really understand if levels of genetic diversity are 'low' or 'reduced' in spring endemic taxa, comparisons to closely related taxa and knowledge of demographic history are needed. For example, Fluker et al. (2010) showed that mDNA based measures of AR were relatively consistent across spring populations of *E. nuchale* (4.41–6.28, mean = 5.22), yet significantly lower compared to its stream-dwelling progenitor *E. swaini* (9.87–12.44, mean = 11.57). Fluker et al. (2010) concluded that the consistently small long-term N_e estimates among *E. nuchale* populations (approx. 25% the size of *E. swaini*) was evidence for small numbers of founders at speciation, with subsequent bottlenecks, and populations have likely persisted in stable spring habitats with historically small N_e. No such clear-cut differences in genetic variation and N_e, were observed between spring populations of *E. phytophilum* and its progenitor *E. parvipinne*, where most values overlapped. These genetic similarities are likely due to their similarity in habitat preference. Although *E. parvipinne* is wide-spread throughout the GCP, it occupies specialized stream habitat, most often found in small springs, seeps, and first-order streams where population numbers are small (Moore and Cross 1950; Robison 1977; Mettee et al. 1989; Boschung and Mayden 2004). These characteristics render populations of *E. parvipinne* highly susceptible to local stochastic events such as drought or habitat alteration and, interestingly, two of the three populations of *E. parvipinne* showed evidence for recent bottlenecks.

The levels of genetic diversity and N_e between Locust Fork (TC, LC) populations of *E. phytophilum* were approximately one-half the values observed in SF *E. phytophilum*. These substantial (but non-significant) differences are likely a product of their independent upland colonization events, in which number and genetic variation of founders may have been quite

different between Locust Fork and SF. That we found no evidence for recent reductions in N_e for any populations of *E. phytophilum* suggests that populations have remained stable throughout recent history or have recovered genetic diversity lost in historic bottlenecks (Akst et al. 2002). As suggested by Fluker et al. (2010), spring populations within the SAH may persist over evolutionary timescales despite low genetic diversity due to the stability of the region and habitat, which is characterized by ridges separating valleys that have potentially hydrologically isolated ancient aquifers (Timpe et al. 2009).

Conservation guidelines for spring endemic species and recommendations for *E. phytophilum*

With the increased demands on groundwater use in the eastern United States (Sun et al. 2008), conservation of spring habitats and their unique taxa will require highly specific planning and broad collaborations that extend beyond the scientific community. Below we adapt the recommendations of Shute et al. (1997), Mirarchi et al. (2004), and George et al. (2009) and provide examples from this study and previously published works to detail comprehensive guidelines (listed in order of priority) for conservation planning of coldwater spring habitats and their associated taxa in the southeastern United States. We recommend that readers supplement each of the guidelines listed below with related information cited above.

1. Communication with land owners, stakeholders, and the public

Conservation planning for spring habitats or species will require broad collaboration between scientists, conservation managers, private land owners, and the public. Cooperation from land owners and approved access to springs is the most important aspect of conserving spring habitats and their species. Scientists should work closely with conservation managers to develop strategies specific to the spring habitat and taxa under consideration. Basic information about strategies must then be communicated to the landowner, as well as assistance with best management practices (BMPs) or other options such as conservation easements (Rissman et al. 2007). Recent events involving *E. nuchale* and *E. phytophilum* provide evidence for the success of conservation strategies when effective communication is in place with land owners, stakeholders, and the public. All populations of *E. nuchale* reside within urbanized areas of the greater Birmingham metropolitan area, and conservation strategies have been well communicated with land owners since discovery of the species in 1964 (Howell and Caldwell

