Phylogeny and Temporal Diversification of Darters (Percidae: Etheostomatinae)

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Abstract.—Discussions aimed at resolution of the Tree of Life are most often focused on the interrelationships of major organismal lineages. In this study, we focus on the resolution of some of the most apical branches in the Tree of Life through exploration of the phylogenetic relationships of darters, a species-rich clade of North American freshwater fishes. With a near-complete taxon sampling of close to 250 species, we aim to investigate strategies for efficient multilocus data sampling and the estimation of divergence times using relaxed-clock methods when a clade lacks a fossil record. Our phylogenetic data set comprises a single mitochondrial DNA (mtDNA) gene and two nuclear genes sampled from 245 of the 248 darter species. This dense sampling allows us to determine if a modest amount of nuclear DNA sequence data can resolve relationships among closely related animal species. Darters lack a fossil record to provide age calibration priors in relaxed-clock analyses. Therefore, we use a near-complete species–sampled phylogeny of the perciform clade Centrarchidae, which has a rich fossil record, to assess two distinct strategies of external calibration in relaxed-clock divergence time estimates of darters: using ages inferred from the fossil record and molecular evolutionary rate estimates. Comparison of Bayesian phylogenies inferred from mtDNA and nuclear genes reveals that heterospecific mtDNA is present in approximately 12.5% of all darter species. We identify three patterns of mtDNA introgression in darters: *proximal mtDNA transfer*, which involves the transfer of mtDNA among extant and sympatric darter species, *indeterminate introgression*, which involves the transfer of mtDNA from a lineage that cannot be confidently identified because the introgressed haplotypes are not clearly referable to mtDNA haplotypes in any recognized species, and *deep introgression*, which is characterized by species diversification within a recipient clade subsequent to the transfer of heterospecific mtDNA. The results of our analyses indicate that DNA sequences sampled from single-copy nuclear genes can provide appreciable phylogenetic resolution for closely related animal species. A well-resolved near-complete species–sampled phylogeny of darters was estimated with Bayesian methods using a concatenated mtDNA and nuclear gene data set with all identified heterospecific mtDNA haplotypes treated as missing data. The relaxed-clock analyses resulted in very similar posterior age estimates across the three sampled genes and methods of calibration and therefore offer a viable strategy for estimating divergence times for clades that lack a fossil record. In addition, an informative rank-free clade-based classification of darters that preserves the rich history of nomenclature in the group and provides formal taxonomic communication of darter clades was constructed using the mtDNA and nuclear gene phylogeny. On the whole, the appeal of mtDNA for phylogeny inference among closely related animal species is diminished by the observations of extensive mtDNA introgression and by finding appreciable phylogenetic signal in a modest sampling of nuclear genes in our phylogenetic analyses of darters. [*Ammocrypta*; *Crystallaria*; divergence times; *Etheostoma*; mitochondrial DNA; *Nothonotus*; nuclear DNA; *Percina*; phylocode; phylogenetic classification; relaxed molecular clock; Teleostei.]

The diversity of freshwater fishes in eastern North America is unrivaled among temperate regions of the world (Briggs 1986; Lundberg et al. 2000). The ability to test evolutionary hypotheses that address the origin and timing of diversification of the species-rich North American freshwater fish fauna is dependent on the availability of resolved species-level phylogenetic hypotheses and robust estimates of divergence times. In addition, resolved phylogenetic trees with dense taxon sampling provide the ideal basis from which to construct species-level phylogenetic classifications (de Queiroz and Gauthier 1990; Cantino et al. 1999; Hillis and Wilcox 2005) and to test the veracity of the traditional pre-phylogenetic classifications that have historically served as the basis for comparative evolutionary studies.

The challenge of estimating comprehensive phylogenies for the North American freshwater fish fauna is daunting because the two most species-rich clades,

darters (Etheostomatinae) and minnows (Cyprinidae), contain an estimated 250 and 320 species (Mayden et al. 1992; Scharpf 2005, 2008), respectively, and previous inferences indicate that these clades exhibit a fairly recent history of diversification, which does not predate the Eocene (Dowling et al. 2002; Near and Keck 2005; Rüber et al. 2007; Hollingsworth and Near 2009). Appropriate data selection strategies are required to resolve the species-level phylogenetic relationships of such large and recent radiations (Wiens et al. 2005; McGuire et al. 2007), and the lack of a fossil record for a clade, as is the case in darters, leads to major challenges in attempting to estimate divergence times using relaxed molecular clock methods (Near and Benard 2004).

