Phylogenetic and Coalescent Strategies of Species Delimitation in Snubnose Darters (Percidae: Etheostoma)

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Abstract.—The rapid accumulation of multilocus data sets has led to dramatic advances in methodologies for estimating evolutionary relationships among closely related species, but relatively less advancement has been made in methods for discriminating between competing species delimitation hypotheses. Multilocus data sets provide an advantage in testing species delimitation scenarios because they offer a direct test of species monophyly and aid in the biological interpretation of such phenomena as allele-sharing and deep coalescent events. Most species tree estimation methods that are designed to analyze multilocus data sets require the a priori assignment of individuals to species categories and therefore do not provide a strategy to directly test competing species delimitation scenarios. An approach was recently proposed that utilizes a coalescent-based species tree estimation method to inform species delimitation decisions by comparing likelihood scores that measure the fit of gene trees within a given species tree. We use a multilocus nuclear and mitochondrial DNA sequence data set to both reexamine a recently proposed species delimitation scenario in the Etheostoma simoterum species complex and test the utility of species tree estimation methods in testing species delimitation hypotheses. Descriptions of species in the E. simoterum species complex of snubnose darters, a group of six teleost freshwater fish species, are based largely on male nuptial coloration. Most of the putative species are nonmonophyletic at every examined locus. Using a novel combination of Bayesian-estimated gene tree topologies, Bayesian phylogenetic species tree inferences, coalescent simulations, and examination of phenotypic variation, we assess the occurrence of shared alleles among species, and we propose that results from our analyses support a three-species rather than a six-species delimitation scenario in the E. simoterum complex. We found that comparing likelihood scores from the species tree estimation approach used across many potential delimitation scenarios resulted in a systematic bias toward over-splitting in the E. simoterum complex and failed to support a species delimitation scenario that was consistent with geographic, phenotype, or any previous species delimitation hypothesis. Despite common expectations, we demonstrate that application of molecular approaches to species delimitation can result in the recognition of fewer, instead of a larger number of species. In addition, our analyses highlight the importance of phenotypic character information in providing an independent assessment of alternative species delimitation hypotheses in the E. simoterum species complex. [Species tree; deep coalescence; nuclear gene; mitochondrial DNA; phylogeography; divergence time.]

The primary functions of systematics are to discover species and estimate their phylogenetic relationships (Simpson 1951; Sites and Marshall 2003; Wiens 2007). Although species discovery is fundamental to the basic understanding of biodiversity, it is phylogenetics, and not species delimitation, that has developed and matured in the context of an extensive analytical framework over the past 30 years (e.g., Felsenstein 2004; Yang 2006). Because of the central role that species occupy in nearly all disciplines of biology, delimitation of species is one of the most important exercises in the effort to improve our understanding of the Tree of Life and can have broad implications for efforts ranging from biological conservation to comparative evolutionary analyses (Daugherty et al. 1990; Agapow et al. 2004; Isaac et al. 2004; Padial and De la Riva 2006). Despite the importance to a fundamental understanding of biodiversity, it is only recently that general and objective methods for delimiting species using comparative phylogenetic data have been proposed (e.g., Puorto et al. 2001; Wiens and Penkrot 2002; Pons et al. 2006; Knowles and Carstens 2007; Leaché et al. 2009; Carstens and Dewey 2010). In systematics, the descriptions and diagnoses of taxa, ranging from species to higher-level clades, serve as testable hypotheses about how populations, species, and major organisinal lineages are related to one another (Wheeler 2004). A hypothesis-testing basis for the discrimination of competing phylogenetic trees has long had a central role in phylogenetic systematics (e.g., Goldman et al. 2000); however, there has been comparatively little effort in treating species designations as testable hypotheses (Baum and Donoghue 1995; Wiens and Servedio 2000; Templeton 2001).

Species delimitation can be especially challenging at early stages of population divergence, when both morphological and molecular characters would be likely to show low magnitudes of differentiation (de Queiroz 2007). Not least among the challenges associated with species discovery and delimitation is the occurrence of incomplete lineage sorting in genetic loci (Hudson and Coyne 2002; Degnan and Rosenberg 2009). Molecular data can provide a wealth of demographic and phylogenetic information, but interpretations of shared alleles among species are complicated by the difficulty in discriminating between incomplete lineage sorting and gene flow between populations or species (Maddison 1997; Slowinski et al. 1997; Shaffer and Thomson 2007). Ancestral genetic polymorphism and gene flow may lead to similar patterns among inferred gene trees, but
these two phenomena have quite different implications for species delimitation.

Phylogeographic studies often discover clear patterns of cryptic species diversity using phylogenies inferred from molecular data (e.g., Egg and Simons 2006). However, very recently diverged species may demonstrate patterns of morphological variation over a backdrop of incomplete lineage sorting and deep coalescent events that can be difficult to interpret for purposes of species delimitation (Leaché et al. 2009). In addition, introgressive hybridization may lead to perceived genetic similarity among phenotypically divergent species, as demonstrated when phylogenies inferred from cytoplasmic genomes reflect a history of hybridization among closely related species and not their true phylogenetic relationships (e.g., Wu and Campell 2005; Bossu and Near 2009; Keck and Near 2010). These types of findings highlight the importance of integrating a multilocus approach to species delimitation.

A new and exciting area in systematic biology is the development of methods that accommodate incomplete lineage sorting and deep coalescent events in a multilocus framework for purposes of species tree estimation (Degnan and Rosenberg 2009; Edwards 2009; Knowles 2009; Kubatko et al. 2009). Recently developed methods avoid some of the problems associated with concatenating multiple genes by accommodating the independent evolutionary histories of unlinked loci in a coalescent framework. Some of the most commonly used species tree methods are minimization of deep coalescent events (Maddison and Knowles 2006), Species Tree Estimation using Maximum Likelihood (STEM) (Kubatko et al. 2009), BEST (Edwards et al. 2007), and *BEAST (Heled and Drummond 2010). Although BEST, *BEAST, and STEM can estimate phylogenetic relationships among species that share ancestral alleles, they are not designed to explicitly test hypotheses of species delimitation because they rely on the a priori assignment of individuals to investigator-defined species categories.

The assignment of individuals to species categories and the recognition of populations as independent evolutionary units in these species tree analyses can be particularly problematic when studying recent radiations that are characterized by uncertain species designations. These assignment decisions have consequences for the resulting species phylogeny, and comparing the results from a series of analyses that have different species delimitation scenarios has been proposed as a quantitative method to test species delimitation hypotheses using multilocus sequence data (Carstens and Dewey 2010). Additionally, coalescent simulations of gene trees provide a method to test empirical data against null expectations of lineage sorting in species trees. In this study, we use phylogenetic and coalescent analyses of a multilocus DNA sequence data set, as well as consideration of phenotypic data, to test specific hypotheses of species delimitation in a clade of darters that has been characterized by taxonomic instability and questions regarding species limits for more than a century.