1965) and subsequent discovery of additional populations through 2002 (Kuhajda 2003). Working together, scientists, state and federal agencies, non-government organizations (NGO), and land owners have provided protection for three of the five known populations. Thomas Spring was transformed into the Watercress Darter National Wildlife Refuge in 1980 (US Fish and Wildlife Service 1992), conservation managers worked closely with the private owners of Seven Springs where BMPs are in place, and Tapawingo Spring (a non-native population) was purchased by a NGO and is maintained as a natural area. *Etheostoma phytophilum* is co-distributed with two other federally endangered darters (*E. chermocki* and *E. nuchale*) within the urban TC drainage. Successful partnerships between NGOs, the city of Pinson, state and federal agencies, and academic institutions led to the development of the Turkey Creek Nature Preserve, which serves as a natural area and offers education programs to inform the public of the unique creek system and its rare inhabitants. However, not all examples for *E. nuchale* are success stories. The Roebuck Spring population in Birmingham recently experienced a fish kill of approximately 11,760 individuals due to the unplanned removal of a small dam by managers of the municipal property (Fluker et al. 2009). While previous agreements with USFWS were in place, the city has not always been sensitive to the species' needs (Stiles 2004).

This example clearly illustrates how communication break-down and mismanagement of spring habitats can erase decades of conservation planning and implementation. As suggested by Fluker et al. (2009), annual meetings between all involved parties including scientists, local, state, and federal agencies, stakeholders, and land owners to review the status of populations and share new information would help ensure successful conservation of spring endemics. In addition, information providing details about the spring habitat and associated species,

conservation and management goals, and emergency protocols with contact information should be distributed to the landowners and posted at appropriate sites.

2. Knowledge and maintenance of aquifer and surface habitat

Spring habitats are unique in that the quality and quantity of their surface habitat is directly related to the underlying aquifer, and the spring-aquifer relationship can be quite complex. For example, aquifers that feed outflows may be small and local or can be quite extensive with recharge zones hundreds of kilometers from the spring (Hubbs 1995). Maintaining spring habitats will require a detailed knowledge of the extent of the aquifer recharge zone and factors that impact the quantity and quality of water re-entering aquifers (Kuhajda 2004). Thus, determination of aquifer recharge areas is a critical step in any spring conservation plan.

The surface habitat of springs is often modified because they provide sources of clear, clean water. Flow retarding structures such as dams, concrete enclosures, and culverts are common in springs of the southeastern United States. For example, all springs containing *E. nuchale* and *E. phytophilum* (with the exception of SF) have flow retarding structures in place that alter natural volumes and flows within spring basins or runs. Where possible, conservation strategies should be implemented to carefully plan and remove flow retarding structures, thus restoring natural flow and volume conditions. Nonpoint-source pollution is another major factor jeopardizing the status of southeastern spring endemic fishes (Etnier 1997). For urban springs such as the TC population of *E. phytophilum* and all *E. nuchale* populations, conservation strategies should minimize the input of stormwater run-off, which often transports industrial and residential toxins and excessive fine sediments. For springs in forested or agricultural settings, such as LC and SF population of *E. phytophilum*, strategies should minimize inputs of excessive

nutrients and fine sediments, which may be detrimental to native aquatic vegetation and water quality. Duncan et al. (2010) showed that abundance of adult *E. nuchale* was highly dependent on within-spring structural diversity, particularly aquatic vegetation. Numerous studies suggest that both *E. nuchale* and *E. phytophilum*, in addition to most spring endemic fishes of the southeastern United States, are highly dependent on aquatic vegetation for cover, reproduction, and food (Ramsey and Suttkus 1965; Williams 1968; Howell and Caldwell 1965; Boschung and Mayden 2004; Duncan et al. 2010; Bart and Taylor 1999). Thus, conservation planning should seek to maximize structural diversity and minimize the removal (mechanical or chemical) of native aquatic vegetation. Restoration and maintenance of riparian vegetation contribute to the stability of spring basin morphology, providing structural diversity and substrate stability for native aquatic vegetation (Mirarchi et al. 2004; Duncan et al. 2010). Studies similar to Duncan et al. (2010) are needed to determine specific habitat types used by *E. phytophilum*.