Over the past few years, there has been progress in the development of phylogenetic hypotheses and molecular divergence time estimates for select North American freshwater fish clades. Near-complete species

sampling, consistent phylogenetic inferences across independent data sets, and relaxed-clock age estimates have been possible for Ictaluridae (Bullhead Catfishes and Madtoms) and Centrarchidae (Sunfishes and Black-basses) because each of these clades contains fewer than 55 species and has a rich fossil record (Hardman and Page 2003; Near et al. 2003, 2004; Near, Bolnick, et al. 2005; Hardman 2004; Hardman M. and Hardman L.M. 2008). A comparison of the time-calibrated phylogenies of these two clades indicates that both Ictaluridae and Centrarchidae originated in the Cenozoic and that the Oligocene and Miocene were periods of their peak diversification (Near, Bolnick, et al. 2005; Hardman M. and Hardman L.M. 2008). Similar relaxed-clock age estimates have been presented for the species-rich North American Cyprinidae (Dowling et al. 2002; Smith et al. 2002; Rüber et al. 2007; Houston et al. 2010), but analyses to date have only included a small fraction of the extant species diversity in this clade.

The inference of time-calibrated phylogenies for species-rich North American freshwater fish clades is complicated by the presence of mitochondrial DNA (mtDNA) introgression among closely related species. An appreciable frequency of heterospecific cytoplasmic genomes is observed in plant and animal species (Chan and Levin 2005), and mtDNA transfer between species has been documented in several disparately related darter clades (e.g., Bossu and Near 2009; Keck and Near 2010). This introgression creates substantial problems because the phylogenetic resolution of the most closely related lineages in species-rich animal clades is typically accomplished using rapidly evolving genes (e.g., Wiens et al. 2005), such as those found in the mtDNA genome. As observed in other teleost fish clades (Scribner et al. 2001), mtDNA introgression in darters typically involves little to no introgression of nuclear-encoded alleles and the introgression is most often asymmetric (Piller et al. 2008; Bossu and Near 2009; Keck and Near 2010). Potential mechanisms that result in the observed asymmetric introgression have not been identified but were outlined in the previous studies (e.g., Bossu and Near 2009; Keck and Near 2010). The study of mtDNA introgression in darters has focused on specific clades of closely related species, and mtDNA transfer among species was detected using comparisons of mtDNA gene trees with phylogenies inferred from nuclear genes and morphology (e.g., Bossu and Near 2009). However, the impact of introgression on deeper portions of mtDNA gene trees inferred for species-rich lineages is not well known (e.g., Shaw 2002). Data matrices consisting of nuclear gene DNA sequences that comprise complete taxon sampling not only provide peace of mind with regard to issues of missing taxa in phylogenetic analyses (e.g., Zwickl and Hillis 2002; Heath et al. 2008), but also provide an opportunity to assess the performance of nuclear genes to infer relationships among closely related species and a set of independent gene trees to determine the occurrence of mtDNA introgression throughout the history of diversification in species-rich lineages.

Darters

An excellent group in which to investigate the history of mtDNA introgression and divergence time estimation in species-rich lineages is the darter clade, which contains approximately 250 species endemic to eastern North America that comprises more than 20% of the entire hyperdiverse North American freshwater fish fauna (Table 1; Lundberg et al. 2000). Darters are classified as Etheostomatinae, which is a subclade of Percidae. In addition to Etheostomatinae, two other major freshwater clades with Holarctic distributions, Percinae and Luciopercinae, comprise Percidae. However, neither Percinae nor Luciopercinae contains more than 10 species (Collette and Banarescu 1977; Song et al. 1998). Darter species differ substantially from those in the two other lineages of Percidae by having a smaller body size, males of many darter species develop elaborate nuptial colors, and the majority of species lack a functional swim bladder (Page 1983; Page and Swofford 1984; Etnier and Starnes 1993).