**Study System: Etheostoma simoterum Species Complex**

Darters are a species-rich clade of approximately 245 freshwater fish species that are endemic to eastern North America and are typically small, brightly colored species that utilize flowing water habitats in rivers and streams (Kuehne and Barbour 1983; Page 1983; Etnier and Starnes 1993; Boschung and Mayden 2004). Systematics and species delimitation in darters have increasingly drawn on progressively finer morphological differences, especially color pattern variation among breeding-condition males, to distinguish and diagnose species (e.g., Suttkus and Bailey 1993; Suttkus et al. 1994; Powers and Mayden 2003). Molecular phylogenetic analyses of darter clades have both corroborated and revised species delimitations based on the morphological traits traditionally used in darter systematics (Page et al. 2003; Page and Near 2007; Bossu and Near 2009), and molecular studies have revealed cryptic evolutionary lineages that have masqueraded as single species (e.g., Near et al. 2001; Switzer and Wood 2002; George et al. 2006; Hollingsworth and Near 2009).

Hypotheses of species delimitation in the *Etheostoma simoterum* species complex have been revised several times since the original species description in the nineteenth century. Species of the *E. simoterum* complex are distributed in the Tennessee, Cumberland, and Duck River systems in the southeastern United States (Fig. 1).

Shortly after *E. simoterum* was described from populations in the upper Tennessee River system (Cope 1868), *E. atripinne* was described from populations in a tributary of the Cumberland River (Jordan 1877). The distinctiveness of *E. atripinne*, relative to *E. simoterum*, was questioned as a result of comparisons of meristic data (Bouchard 1977; Etnier 1980a, 1980b). Later authors treated *E. atripinne* as a junior synonym of *E. simoterum* (Page and Mayden 1981), while still referring to it as a subspecies, and suggested that populations representing intergrades between *E. s. simoterum* and *E. s. atripinne* were present in the lower Tennessee River system between the mouths of the Paint Rock and Duck rivers (Fig. 1a) (Etnier and Starnes 1993). More recently, Powers and Mayden (2007) described four new species and recognized a total of six species in the *E. simoterum* complex (Fig. 1b). The species were delimited and diagnosed almost entirely on the basis of color patterns of breeding-condition males, particularly the pattern and extent of coloration on the dorsal fin.

Powers and Mayden (2007) recognized three species in the Cumberland River system (*Etheostoma atripinne, E. occidentale*, and *E. orientale*), one in the Duck River system (*E. planasaPixie*) and two in the Tennessee River system (*E. simoterum* and *E. tennesseense*) (Fig. 1b). Principal components analysis of morphometric and meristic data showed largely overlapping principal component scores among all the six recognized species of the *E. simoterum* complex. A phylogenetic analysis of DNA sequences sampled from the mitochondrial cytochrome *b* (cytb) gene tree resulted in a pattern of paraphyly in four of the six recognized species in the
FIGURE 1. Maps detailing the geographic distribution of species in the *Etheostoma simoterum* species complex and illustrating various species delimitation hypotheses. 

a) *E. simoterum* distributed throughout the Tennessee River system, *E. atripinne* in the Cumberland and Duck, and intergrades in the lower Tennessee. 

b) The six-species scenario as proposed by Powers and Mayden (2007), sampled specimen localities for this study are marked with black dots. 

c) Populations that correspond to strongly supported clades that we interpret as species in our multilocus phylogenetic and coalescent analyses.
E. simoterum complex (Powers and Mayden 2007). The lack of monophyly was hypothesized by Powers and Mayden (2007) to have resulted from historically large genetic effective population sizes that led to widespread ancestral polymorphism and incomplete lineage sorting, and the phylogeny was not used in the process of species delimitation in the E. simoterum complex.

In light of the historical differences in the interpretation of the taxonomic significance of morphological variation among the proposed species of the E. simoterum complex, our goal is to use gene trees sampled from multiple loci to test multiple species delimitation hypotheses proposed for this clade of darters. Our approach to species delimitation is designed to integrate phylogenetic and coalescent analyses of DNA sequence data sampled from mitochondrial and nuclear genes with patterns of morphological differences in characters traditionally used in the description and diagnosis of ray-finned fish species. Our approach to the problem is general in that it offers an objective strategy to empirically investigate questions of species delimitation not only in teleost fishes but also for a broad range of clades in the Tree of Life.

MATERIALS AND METHODS

Specimen Sampling, DNA Sequencing, and Alignment

Specimens of the E. simoterum species complex were collected from 75 sites throughout their range in the Cumberland, Duck, and Tennessee River systems and from the McClure River (Ohio River Drainage) using a seine net (Fig. 1b). Specimens were anesthetized using MS-222, and tissue samples for DNA extraction were taken from right pectoral fins and catalogued in the Yale Fish Tissue Collection. Voucher specimens were fixed in formalin before being transferred to 70% ethanol and deposited in fish collections at the Yale Peabody Museum of Natural History or the David A. Etnier University of Tennessee Research Collection of Fishes.

The Qiagen DNeasy Tissue kit was used to isolate DNA from tissue biopsies following the manufacturer’s protocol. The following genes were polymerase chain reaction (PCR) amplified using primers and reaction conditions described in the previous studies: mitochondrial cytb (Near et al. 2000) and three nuclear genes, recombination activating gene 1 exon 3 (RAG1) (Lopez et al. 2004), mixed lineage leukemia (MLL) (Bossu and Near 2009), and S7 ribosomal protein intron 1 (S7) (Chow and Hazama 1998). Sequences were obtained for the mitochondrial cytb gene from all sampled specimens (260), and a subset of approximately 100 individuals (at least one individual per sampling locality) was sequenced for all three nuclear genes. Subsequent analyses of multilocus data were performed on a subset of 37 specimens that spanned the broadest observed genetic diversity for each of the six hypothesized species. Gene sequences from E. barrenense and E. rafinesquei were sampled as outgroups.

PCR products for each gene were purified by polyethylene glycol precipitation. These cleaned PCR products were used as template for Big Dye (Applied Biosystems) cycle sequencing on an ABI 3100 automated sequencer at the Molecular Systematics and Conservation Genetics Laboratory (Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT, USA). Mitochondrial cytb was sequenced in three segments using primers Glu2, 658Fsim (5′ TTATAACTCCGAGGCCGA-3), and 517R1. Contiguous sequences were created and edited using Sequencher (GeneCodes, Ann Arbor, MI) and aligned manually using Se-Al v. 2.0 (http://tree.bio.ed.ac.uk/software/seal/).