3. Knowledge of genetic distinctiveness and diversity

Resolving taxonomic uncertainty and population genetic structure is a key component of conservation planning for imperiled species (Frankham et al. 2002; Allendorf and Luikart 2007). The current taxonomic status of *E. phytophilum* warrants further attention as populations in the Locust Fork and SF are potentially distinct species. Bart and Taylor (1999) noted slight morphological differences between SF and Locust Fork (TC, LC) populations in their original description. Our phylogenetic analysis showed that SF and Locust Fork populations were the result of independent colonization events from distinct lineages of *E. parvipinne*. However, these results were not corroborated by independent analysis of nuclear S7 data (not shown), in which we found no phylogenetic resolution among focal taxa (clade 3; Fig1A). Upland populations of *E. phytophilum* are relatively young (< 2.7 Mya), therefore insufficient time may have passed for

sorting of both nDNA and mtDNA alleles (Funk and Omland 2003). Regardless of species status, we identify three genetically distinct populations for *E. phytophilum* that should be treated independently in conservation and management practices: 1) Sipsey Fork (Mill Creek and Wildcat Branch); 2) Turkey Creek of the Locust Fork; and 3) Little Cove Creek of the Locust Fork.

Understanding the genetic and demographic history of imperiled species and their populations is a central goal because of the potential to identify processes that contributed to their current endangered status (DeSalle and Amato 2004; Allendorf and Luikart 2007). For example, spring populations that are presently genetically structured and exhibit low genetic diversity may have 1) historically small, stable population sizes or 2) demographic events may have contributed to recent declines in population size. Uncovering the former scenario may suggest that spring populations are relatively stable and require only simple habitat protection, whereas the latter would suggest the need for more intensive strategies to recover genetic diversity. Results here suggest that levels of genetic diversity in *E. phytophilum* are not significantly different from its widespread progenitor *E. parvipinne*. Further, we found no evidence for recent reductions in N_e, suggesting that contemporary events have likely had little effects on genetic diversity within populations of *E. phytophilum*. Knowledge of the levels of population genetic diversity for *E. phytophilum* do, however, allow the prioritization of populations based on their evolutionary potential (Frankel 1974; Spielman et al 2004; Frankham 2005). Populations in the Locust Fork, specifically Turkey Creek, showed the lowest levels of genetic diversity, which increases their susceptibility to stochastic events and environmental changes, thus conservation efforts should be prioritized ahead of Sipsey Fork.

4. Guidelines for propagation, translocation, reintroduction, and augmentation (PTRA, George et al. 2009)

Conservation planning for imperiled species often includes some form of PTRA and, if conducted properly, can be an integral component of recovery strategies for spring endemic taxa. However, PTRA is often considered or implemented for imperiled taxa well before key prerequisites are in place, which can be detrimental to conservation efforts. For example, from 1965–1988 three translocations of *E. nuchale* were conducted in an attempt to establish new populations in nearby springs, only one of which was successful in 1988 in Tapawingo Spring, a tributary to TC of the Locust Fork (US Fish and Wildlife Service 1992). Unfortunately, this transplantation was established outside of the native distribution of *E. nuchale*, where its success has likely led to the extirpation of the native spring endemic in Tapawingo Spring *E. phytophilum* (Bart 2004; George et al. 2009; Fluker et al. 2010). Based on such examples, PTRA strategies for spring endemic taxa will require considerable planning to be successful. First, any and all PTRA strategies (especially fishes) should follow the guidelines of George et al. (2009). Second, PTRA strategies for spring endemic taxa should only be pursued if no other solutions exist to recover species or their populations. However, we suggest that pilot captive propagation studies using stocks from surrogate species or healthy populations of the target species would be useful to determine the efficacy of PTRA programs if needed in the future. Finally, if PTRA programs are deemed necessary, steps 1–3 of the guidelines herein should be in place. Without the proper communication between all involved parties, knowledge of the status of aquifer and surface habitats, and knowledge of genetic characteristics of the species and its populations, implementation of PTRA strategies may prove more detrimental than helpful.

Pilot captive propagation studies have already been conducted for *E. phytophilum*, showing that the species is easily cultured in aquaria (Rakes and Shute 2005; Petty and Rakes 2009). Because spring sites within the TC population are heavily impacted by urbanization and have the lowest genetic diversity of any *E. phytophilum* population, it would be highest priority if PTRA strategies were put into place. In particular, the Tapawingo Spring population would be a likely candidate for re-introduction of *E. phytophilum*, but would currently fail if the non-native *E. nuchale* population is not controlled, as it is likely a superior competitor for resources (George et al. 2009).