Recent molecular phylogenetic analyses of darters have revealed several instances of incongruence between inferred phylogenetic trees and traditionally recognized genera and subgenera. For example, gene trees inferred from mtDNA sequences suggest that the species-rich genus *Etheostoma* is not monophyletic because the subgenus *Nothonotus* and the species *Etheostoma cinereum* are each more closely related to *Percina* or to a clade containing *Ammocrypta* and *Crystallaria* (Song et al. 1998; Sloss et al. 2004; Mayden et al. 2006; Lang and Mayden 2007; Bossu and Near 2009). These results prompted Near and Keck (2005) to consider the subgenus *Nothonotus* as a clade distinct from *Etheostoma*, and, in the context of Linnaean-ranked taxonomy, elevated it to the rank of genus. In addition, among the 20 recognized polytypic subgenera of *Etheostoma* and *Percina* recognized in Page (2000), 11 were not supported as monophyletic in molecular phylogenetic analyses of mtDNA and nuclear genes (Near 2002; Mayden et al. 2006; Lang and Mayden 2007) and 4 were not monophyletic in phylogenies inferred from discretely coded morphological characters (Ayache and Near 2009). A subgeneric classification of darters representing conclusions proposed by Bailey and Etnier (1988) and Page (2000) is presented in Table 1.

Objectives for the Investigation of Darter Phylogeny

In this study, we investigate the phylogenetic relationships of darters using a molecular data set that contains DNA sequences from the mitochondrial gene cytochrome *b* (*cytb*) and two nuclear genes: the first intron of the *S7* ribosomal protein (*S7*) and exon 3 of the recombination activating gene-1 (*RAG1*). These genes were selected because they span the spectrum of slow- to fast-evolving genomic elements; nucleotides in the mitochondrial genome have relatively high rates of substitution, whereas nuclear protein-coding genes exhibit relatively lower rates of substitution. Nuclear introns are less functionally constrained and are therefore

TABLE 1. Traditional classification of darters that combines proposals by Bailey and Etnier (1988), Page (2000), and Near and Keck (2005)

Genus Percina

Subgenus Alvordius*
P. crassa	Piedmont Darter
P. gymnocephala	Appalachian Darter
P. kusha	Bridled Darter
P. cf. kusha[a]	Etowah Bridled Darter
P. macrocephala	Longhead Darter
P. maculata	Blackside Darter
P. nevisense	Chainback Darter
P. notogramma	Stripeback Darter
P. pantherina	Leopard Darter
P. peltata	Shield Darter
P. roanoka	Roanoke Darter
P. sipsi	Bankhead Darter
P. smithvanizi	Muscadine Darter
P. williamsi	Sickle Darter

Subgenus Cottogaster
P. aurora	Pearl Darter
P. brevicauda	Coal Darter
P. copelandi	Channel Darter

Subgenus Ericosma*
P. evides	Gilt Darter
P. cf. evides[a]	Western Gilt Darter
P. palmaris	Bronze Darter
P. cf. palmaris[a]	Copper Darter

Subgenus Hypohomus
P. aurantiaca	Tangerine Darter

Subgenus Imostoma
P. antesella	Amber Darter
P. shumardi	River Darter
P. tanasi	Snail Darter
P. uranidea	Stargazing Darter
P. vigil	Saddleback Darter

Subgenus Hadropterus*
P. apristis	Guadalupe Darter
P. aurolineata	Goldline Darter
P. crypta	Halloween Darter
P. lenticula	Freckled Darter
P. nigrofasciata	Blackband Darter
P. sciera	Dusky Darter
P. westfalli[b,c]	Eastern Blackbanded Darter

Subgenus Percina
P. austroperca	Southern Logperch
P. bimaculata	Chesapeake Logperch
P. burtoni	Blotchside Logperch
P. caprodes	Logperch
P. cf. caprodes[a]	Driftless Logperch
P. carbonaria	Texas Logperch
P. fulvitaenia	Ozark Logperch
P. jenkinsi	Conasauga Logperch
P. kathae	Mobile Logperch
P. macrolepida	Bigscale Logperch
P. rex	Roanoke Logperch
P. suttkusi	Gulf Logperch

Subgenus Odontopholis
P. cymatotaenia	Bluestripe Darter
P. stictogaster	Frecklebelly Darter

Subgenus Swainia
P. nasuta	Longnose Darter
P. cf. nasuta[a]	Ouachita Darter
P. oxyrhynchus	Sharpnose Darter
P. phoxocephala	Slenderhead Darter
P. squamata	Olive Darter