Bayesian Estimation of Gene Trees

Phylogenies were estimated from each of the sampled loci using a partitioned Bayesian strategy executed in the computer program MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Ten data partitions were identified that correspond to the three codon positions in each of the three sampled protein-coding genes (cytb, RAG1, and MLL) and with a single partition for the S7 intron. The most parameterized molecular evolutionary model was chosen that best fit each of the data partitions using the Akaike information criterion (AIC) as implemented in the computer program MrModeltest version 2.3 (Nylander, 2004). The optimal partitioning scheme for each of the three protein-coding genes was selected from the comparison of Bayes factors that were calculated from the log of the harmonic mean of the likelihood values sampled from the posterior distributions of the two compared MrBayes runs (Newton and Raftery 1994; Nylander 2004; Brandley et al. 2005). Posterior trees and model parameters were sampled from MrBayes runs of 20 million generations with four simultaneous chains in the analysis of each gene region. Stationarity of the chains and convergence of the trees and parameter values were determined by plotting the likelihood score and all other model parameter values against the generation number using the computer program Tracer v1.5 (Rambaut and Drummond 2003). Convergence of the MrBayes runs was also assessed by monitoring the average standard deviation of the split frequencies between the two independent runs, assuming that stationarity of the chains was achieved when this value was less than 0.005, and by monitoring the potential scale reduction factors between independent runs. Burn-in was set at 1 × 10^6 generations, discarding all trees and parameter values sampled before the burn-in.

Species Tree Estimation

We were primarily interested in evaluating species delimitation scenarios and relationships for the species-level taxa that have been proposed in the literature. These alternatives consist of a single species, two species (E. simoterum in the Tennessee River system, E. atripinne in the Cumberland and Duck systems), the three
species that are inferred and strongly supported in the mitochondrial gene tree (E. simoterum, E. planasaxatile, and E. atripinne) (Fig. 1c), and the six-species scenario proposed by Powers and Mayden (2007) (Fig. 1b). Using *BEAST (Heled and Drummond 2010), we generated species trees for two species delimitation scenarios within the E. simoterum complex: the six-species scenario proposed by Powers and Mayden (2007) and a three-species scenario that includes E. simoterum, E. atripinne, and E. planasaxatile. For this analysis, we used sequence data from all four loci sampled from 37 individuals that represented a subsample of the individual gene sequences used in the MrBayes inference of gene trees. We incorporated AIC-identified optimal molecular evolutionary models for each partition within each gene. For each species tree delimitation scenario, eight *BEAST analyses were run for 60 million generations each, with a burn-in of 20 million for each run before runs were combined using the computer program LogCombiner v. 1.53 (http://beast.bio.ed.ac.uk/LogCombiner).

Divergence Time Estimation

Our coalescent analyses required knowledge of divergence times among species in the E. simoterum complex. We utilized a Bayesian strategy that relied on external fossil calibrations in the perciform fish clade Centrarchidae to time-calibrate the cytb gene tree. A similar strategy has been used in several investigations of divergence times in darter clades (Near and Benard 2004; Near and Keck 2005; Hollingsworth and Near 2009). Divergence times were estimated using an uncorrelated lognormal (UCLN) model of molecular evolutionary rate heterogeneity using the Bayesian method in the computer program BEAST v. 1.53 (Drummond et al. 2006; Drummond and Rambaut 2007). The optimal molecular evolutionary models were identified using AIC, and the data-partitioning schemes were assessed using Bayes factor comparisons. The UCLN model was used in BEAST to estimate the posterior density of divergence times. A birth–death speciation prior was used for the branching rates in the phylogeny. The calibration priors consisted of five centarchid fossil ages that were identified as producing internally consistent age estimates using a fossil cross-validation analysis (Near et al. 2005). The fossil age estimates were treated as probability distribution–based calibrations, using a lognormal distribution with a zero-point minimal bound reflecting the estimated geological age of the fossil (Ho 2007). Previous fossil cross-validation analyses and the temporal bounds of geological chrons and Land Mammal Age intervals associated with the formations bearing the fossils were used to estimate the uncertainty of the zero-point lower bound in the calibration age priors (Woodburne 2004; Near et al. 2005). Information on the age, taxonomic identity, and specific of lower and upper bound ages used in the calibration priors are given in Near et al. (2005) and Hollingsworth and Near (2009). The phylogenetic placement of the taxa represented as fossils in the context of extant species of Centrarchidae reflected that discussed and used in Near et al. (2005). The BEAST analyses were run three times, and each run consisted of $3.0 \times 10^7$ generations. The resulting trees and parameter values from each run were combined using the computer program LogCombiner v. 1.53 (http://beast.bio.ed.ac.uk/LogCombiner). Convergence of model parameter values and estimated ages of nodes to optimal posterior distributions were assessed by plotting the marginal posterior probabilities using Tracer v. 1.5 (http://beast.bio.ed.ac.uk/Tracer). The posterior probability density of the combined tree files was summarized using the computer program Tree Annotator v. 1.5.3 (http://beast.bio.ed.ac.uk/TreeAnnotator). The mean and 95% highest posterior density (HPD) estimates of divergence times were visualized as a chronogram using the computer program FigTree v. 1.2.3 (http://beast.bio.ed.ac.uk/FigTree). In an effort to assess the influence of the calibration priors on the posterior divergence time estimates, BEAST was also run using an empty alignment.

Coalescent Species Tree Estimation and Testing Species Delimitation Hypotheses

We implemented two coalescent-based approaches to testing alternative hypotheses of species delimitation. The first utilizes STEM as outlined in Carstens and Dewey (2010) in which likelihood scores are compared among species trees generated under alternative species delimitation scenarios. Because STEM requires the a priori assignment of individuals to species categories, we performed a set of analyses where individuals were assigned to a series of alternative species categories, ranging from a single to 10 species. The alternative delimitation scenarios applied to the E. simoterum complex included those we identified as viable (two, three, and six species as discussed previously) because they are previous hypotheses based on the analyses of phenotype and geographic distribution. We also tested a series of species delimitations that have never been proposed in order to investigate the general utility of species tree methods for informing species delimitation scenarios. These additional species delimitation scenarios were generated without regard to geography or phenotype but rather based on clusters of specimens identified in an unWeighted Pair Group Method with Arithmetic Mean (UPGMA) phenogram constructed from a matrix of Kimura 2-parameter–corrected genetic distances calculated using the concatenated nuclear and mitochondrial DNA (mtDNA) gene data set (Fig. 2a).