5. Documentation

Complete and accurate documentation of any and all activities involving spring habitats and their associated taxa are critical to develop sound, effective conservation plans. These data serve as starting points in the development of recovery plans for endangered species and their critical habitat in addition to providing a means of measuring success or failure of the implemented plans.

Results presented here on *E. phytophilum* contribute to a growing body of knowledge that spring endemic taxa of the southeastern United States show high levels of genetic distinctiveness on fine geographical scales (Mayden et al. 2005; Timpe et al. 2009; Fluker et al. 2010). Because spring habitats are frequently exploited for their rich sources of clean, clear water (Hubbs 1995; Etnier 1997; Warren et al. 2000), preserving these habitats and their unique inhabitants will require planning that goes beyond conservation genetic studies. More importantly, conservation of spring and headwater stream habitats (i.e. intermittent first- and second-order streams) has broader implications to entire river networks as these habitats provide a myriad of benefits to residents and migrants by: offering refuge from temperature and flow extremes, predators, and

competitors; providing spawning sites, rearing areas, and rich food sources; and creating migration corridors throughout the landscape (Meyer et al. 2007). The proposed guidelines herein establish a framework in which to preserve these unique habitats and their invaluable contributions to watershed health and regional biodiversity.

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Table 1 Details of *Etheostoma phytophilum* and *E. parvipinne* specimens used in this study, ordered by longitude, showing number of individuals genotyped for microsatellite loci (*N*m), sequenced for nuclear intron S7 (*N*n), and sequenced for three mitochondrial DNA regions (*N*mt). Composite mtDNA haplotype codes are followed by museum catalog numbers. Map ID corresponds to Fig. 1B

Species/Locality	Map ID	River System	River Drainage	Latitude	Longitude	*N*m	*N*n	*N*mt	Haplotype	Catalog number
Etheostoma phytophilum										
Wildcat Branch	SF	Sipsey Fork	Black Warrior	34.06528	-87.43555	9		3	RU1,RU2	10645.01
Mill Creek	SF	Sipsey Fork	Black Warrior	34.07778	-87.39000	14	1	6	RU1,RU2	14796.01
Beaver Cr. tributary	TC	Locust Fork	Black Warrior	33.66750	-86.70833	17				15140.01
Beaver Cr. tributary	TC	Locust Fork	Black Warrior	33.66833	-86.70500	6		5	RU3	14920.01
Unnamed spring	TC	Locust Fork	Black Warrior	33.67667	-86.69388	4	1	4	RU3	11354.01, 14919.01
Little Cove Creek	LC	Locust Fork	Black Warrior	34.09250	-86.29222	22		4	RU4	15147.01
Cove Spring	LC	Locust Fork	Black Warrior	34.10778	-86.26083	1	1	1	RU5	14921.02
E. parvipinne										
Kelly Bayou	RR[a]	Red River	Mississippi	33.03490	-93.91690		1	2	GS1,GS2	15122.02
Mill Creek	HA	Hatchie River	Mississippi	35.34030	-88.94225			1	GS3	STL168.05
Browns Creek	PR	Chickasawhay R	Pascagoula	32.24583	-88.77028	26		1	GS4	15175.02, 15736.01
Sand Creek	NX	Noxubee River	Upper Tombigbee	32.95514	-88.64634		1	1	GS5	15223.01
Cane Creek	OB[a]	Obion River	Mississippi	35.76278	-88.52417			1	GS6	13199.09
Little Mill Creek	LT	–	Lower Tombigbee	31.87560	-88.32090			1	GS7	STL856.09
Buck Branch	TN[a]	Bear Creek	Tennessee	34.83089	-88.05843			1	GS8	GSA2192
Wards Mill Creek	SR	Sipsey River	Upper Tombigbee	33.31833	-87.71889			1	GS9	10832.02
Carroll Creek	CC	North River	Black Warrior	33.33639	-87.63472		1	1	GS10	14446.04
Mill Creek	MC	–	Black Warrior	33.23778	-87.60250			1	GS11	15138.01
Cypress Creek	CY	–	Black Warrior	33.16917	-87.55944			1	GS12	13573.01
Little Tyro Creek	TY	North River	Black Warrior	33.58917	-87.53139	13		1	GS13	15280.01, 15591.01
Rice Mine Creek	RM	–	Black Warrior	33.23670	-87.51310			1	GS11	15067.01
Cottondale Creek	CD	–	Black Warrior	33.20040	-87.44630			1	GS14	15123.02
Unnamed spring	CR	Cahaba River	Alabama	32.96769	-87.31127	8	1	2	GS15	15121.01
Lick Branch	CR	Cahaba River	Alabama	32.97070	-87.28830	2				15589.01, 15725.01
Lick Br. tributary	CR	Cahaba River	Alabama	32.97818	-87.28651	1				15654.01
Cahaba River	CR	Cahaba River	Alabama	32.66500	-87.24083	1		1	GS16	15013.01
Gully Creek	CR	Cahaba River	Alabama	32.90037	-87.12399	4		1	GS16	STL359.03, 15727.01
Noland Creek	AL	–	Alabama	32.44070	-86.52560			1	GS17	STL364.02
Corn Creek	CO	Coosa River	Alabama	32.56900	-86.19900			1	GS18	STL366.01
Peterman Creek	CH	Chattahoochee R	Apalachicola	31.60620	-85.13110			1	GS19	15779.01

[a]Not shown in Fig. 1B; museum catalog numbers are University of Alabama Ichthyological Collection (UAIC) unless listed otherwise; St. Louis University Ichthyological Collection (STL); Geological Survey of Alabama (GSA)

Table 2 GenBank accession numbers for outgroup taxa used in MrBayes and BEAST analyses

Analysis/taxon/individual ID	d-loop	Cyt *b*	ND2	S7
MrBayes				
Percina caprodes	EF587845	DQ493490	EF027178	–
Etheostoma edwini	EEU77006	AY374267	EF027193	–
BEAST				
P. caprodes	–	–	EF027178	EF035498
E. asprigene	–	–	EF027180	EF035500
E. collis	–	–	EF027190	EF035510
E. edwini	–	–	EF027193	EF035513
E. nuchale				
Glenn Spring	–	–	HM856125	HM856115
Roebuck Spring	–	–	HM856127	HM856115
E. swaini				
Alabama River	–	–	HM856132	HM856119
Wolf Creek	–	–	HM856131	HM856118
E. trisella	–	–	EF027226	EF035546

Mitochondrial control region (d-loop); mitochondrial cytochrome *b* gene (cyt *b*); mitochondrial NADH subunit 2 (ND2); first intron of the nuclear ribosomal protein S7 (S7)

Table 3 Models of DNA substitution for the seven partitions of mitochondrial DNA used in the MrBayes analysis. Substitution models are followed by settings to define the structure of the model in MrBayes runs

		lset	
Partition	Substitution model	nst	rates
Control region	K81uf+I+Γ	6	invgamma
Cytochrome *b*			
1st codon	TrNef+I	6	propinv
2nd codon	F81	1	equal
3rd codon	GTR+Γ	6	gamma
NADH subunit 2			
1st codon	HKY+Γ	2	gamma
2nd codon	HKY+I	2	propinv
3rd codon	TrN+Γ	6	gamma

Table 4 Percentage of mitochondrial DNA sequence divergence (above diagonal) and microsatellite based pairwise population fixation indices (F_{st}, below diagonal) among populations of *Etheostoma phytophilum* and focal populations of *E. parvipinne*. Population identifier (ID) described and mapped in Table 1 and Fig. 1B

Species	ID	SF	TC	LC	PR	TY	CR
E. phytophilum	SF	–	2.03	1.87	1.42	1.38	1.61
E. phytophilum	TC	0.33	–	1.40	2.17	2.13	1.97
E. phytophilum	LC	0.34	0.50	–	2.15	2.12	1.74
E. parvipinne	PR	0.26	0.39	0.28	–	0.14	1.76
E. parvipinne	TY	0.37	0.51	0.35	0.15	–	1.72
E. parvipinne	CR	0.32	0.47	0.34	0.09	0.21	–