Species tree analyses were performed using the computer program STEM (Kubatko et al. 2009) with maximum likelihood gene trees estimated for each locus using GARLI v0.951 (Zwickl 2006). We used a subsample of 37 individuals consisting of approximately six individuals sampled from each of the six (Powers and Mayden 2007) species and E. rafinesquei as an outgroup. STEM requires fully resolved gene trees, so polytomies
FIGURE 2. STEM species tree analysis. a) UPGMA phenogram used to guide the progressive assignment of individuals to species categories, with numbers next to each node indicating the order in which individuals were assigned to new species categories. b) Log-likelihood values generated by STEM for each species delimitation scenario. Triangles indicate the credible species hypotheses, and circles indicate species hypotheses based on the UPGMA phenogram.

were resolved randomly and internode branch lengths were set to $1.0 \times 10^{-8}$ with TreeEdit v1.0 (Rambaut and Charleston 2002). Individuals were chosen based on the possession of unique haplotypes that were identified using the mitochondrial gene tree. Branch lengths of each gene tree were scaled relative to the estimated mutation rates for each gene, and theta was based on values of effective population size calculated using IMa2 (described below). Maximum likelihood scores for each species tree generated using STEM were evaluated using an information-theoretic approach, as outlined in Carstens and Dewey (2010).

The second coalescent analysis used gene tree simulations to generate null expectations for the extent of incomplete lineage sorting across species boundaries, given a particular species tree. We are primarily interested in evaluating species delimitation hypotheses that have previously been proposed and are focusing on the six-species scenario of Powers and Mayden (2007) and a three-species scenario that resulted from assessment of geographic distribution and observed species monophyly in the mitochondrial gene tree (one species each in the Tennessee, Duck, and Cumberland Rivers). We constructed phylogenies for both the six-species and the three-species delimitation scenarios from the tree topologies resulting from the *BEAST multispecies coalescent species tree analyses. Species tree branch lengths were converted to numbers of generations. Estimates of the number of generations between nodes were calculated from the divergence time estimates resulting from the BEAST analyses using the external Centrarchidae fossil calibrations. For the coalescent simulations, we assumed one generation per year based on life history observations (Page and Mayden 1981; Etnier and Starnes 1993). A range of values was used for the effective population size, and the effective population size for each species was scaled relative to their respective haplotype diversity. Using the Coalescence package in Mesquite version 2.72 (Maddison W.P. and Maddison D.R. 2009), we generated a set of gene trees (with six genes per species) contained within the species tree using a neutral coalescent process. For each gene tree, a deep coalescent penalty (DCP) (Maddison 1997) and Slatkin’s $s$ score (Slatkin and Maddison 1989) were calculated and used as metrics for assessing its fit to the species tree. These scores are essentially counts of the number of deep coalescent occurrences on a tree—in other words, the number of instances where a haplotype of one species coalesces with members of another species before coalescing with members of its own species. A set of 200 gene trees was generated for each value of effective population size tested, ranging from an $N_e$ of 1000 and increasing by one order of magnitude to 100,000,000. In order to compare the DCP and $s$ values of the simulated gene trees with our observed data, we calculated the DCP and $s$ score for the mitochondrial cytb gene tree that was pruned down to include the same number of individuals per species as in the simulations. Six individuals per species were selected that spanned the node that subtends the most recent common ancestor of each species. The observed DCP and Slatkin’s $s$ value of the cytb gene tree were compared with the generated distribution of DCP and $s$ values for each of the effective population size values.

The gene tree simulation analysis was conducted over a range of effective population sizes. In order to determine which effective population size range is a best fit for species in the *E. simoterum* complex, we estimated the effective population size for each species using the entire multilocus data set and the computer program IMa2 (Hey 2010). A series of preliminary IMa2 analyses were performed in order to ensure adequate mixing and convergence of the Markov chain Monte Carlo (MCMC) runs and that posterior distributions of parameters would generate unimodal distributions. Once appropriate priors were established, we ran three MCMC runs...
analyses with different starting seeds in order to ensure convergence of the samples on the same estimates. The population parameter estimates and sequence mutation rates across loci were required for the IMa2 analysis. For these priors, we used Watterson’s $\theta$ calculated using the computer program DNAsp (Librado and Rozas 2009) and mutation rates estimated for each locus using external centrarchid fish fossil calibrations (Near et al. 2011).

Assessment of Variation in Diagnostic Color and Pigmentation Characters

The variation in color patterns that was used by Powers and Mayden (2007) to diagnose species of the E. simoterum complex was analyzed using color photographs of freshly captured specimens taken in the field. Anesthetized specimens were photographed using a small “squeeze tank” filled with distilled water that was constructed with 0.5-cm-thick window glass and silicone aquarium sealant. The dimensions of the tank are given in Near et al. (2007). A tripod mounted Nikon Coolpix 4300 or a Cannon Rebel XT1 with a 60-mm macro lens was used to photograph the left side of the specimens under ambient daylight conditions. One hundred and four specimens were photographed for analysis of diagnostic color patterns. Eleven color characteristics presented by Powers and Mayden (2007) as diagnostic for their hypothesized six species were scored from each photographed specimen. These characters include the predominant color of the head, mouth, breast, belly, and ventral portion of the caudal peduncle; whether a line of ventrolateral orange scales is continuous or broken; lateral blotch color and shape; presence of a lateral green cast; and the extent and pattern of red color on the first dorsal fin. For each of the 11 diagnostic color traits, every individual was scored as either exhibiting the correct trait or exhibiting a pattern that does not match the description (either possessing the diagnostic trait of another species or a trait that does not match any of the descriptions).

RESULTS

Bayesian Phylogenetic Analyses

Specimens used for molecular and diagnostic color trait analysis are listed in Supplementary Table 1 (available from http://www.sysbio.oxfordjournals.org/). The number of sites in the aligned gene sequences of each locus were 1140 for cytb, 1285 for RAG1, 757 for MLL, and 575 from the S7 intron 1. All new DNA sequences generated for this study were submitted to GenBank (JK497168–JF497771), and all data matrices and resulting tree files were submitted to TreeBASE (http://www.treebase.org; S11289). Optimal molecular evolutionary models as determined using AIC for each locus and locus-specific data partitions are given in Table 1. Bayes factor analyses of data-partitioning schemes for each of the protein-coding loci are presented in Table 2.

| Table 1. Substitution models for nucleotide data partitions selected using the AIC |

<table>
<thead>
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<th>Data partition</th>
<th>Model</th>
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</tr>
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</table>

Bayesian phylogenetic analysis of the mitochondrial cytb gene resulted in three strongly supported monophyletic groups that correspond to specimens sampled from each of the Tennessee, Duck, and Cumberland systems, and only one of the six species, E. planasaxatile, as recognized in Powers and Mayden (2007) was monophyletic in the cytb gene tree (Fig. 3). The Duck River clade (E. planasaxatile) was sister to the reciprocally monophyletic Tennessee (E. simoterum and E. tennessense) and Cumberland River (E. occidentale, E. atripinne, and E. orientale) clades (Fig. 3). This pattern was similar to the cytb gene tree presented in Powers and Mayden (2007), with the notable exception that they observed an E. planasaxatile individual sampled from the type locality of the species with a haplotype nested within the Cumberland River (e.g., E. atripinne) clade. We genotyped 47 specimens from this locality, and none of these specimens exhibited mtDNA haplotypes that were phylogenetically nested within the Cumberland River clade (Fig. 3). Given the very strong posterior probability support for monophyly of species distributed in each of the three major river systems (Fig. 3), we believe that a more likely explanation for their observation of an E. planasaxatile haplotype nested within all Cumberland River species haplotypes is laboratory error (e.g., mislabeled sample), rather than an example of incomplete lineage sorting, as suggested in Powers and Mayden (2007).