All F_{st} values significant ($P < 0.0001$)

Table 5 Divergence time estimates among members of *Fuscatelum* and the *E. asprigene* species group as posterior mean age in millions of years (Mya). Credibility intervals are based on the 95% highest posterior density (HPD). Nodes correspond to pruned chronograms shown in Fig. 2

Node	Posterior mean age (Mya)	95% HPD
A	6.5	4.2, 9.4
B	2.2	1.5, 3.1
C	1.3	0.8, 1.9
D	0.7	0.3, 1.1
E	1.9	1.2, 2.7
F	1.2	0.6, 1.8
G	9.5	6.9, 12.6
H	7.0	4.9, 9.5
I	4.6	2.9, 6.4
J	1.3	0.7, 2.2

Table 6 Sample sizes and genetic diversity estimates (averaged over seven microsatellite loci) for *Etheostoma phytophilum* and focal populations of *E. parvipinne*. Genetic diversity estimates are followed by *P*-values from the H_e excess tests as performed in BOTTLENECK (Cornuet and Luikart 1996) and critical *M* values, *M*-ratios, and associated significance as performed using the methods of Garza and Williamson (2001). Significant values ($P < 0.05$) shown in bold. Population identifier (ID) described and mapped in Table 1 and Fig. 1B

Species/Population	ID	*N*	*NA*	*A*	AR	PA	H_o	H_e	*P*-value (H_e excess)	Critical *M*	*M*-ratio	*P*-value (*M*-ratio)
E. phytophilum		73	95	13.571	12.749	5.331	0.540	0.791	–	–	–	–
Sipsey Fork	SF	23	58	8.286	7.149	2.382	0.646	0.668	0.945	0.631	0.694	0.198
Turkey Creek	TC	27	28	4.000	3.277	1.263	0.450	0.429	0.953	0.629	0.869	0.908
Little Cove Creek	LC	23	34	4.857	4.336	1.077	0.540	0.566	0.531	0.641	0.698	0.194
Population mean			40	5.714	4.921	1.574	0.545	0.554	–	–	–	–
E. parvipinne		55	102	14.571	14.571	7.153	0.668	0.755	–	–	–	–
Browns Creek	PR	26	87	12.429	9.916	2.364	0.747	0.774	**0.039**	0.642	0.850	0.884
Tyro Creek	TY	13	32	4.571	4.571	0.717	0.571	0.553	0.996	0.595	0.421	**0.000**
Cahaba River	CR	16	37	5.286	5.022	0.722	0.616	0.642	0.422	0.605	0.669	0.201
Population mean			52	7.429	6.503	1.268	0.645	0.656	–	–	–	–

Number of individuals (*N*); total number of alleles (*NA*); mean number of alleles per locus (*A*); allelic richness (AR); private allelic richness (PA); heterozygosity observed (H_o); heterozygosity expected (H_e)

Table 7 Analysis of molecular variance (AMOVA) for different hierarchical groupings of *Etheostoma phytophilum* and *E. parvipinne* based on seven microsatellite loci

Source of variation	d.f.	SS	% of variance	Fixation index	*P*-value
Both species					
Among populations	5	242.07	34.09	F_{st} = 0.34	<0.00001
Among individuals within populations	122	263.33	1.19	F_{is} = 0.02	0.185
Within individuals	128	266.50	64.72	F_{it} = 0.35	<0.00001
E. phytophilum					
Among populations	2	127.77	39.99	F_{st} = 0.40	<0.00001
Among individuals within populations	70	135.84	0.79	F_{is} = 0.01	0.347
Within individuals	73	138.00	59.22	F_{it} = 0.41	<0.00001
E. parvipinne					
Among populations	2	31.93	13.90	F_{st} = 0.14	<0.00001
Among individuals within populations	52	127.49	2.07	F_{is} = 0.02	0.198
Within individuals	55	128.50	84.03	F_{it} = 0.16	<0.00001

Degrees of freedom (d.f.); sum of squares (SS)

Table 8 Microsatellite based estimates of theta (Θ) and 95% intervals calculated in LAMARC, the corresponding estimates of long-term effective population size (N_e), and current N_e with 95% intervals as estimated using the LDNe method. Population identifier (ID) described and mapped in Table 1 and Fig. 1B

Species/population	ID	Theta		Long-term N_e		Current N_e	
E. phytophilum							
Sipsey Fork	SF	6.925	(6.114, 7.340)	3463	(3057, 3670)	920	(44, ∞)
Turkey Creek	TC	3.108	(2.548, 4.046)	1554	(1274, 2023)	–	–
Little Cove Creek	LC	2.404	(1.961, 2.675)	1202	(981, 1337)	45	(14, ∞)
E. parvipinne							
Browns Creek	PR	9.603	(8.503, 9.809)	4802	(4252, 4905)	175	(47, ∞)
Tyro Creek	TY	3.094	(2.114, 3.826)	1547	(1057, 1913)	12	(3, 141)
Cahaba River	CR	4.613	(3.190, 5.509)	2307	(1595, 2755)	30	(10, ∞)

Infinity (∞)

Table 9 Microsatellite based estimates of migration (*M*), number of effective migrants per generation (4*N*m), as estimated in LAMARC, among populations of *Etheostoma phytophilum* and *E. parvipinne*, respectively. The corresponding 95% intervals are shown in parentheses. Populations described and mapped in Table 1 and Fig. 1B

Species/Population	*M*		4*N*m	
E. phytophilum				
SF into TC	0.071	(0.025, 0.135)	0.221	(0.078, 0.420)
SF into LC	0.043	(0.017, 0.086)	0.103	(0.041, 0.207)
TC into LC	0.016	(0.012, 0.042)	0.038	(0.029, 0.101)
LC into TC	0.022	(0.013, 0.060)	0.068	(0.040, 0.186)
LC into SF	0.025	(0.014, 0.056)	0.173	(0.097, 0.388)
TC into SF	0.038	(0.015, 0.063)	0.263	(0.104, 0.436)
E. parvipinne				
PR into TY	0.192	(0.096, 0.339)	0.594	(0.297, 1.049)
PR into CR	0.173	(0.096, 0.309)	0.798	(0.443, 1.425)
TY into CR	0.095	(0.036, 0.158)	0.438	(0.166, 0.729)
CR into TY	0.119	(0.038, 0.195)	0.368	(0.118, 0.603)
CR into PR	0.099	(0.039, 0.143)	0.951	(0.375, 1.373)
TY into PR	0.090	(0.042, 0.133)	0.864	(0.403, 1.277)

$M = m/\mu$, where m is the per-generation migration rate and μ is the mutation rate

Fig. 1 (a) Fifty percentage majority-rule consensus phylogram for Bayesian analysis of the concatenated mitochondrial DNA data set. Nodes with mean posterior probabilities ≥ 0.95 are black and nodes without significant support (< 0.95) are pink. Node numbers are referenced in text. Taxon labels show locality and haplotype codes for *Etheostoma phytophilum* (squares) and *E. parvipinne* (circles) that correspond to Table 1. Outgroup taxa not shown for simplicity. (b) Map showing sample locations for *E. phytophilum* and focal populations of *E. parvipinne* from clade 3. Physiographic provinces are indicated by color. Inset shows map of the United States with the focal area of the study in black. (c) STRUCTURE bar plot of $K = 4$ and (d) the most likely genetic structure ($K = 6$) for all samples of *E. phytophilum* and closely related populations of *E. parvipinne* utilized for microsatellites. Bars correspond to multilocus genotypes of individuals and colors represent the probability of ancestry to each cluster (K). Locality codes correspond to Table 1 and Fig. 1A, B