Table 2. Bayes factor scores calculated the harmonic mean of the posterior likelihood scores resulting from MrBayes analyses with different data-partitioning strategies

<table>
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<th>3 Partitions</th>
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<tbody>
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<td>5.46 2 Partitions</td>
</tr>
</tbody>
</table>
FIGURE 3. Gene tree of the *Etheostoma simoterum* species complex inferred from a Bayesian analysis of the mitochondrial cytochrome *b* gene. Identical haplotypes were removed prior to analysis. To the right of the Tennessee River clade, haplotypes and haplotype groups are designated as Upper Tennessee (UT) and Lower Tennessee (LT), referring to whether they were collected from either upstream or downstream, respectively, of the confluence of the Tennessee and Paint Rock River systems. Bayesian posterior probabilities are indicated to the left of major nodes.
Within the Tennessee and Cumberland River clades, there were no strong patterns of geographic correlation between the mtDNA gene tree and the described species boundaries. In both the Tennessee and the Cumberland River clades, there were dramatic examples of closely related haplotypes exhibiting widespread “upstream” and “downstream” geographic distributions within each of these major river systems. For instance, populations sampled from the Clinch River (in the far upper portion of the Tennessee River drainage) had haplotypes that were related to those collected from Sequatchie River (middle Tennessee drainage) and Beech and Cedar Creeks (extreme lower Tennessee drainage portion of *E. tennesseense*’s range). Within the Tennessee River system, both *E. simoterum* and *E. tennesseense* were paraphyletic relative to each other. Similar patterns were observed within the Cumberland River system, with observations of shared haplotypes between *E. atripinne* and both *E. occidentale* and *E. orientale* (Fig. 3).

Each of the three nuclear gene trees was characterized by limited phylogenetic resolution and very few nodes supported with significant Bayesian posterior probabilities (Fig. 4). The maximum observed uncorrected genetic distance among individuals for any of the three nuclear genes was never more than 1%, as compared with approximately 6% maximum uncorrected genetic divergence observed between the clades that correspond to the Tennessee, Cumberland, and Duck River systems at the mitochondrial cytb gene. Although there was shallow genetic divergence at each nuclear gene, and alleles were shared between species, each nuclear gene exhibited a slightly different pattern of coalescence for the six putative species (Fig. 4).

**Species Tree Analyses**

*BEAST* analyses resulted in a three-species scenario species tree where *E. planasaxatile* was sister to a clade containing *E. atripinne* and *E. simoterum*. The six-species scenario *BEAST* species tree had a similar topology, but *E. orientale* was sister to an *E. atripinne–E. occidentale* clade (Fig. 5). Both the three- and the six-species scenario *BEAST* analyses did not show significant posterior probability support for the node representing the most recent common ancestor of the Tennessee and Cumberland River species clade (Fig. 5).

**STEM Analysis of Species Delimitation Hypotheses**

We used the protocol presented in Carstens and Dewey (2010) that uses the computer program STEM to generate a species tree from a given set of gene trees under different species delimitation scenarios. Species tree maximum likelihood scores were compared among all the examined delimitation scenarios. Using the same set of gene trees, we tested a wide range of possible species delimitation scenarios for the *E. simoterum* species complex, including those that have been proposed in the literature based on phenotypic variation and geographic distribution, as well as a number of contrived scenarios that have not been considered. These other delimitations were based on clusters observed in a UPGMA phenogram (constructed using the concatenated data set) and included up to 10 species. Maximum likelihood scores of the species trees estimated using STEM increased with increasing numbers of species (Fig. 2b). An information-theoretic approach to model selection, which penalizes the likelihood scores by the number of parameters (i.e., the number of delimited species), strongly supported a contrived eight-species scenario over the set of credible species delimitations based on gene tree monophyly, phenotypic disparity, and geographic distribution (Table 3). Among the viable species delimitation scenarios (two, three, and six species), the STEM analysis greatly favored the three-species delimitation for the *E. simoterum* complex (Fig. 2b and Table 3).

**Gene Tree Simulation Analysis**

The mean posterior age of the most recent common ancestor of the *E. simoterum* complex using the centrarchid fossil calibration priors in a BEAST analysis was 3.7 Ma, 95% HPD [2.1, 5.3]. Divergence time estimates for species within the Cumberland clade were all less than 1.0 Ma, and the age of the most recent common ancestor of the Tennessee and Cumberland clade was 2.9 Ma, 95% HPD [1.7, 4.2]. The age of the most recent common ancestor of Centrarchidae in this analysis was 35.2 Ma, 95% HPD [27.4, 43.9]. This age estimate is very similar to 32.6 Ma, 95% HPD [25.3, 39.9], estimated using a combined mtDNA and nuclear gene data set in Hollingsworth and Near (2009) and 33.6 ± 3.6 Ma resulting from a penalized likelihood analysis of multiple mitochondrial and nuclear genes (Near et al. 2005).

The IMa2 Bayesian analyses of population parameters of the *E. simoterum* complex provided estimates of effective population size (*N_e*) under both a three- and a six-species delimitation scenario that were used in gene tree simulation analyses. Bayesian estimates of *N_e* for the three-species scenario tended to be slightly higher, although estimates of *N_e* exhibited overlapping 95% HPD estimates between the two contrasted species delimitation scenarios (Table 4).

The mitochondrial cytb gene tree had a DCP and Slatkin’s *s* score of 21 and 13, respectively, for the six-species scenario *E. simoterum* complex species tree. These values are significantly higher than the range of DCP and *s* for gene trees simulated at an *N_e* equal to 10^8 (Table 5). The DCP and *s* values calculated for the observed six-species scenario cytb gene tree was within the distribution generated assuming a value of *N_e* equal to 10^8 and was well outside the generated distributions assuming a value of *N_e* equal to 10^8 (Table 5). The three-species scenario, in which each river system contains a monophyletic species, had minimal DCP and *s* values (DCP of 0, *s* of 3). Gene tree simulations on a three-species scenario tree required an effective population size of 10^9 before instances of incomplete lineage sorting among the three species were observed. The
FIGURE 4. Gene trees of the *Etheostoma simoterum* species complex inferred from Bayesian analyses of each sampled nuclear gene. a) S7. b) MLL. c) RAG1. Redundant alleles were removed prior to analysis. Bayesian posterior probabilities are indicated to the left of major nodes. This figure is available in black and white in print and in color at *Systematic Biology* online.
Only form of deep coalescence observed in the three-species simulations at the three species scenario was due to differences among the relationships of the three species, instead of nonmonophyly among the species.

Variation in Diagnostic Color and Pigmentation Characters

Powers and Mayden’s (2007) presentation of morphometric and meristic variation showed broad overlap in multivariate morphological space among species within the E. simoterum complex, and diagnoses of species were based largely on the variation in color patterns across the Cumberland and Tennessee River systems. The nuptial-condition males of the E. simoterum species complex included in our analysis showed substantial intra- and interspecific variation in the color patterns useful for species diagnosis (Supplementary Fig. 1 provides example photographs of each species). Our examination of the 11 color traits that were emphasized for the diagnosis of species demonstrates that many of these traits do not correspond with the geographic ranges of the described species. Although most individuals exhibited the correct pattern of banding and vermiculation on the first dorsal fin, approximately 24% of individuals we examined had patterns that were characteristic of other species within the same river system (e.g., between E. occidentale and E. atripinne or E. simoterum and E. tennesseense) (Table 6). Some color characters, such as head color and lateral green cast, failed to correspond to the designated species at a high frequency, whereas other characters, such as the presence of a continuous line of ventrolateral orange scales, performed well as a diagnostic trait in some species but very poorly in others. For example, 28% of E. planasaxatile examined had a broken, instead of the purportedly diagnostic continuous line of ventrolateral orange scales (Table 6).

### Table 3. Likelihood scores and information-theoretic comparisons for STEM analysis of species delimitation scenarios in the Ethoestoma simoterum species complex

| Number of species | −lnL | k | AIC | ΔI | L(Model|Data) | w_{i} |
|-------------------|------|---|-----|----|----------|--------|
| 8                 | −5137.8985 | 9 | 10293.797 | 0.000 | 1.000 | 0.663 |
| 9                 | −5137.8935 | 10 | 10295.787 | 1.9900 | 0.370 | 0.245 |
| 10                | −5137.8927 | 11 | 10297.7854 | 3.9884 | 0.136 | 0.090 |
| 5                 | −5325.8982 | 6 | 10663.7963 | 369.9993 | 0.000 | 0.000 |
| 6 (contrived)     | −5325.8890 | 7 | 10665.7780 | 371.9810 | 0.000 | 0.000 |
| 7                 | −5325.8783 | 8 | 10667.7866 | 373.9596 | 0.000 | 0.000 |
| 4                 | −5403.8169 | 5 | 10817.6338 | 789.3223 | 0.000 | 0.000 |
| 3                 | −5537.5598 | 4 | 11083.1196 | 798.2581 | 0.000 | 0.000 |
| 6                 | −6217.7449 | 7 | 12449.4898 | 2155.6928 | 0.000 | 0.000 |
| 2                 | −6528.0275 | 3 | 13062.0551 | 3768.2581 | 0.000 | 0.000 |

Notes: Columns (from left) indicate the number of species, the log-likelihood of the species tree (−lnL), number of parameters (k), AIC, AIC difference (ΔI), relative likelihood of model given the data (L), and the model probabilities (w_{i}). Rows are ordered by the respective AIC values for each delimitation scenario.
(Nixon and Wheeler 1990). In contrast, a history-based phylogenetic species concept relies on information that can be interpreted in an evolutionary context, such as monophyly in gene trees (Baum and Donoghue 1995; Hudson and Coyne 2002). Powers and Mayden (2007) cite the character-based apomorphy phylogenetic species concept (Nixon and Wheeler 1990); however, our analyses clearly demonstrate that the multiple species they recognize in the Tennessee (E. simoterum and E. tennesseense) and Cumberland River systems (E. atripinne, E. occidentale, and E. orientale) exhibit neither monophyly nor consistent diagnostic morphological apomorphies. The species delimitation scenario proposed in Powers and Mayden (2007) is not supported in either the character-based or the history-based phylogenetic species concepts.

We are not advocating a strict genealogical species concept (Baum and Shaw 1995); however, if one assumes that the six hypothesized species in the E. simoterum complex were reproductively isolated from the time of speciation, long periods of time would have to elapse before observing monophyly of species at a large number of nuclear loci. We expect that when dealing with very recently diverged species, as in the E. simoterum species complex, gene trees from most nuclear loci will provide limited phylogenetic resolution and coalescent information. In general, genes from cytoplasmic genomes will reach monophyly much more quickly than any sampled set of nuclear loci because they have a smaller effective population size and generally much higher mutation rate (Moore 1995; Hudson and Coyne 2002). There are a couple of potential weaknesses of relying largely on mitochondrial loci for inferring species delimitations, such as the potential for introgression across species boundaries and the effects of direct or indirect selection (Ballard and Whitlock 2004; Bossu and Near 2009; Keck and Near 2010), and overcoming these potential pitfalls requires the discovery of multiple nuclear loci with sufficiently high mutation rates to allow for reconstruction of demographic and coalescent patterns. With the observed monophyly in the mitochondrial gene tree of three species with a clear geographic pattern of allopatry and the failure of morphological traits to diagnose the six species proposed by Powers and Mayden (2007), we are confident that our phylogenetic and coalescent analyses provide substantial support for recognition of three history-based phylogenetic species in the E. simoterum complex, with E. simoterum in the Tennessee River, E. planasaxatile in the Duck, and E. atripinne in the Cumberland River system (Fig. 1c). The observed pattern of nonmonophyly of the described species within the Cumberland and Tennessee River systems is likely due to gene flow, and the multiple described species within the respective river systems likely represent populations of contiguous evolutionary lineages.

The recognition of three phylogenetic species in the E. simoterum complex that correlates with their geographic distribution in the Duck, Cumberland, and Tennessee River systems appears the best-supported delimitation scenario from both morphological and molecular data and is concordant with paleogeographic

### Table 4. Bayesian estimates of effective population size estimates ($N_e$) and the 95% HPD for the *Etheostoma simoterum* complex

<table>
<thead>
<tr>
<th>Species</th>
<th>$N_e$, six species</th>
<th>95% HPD</th>
<th>$N_e$, three species</th>
<th>95% HPD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Etheostoma atripinne</em></td>
<td>60,309</td>
<td>24,751, 163,160</td>
<td>293,259</td>
<td>81,370, 388,950</td>
</tr>
<tr>
<td><em>Etheostoma occidentale</em></td>
<td>32,376</td>
<td>120,64, 88,243</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Etheostoma orientale</em></td>
<td>13,526</td>
<td>6983, 41,260</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Etheostoma simoterum</em></td>
<td>71,735</td>
<td>36,663, 121,260</td>
<td>811,491</td>
<td>402,652, 1,271,159</td>
</tr>
<tr>
<td><em>Etheostoma tennesseense</em></td>
<td>269,834</td>
<td>178,409, 382,762</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Etheostoma planasaxatile</em></td>
<td>22,232</td>
<td>9522, 75,558</td>
<td>34,873</td>
<td>15,726, 799,336</td>
</tr>
</tbody>
</table>

Note: Estimates are presented for both a six-species and a three-species delimitation scenario.