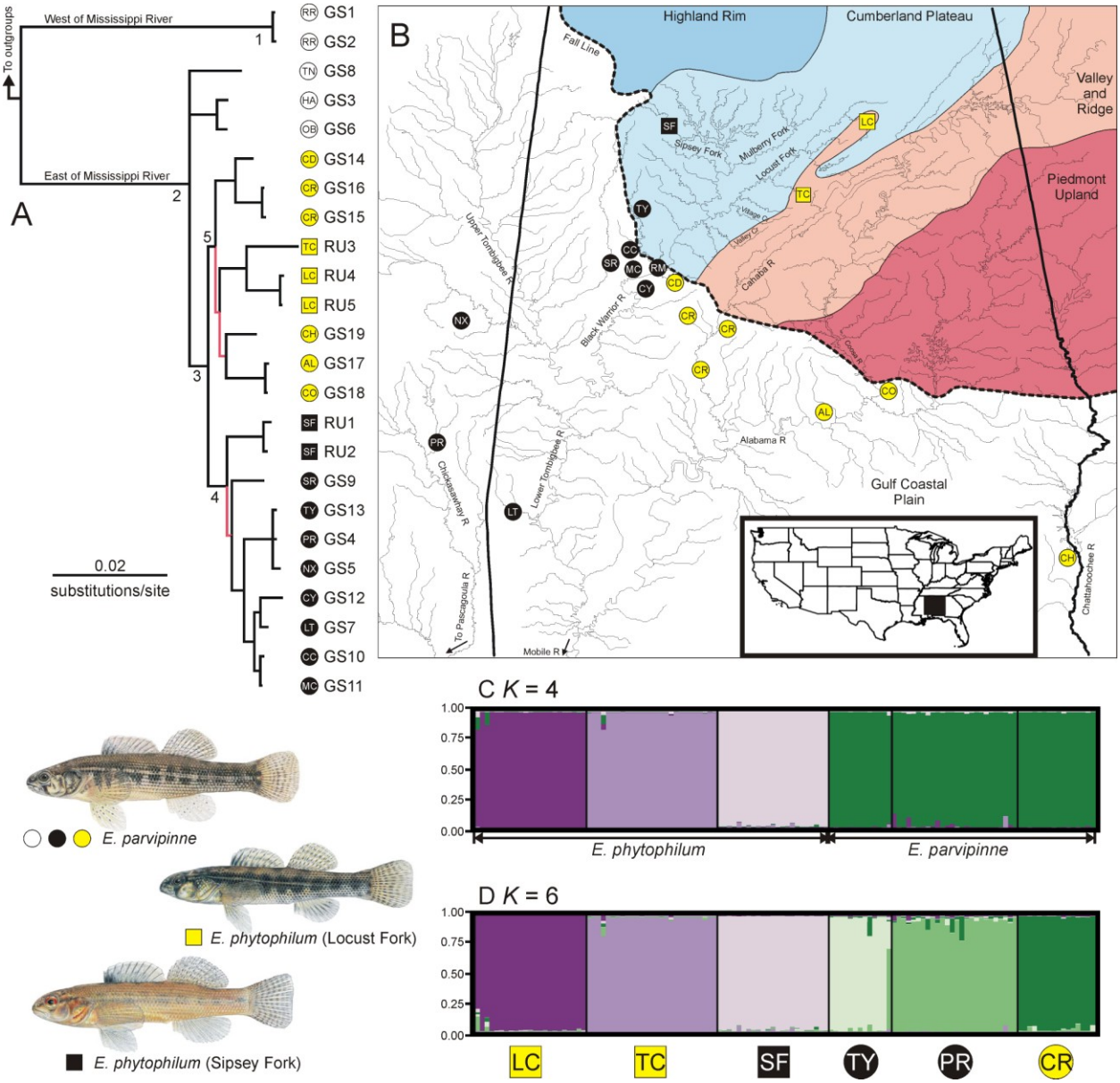

Fig. 2 Chronograms for *Fuscatelum* and *E. asprigene* groups (pruned from the BEAST analysis with centrarchid and darter outgroups) based on the combined analysis of mitochondrial (ND2) and nuclear (S7) gene sequences. Letters at nodes correspond with age estimates in Table 5. Gray bars at nodes represent the 95% highest posterior density of age estimates. Taxon names are followed by locality and haplotype codes corresponding to Table 1 and Fig. 1. Shaded vertical bars represent the estimated age of *E. nuchale* (blue) and *E. phytophilum* (purple), and the length of time that populations of both species have been diversifying within the upper Black Warrior River (dark gray)