### Table 5. DCP and Slatkin’s $s$ coalescent penalties for mitochondrial *cytb* gene tree simulations on a six-species tree, with $P$ values for the DCP and $s$ of the observed gene tree

<table>
<thead>
<tr>
<th>Statistic</th>
<th>$N_e^a$</th>
<th>Mean</th>
<th>SD$^b$</th>
<th>95% CI$^c$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00–0.000</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>10,000</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00–0.000</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>100,000</td>
<td>0.36</td>
<td>0.57</td>
<td>0.00</td>
<td>0.00–1.487</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>1,000,000</td>
<td>8.10</td>
<td>2.32</td>
<td>0.00</td>
<td>3.459–12.731</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>10,000,000</td>
<td>30.80</td>
<td>4.40</td>
<td>0.00</td>
<td>21.96–39.569</td>
<td>0.02</td>
</tr>
<tr>
<td>100,000,000</td>
<td>63.90</td>
<td>5.81</td>
<td>0.00</td>
<td>52.27–75.512</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>6.00</td>
<td>0.00</td>
<td>0.00</td>
<td>6.000–6.000</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>10,000</td>
<td>6.00</td>
<td>0.00</td>
<td>0.00</td>
<td>6.000–6.000</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>100,000</td>
<td>6.03</td>
<td>0.16</td>
<td>0.00</td>
<td>5.717–6.343</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>1,000,000</td>
<td>8.65</td>
<td>1.02</td>
<td>0.00</td>
<td>6.608–10.692</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>10,000,000</td>
<td>15.60</td>
<td>3.58</td>
<td>0.00</td>
<td>12.00–19.201</td>
<td>&lt;0.13</td>
</tr>
<tr>
<td>100,000,000</td>
<td>22.60</td>
<td>3.58</td>
<td>0.00</td>
<td>19.03–26.120</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

Notes: $^a$Effective population size ($N_e$).

$^b$SD, standard deviation.

$^c$CI, confidence interval.

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These rivers share dynamic patterns of connection over the last 2–10 myr that could have been important factors in the geography of speciation in the *E. simoterum* complex (Starnes and Etnier 1986). In addition, several other teleost species and species groups share a similar pattern of geographic distribution and may exhibit divergence times similar to those estimated for the *E. simoterum* complex (Powers et al. 2004; George et al. 2006; Berendzen et al. 2008).

Many species of *Etheostoma* exhibit dramatic sexual dichromatism, with males often being colored with brilliant shades of blue, green, and red. Descriptions of darter species have historically relied entirely on discovery of morphological characters, especially color patterns of adult breeding-condition males and meristic counts (Page et al. 1992; Suttkus et al. 1994; Ceas and Page 1997). The observations of geographic association with color pattern variation among species of the *E. simoterum* complex are intriguing; however, our analyses demonstrate that color pattern variation attributed to species boundaries was not consistent with the geographic boundaries of a six-species scenario. Several of the color characteristics that were proposed as diagnostic differences between breeding males of *E. tennesseense* and *E. simoterum* in the Tennessee River and *E. atripinne*, *E. occidentale*, and *E. orientale* in the Cumberland River were not consistently associated with these described species. We observed considerable overlapping variation in color characteristics that were intended to be diagnostic for species in the *E. simoterum* complex (Table 6). Reliance on such variable characters for species delimitation makes these species difficult to identify in practice and seems to impart a biological or evolutionary importance on these characters with little or no empirical basis.

Principal components analyses of both meristic and morphometric characters showed broad overlap among most species (Powers and Mayden 2007, table 4, figures 6, 7, and 8), with scale counts heavily weighting the separation of populations of *E. simoterum* from those of the other species in the complex. Scale counts are highly variable within the Tennessee River system. Data presented in Powers and Mayden (2007, table 4) show a bimodal distribution of scale counts within *E. tennesseense*. Bouchard (1977) reported a potential cline in scale counts within the Tennessee River, with populations in upper Tennessee tributaries having lower mean scale counts than those in tributaries in the lower Tennessee system. Scale count data in Powers and Mayden (2007) were grouped by species and not by population, and it is difficult to determine if populations of *E. tennesseense* that are geographically more proximal to *E. simoterum* have scale counts more similar to them than other *E. tennesseense* populations. Scale counts and other meristic measurements are a nearly ubiquitous feature of teleost fish species descriptions and are frequently used as characters for distinguishing between closely related species. However, it is not clear how to interpret meristic variation in *E. simoterum*. In the mitochondrial *cytb* gene tree, there was no clear association between geography

### Table 6. Percentage of specimens examined that exhibit diagnostic color and pigmentation patterns presented in Powers and Mayden (2007)

<table>
<thead>
<tr>
<th>Character</th>
<th>Etheostoma atripinne</th>
<th>Etheostoma occidentale</th>
<th>Etheostoma orientale</th>
<th>Etheostoma planasaxatile</th>
<th>Etheostoma simoterum</th>
<th>Etheostoma tennesseense</th>
<th>Species total average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head color</td>
<td>37.5</td>
<td>42.9</td>
<td>73.7</td>
<td>74.5</td>
<td>76.6</td>
<td>89.0</td>
<td>79.9</td>
</tr>
<tr>
<td>Mouth color</td>
<td>50.0</td>
<td>71.4</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>76.3</td>
<td>83.0</td>
</tr>
<tr>
<td>Breast color</td>
<td>37.5</td>
<td>100.0</td>
<td>100.0</td>
<td>85.7</td>
<td>100.0</td>
<td>100.0</td>
<td>87.2</td>
</tr>
<tr>
<td>Belly color</td>
<td>37.5</td>
<td>100.0</td>
<td>100.0</td>
<td>21.4</td>
<td>100.0</td>
<td>100.0</td>
<td>76.5</td>
</tr>
<tr>
<td>Caudal peduncle</td>
<td>25.0</td>
<td>85.7</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>85.1</td>
</tr>
<tr>
<td>Venter color</td>
<td>85.1</td>
<td>87.5</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>74.5</td>
</tr>
<tr>
<td>Line of ventrolateral orange scales</td>
<td>87.5</td>
<td>100.0</td>
<td>100.0</td>
<td>71.4</td>
<td>100.0</td>
<td>81.6</td>
<td>81.8</td>
</tr>
<tr>
<td>Color of blotches on body</td>
<td>87.5</td>
<td>100.0</td>
<td>20.0</td>
<td>57.1</td>
<td>94.7</td>
<td>71.1</td>
<td>71.7</td>
</tr>
<tr>
<td>Shape of blotches on body</td>
<td>50.0</td>
<td>85.7</td>
<td>40.0</td>
<td>71.4</td>
<td>57.9</td>
<td>73.7</td>
<td>63.1</td>
</tr>
<tr>
<td>Lateral green cast on body</td>
<td>25.0</td>
<td>28.6</td>
<td>40.0</td>
<td>57.1</td>
<td>100.0</td>
<td>0.0</td>
<td>41.8</td>
</tr>
<tr>
<td>First dorsal fin red pigment</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>42.1</td>
<td>100.0</td>
<td>90.4</td>
</tr>
<tr>
<td>Total average</td>
<td>52.3</td>
<td>81.8</td>
<td>74.5</td>
<td>76.6</td>
<td>88.0</td>
<td>79.9</td>
<td></td>
</tr>
</tbody>
</table>
and haplotype distribution within the Tennessee River system (Fig. 3), making the identification of phylogenetic lineages within the Tennessee River system potentially masquerading as E. simoterum difficult. For example, haplotypes sampled from E. simoterum populations in the Clinch and French Broad Rivers were distributed in phylogenetically disparate lineages in the cytb gene tree (Fig. 3). Moreover, nearly every major clade within the E. simoterum/tennesseense region of the cytb gene tree contained haplotypes sampled from populations in both upper and lower portions of the Tennessee River system. The geographic distribution of E. simoterum in the Tennessee River system spans several distinct physiographic regions, characterized by colder, higher gradient streams in the upper Tennessee system and lower gradient, warmer streams in the lower Tennessee River system. The pattern of meristic variation among E. simoterum populations throughout the Tennessee River system and the lack of a clear phylogeographic signal among E. simoterum populations in the cytb gene tree do not lend itself as primary evidence for the delimitation of multiple species of the E. simoterum complex within the Tennessee River system.

Species Tree and Coalescent Methods for Species Delimitation

There are a variety of species tree inference methods (e.g., BEST, *BEAST, and STEM) that can accommodate the unique evolutionary histories of unlinked loci, and because these programs can accommodate many individuals sampled from populations or recently diverged species, there will likely be an overlap between a user’s desire to understand the significance between shared alleles among populations and the genetic structure within species for purposes of defining distinct evolutionary units in phylogenetic analyses. Unfortunately, these methods do not provide an explicit test of hypotheses concerning species delimitation. We have shown that coalescent-based species tree methods may be biased toward over-splitting in the comparison of species delimitation hypotheses in the E. simoterum complex.

The observed behavior of the STEM-based information-theoretic strategy presented by Carstens and Dewey (2010), which supports delimitation of too many species, is largely due to the fact that species tree coalescent maximum likelihood scores are a function of the number of lineages within each branch of the species tree, the amount of time these lineages persist in the branch, and the effective population size (Rannala and Yang 2003; Kubatko et al. 2009). The maximum likelihood score of the species tree will increase as the number of lineages in each branch of the tree decreases. Dividing a sample into a greater number of delimited species will increase the number of branches in the species tree and decrease the average number of lineages in each branch. STEM uses a global theta, and without adjusting theta on each branch as a result of increasing the number of species, likelihood scores will increase as the number of taxa increases. This issue could be addressed by using the same number of genes sampled per delimited species and making appropriate adjustments to theta for each delimitation scenario. Although the AIC test does penalize for additional parameters, such as increasing the number of delimited species in the analysis, an information-theoretic approach to species delimitation in the E. simoterum complex did not provide an effectively conservative penalty against partitioning the sample into a biologically unrealistic number of species. Our observation that the STEM-based strategy of species delimitation leads to an over-splitting of Etheostoma species suggests that this class of species tree methods may be most appropriately used to assess differences in maximum likelihood scores among a set of competing species tree hypotheses that each contain the same number of delimited species.

Our coalescent simulation analyses do not support the hypothesis that the observed nonmonophyly of species in the six-species scenario is due to incomplete lineage sorting, given reasonable values of effective population size. The effective population size would need to be in the tens of millions in order to produce the observed pattern of allele sharing by incomplete lineage sorting. Unfortunately, there is no information on measures of genetic effective population size or census population size for darter species. Our observed gene tree produced a DCP and s score for the six-species scenario that was consistent with an effective population size on the order of tens of millions per species. If species in the E. simoterum species complex actually exhibit a Ne/N ratio equal to 0.1 (as observed in most nongame teleosts; Frankham 2007; Turner et al. 2007), there would need to be a census size of approximately 100,000,000 individuals for each of the six species to generate the observed pattern of deep coalescence and incomplete lineage sorting observed in the cytb gene tree. Instead, our calculations of effective population size produced estimates that are orders of magnitude lower than what is required by the simulations to produce observed patterns of deep coalescence across species boundaries in the six-species scenario. The largest estimated Ne was approximately 270,000, whereas estimates of Ne for the other species ranged between approximately 13,000 and 72,000 (Table 4).

This coalescent simulation analysis seems ideally suited for testing individual hypotheses of species delimitations. It may be advantageous compared with other methods in that it is not biased toward splitting or lumping of taxa because empirical data are compared with null distributions generated for each delimitation scenario. An obvious limitation to this approach is that it requires prior estimations of divergence times and effective population sizes, for which there likely will be some uncertainty. In spite of this drawback, it provides a useful way to interpret the frequency of shared alleles among species in a coalescent framework.
CONCLUSIONS

Despite a perceived paucity of analytical methods for objective approaches to species delimitation using genetic sequence data, our analyses bring together several strategies that comprise Bayesian estimation of gene trees, Bayesian estimation of divergence times, coalescent analyses, and species tree estimation in the context of a history-based phylogenetic species concept to discriminate between competing hypotheses of species delimitation. We find that a recently introduced information-theoretic strategy to compare species trees may be susceptible to over-splitting of species in the *E. simoterum* complex. With the growing resolution of the shape and dimensions of the Tree of Life, there is a critical need for a set of analytical protocols for species delimitation that incorporates multilocus data in a coalescent framework. Such efforts will reveal the magnitude of shared alleles among species and genetic structure within species and specifically provide important insight on whether a species delimitation hypothesis is a reasonable fit for the observed population genetic structure, patterns of gene tree deep coalescence, and phenotypic disparity. In the meantime, it may be best to consider species delimitation as a hands-on process, whereby patterns that emerge from the analyses of phenotype, genotype, and correlation of genetic and phenotypic variation with geographic distribution are considered in the generation and testing of species delimitation hypotheses.

SUPPLEMENTARY MATERIAL

Supplementary material, including data files and/or online-only appendices, can be found at http://www.sysbio.oxfordjournals.org/.

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