TABLE 1. *Continued*

Genus Crystallaria
C. asprella	Crystal Darter
C. cincotta	Diamond Darter

Genus Ammocrypta
A. beanii	Naked Sand Darter
A. cf. beanii[a]	Mobile Sand Darter
A. bifascia	Florida Sand Darter
A. clara	Western Sand Darter
A. meridiana	Southern Sand Darter
A. pellucida	Eastern Sand Darter
A. vivax	Scaly Sand Darter

Genus Nothonotus
N. acuticeps	Sharphead Darter
N. aquali	Coppercheek Darter
N. bellus	Orangefin Darter
N. camurus	Bluebreast Darter
N. chlorobranchius	Greenfin Darter
N. chuckwachatte	Lipstick Darter
N. denoncourti	Golden Darter
N. douglasi	Tuskaloosa Darter
N. etowahae	Etowah Darter
N. jordani	Greenbreast Darter
N. juliae	Yoke Darter
N. maculatus	Spotted Darter
N. microlepidus	Smallscale Darter
N. moorei	Yellowcheek Darter
N. rubrus	Bayou Darter
N. rufilineatus	Redline Darter
N. sanguifluus	Bloodfin Darter
N. cf. sanguifluus[a]	Caney Fork Darter
N. tippecanoe	Tippecanoe Darter
N. vulneratus	Wounded Darter
N. wapiti	Boulder Darter

Genus Etheostoma*
Subgenus Allohistium
E. cinereum	Ashy Darter

Subgenus Belophlox*
E. fricksium	Savannah Darter
E. mariae	Pinewoods Darter
E. okaloosae	Okaloosa Darter

Subgenus Boleosoma
E. longimanum	Longfin Darter
E. nigrum	Johnny Darter
E. olmstedi	Tessellated Darter
E. perlongum	Waccamaw Darter
E. podostemone	Riverweed Darter
E. susanae	Cumberland Darter

Subgenus Catonotus*
E. brevispinum	Carolina Fantail Darter
E. flabellare	Fantail Darter
E. humerale[b]	Chesapeake Fantail Darter
E. kennicotti	Stripetail Darter
E. lemniscatum	Tuxedo Darter
E. marmorpinnum	Marbled Darter
E. percnurum	Duskytail Darter
E. sitikuense	Citico Darter
E. barbouri	Teardrop Darter
E. basilare	Corrugated Darter
E. cf. basilare[a]	Collins Darter
E. cf. basilare[a]	Calfkiller Darter
E. cf. basilare[a]	Cane Darter
E. cf. basilare[a]	Volunteer Darter
E. cf. basilare[a]	Mountain Darter
E. cf. basilare[a]	Hickory Darter
E. derivativum	Stone Darter
E. obeyense	Barcheek Darter
E. smithi	Slabrock Darter

Continued

TABLE 1. *Continued*

E. striatulum	Striated Darter
E. virgatum	Stripped Darter
E. cf. virgatum[a]	Buck Darter
E. chienense	Relict Darter
E. corona	Crown Darter
E. crossopterum	Fringed Darter
E. forbesi	Barrens Darter
E. neopterum	Lollypop Darter
E. nigripinne	Blackfin Darter
E. olivaceum	Sooty Darter
E. oophylax	Guardian Darter
E. cf. oophylax[a]	Clarks Darter
E. pseudovulatum	Egg-mimic Darter
E. squamiceps	Spottail Darter
Subgenus *Doration*	
E. akatulo	Bluemask Darter
E. jessiae	Blueside Darter
E. meadiae	Bluespar Darter
E. stigmaeum	Speckled Darter
E. cf. stigmaeum[a]	Bluegrass Darter
E. cf. stigmaeum[a]	Longhunt Darter
E. cf. stigmaeum[a]	Clown Darter
E. cf. stigmaeum[a]	Highland Darter
Subgenus *Etheostoma**	
E. blennioides	Greenside Darter
E. blennius	Blenny Darter
E. gutselli	Tuckasegee Darter
E. histrio	Harlequin Darter
E. inscriptum	Turquoise Darter
E. lynceum	Brighteye Darter
E. newmani	Highlands Greenside Darter
E. cf. newmani[a]	Arkansas Greenside Darter
E. pholidotum	Central Greenside Darter
E. rupestre	Rock Darter
E. cf. rupestre[a]	Central Rock Darter
E. cf. rupestre[a]	Eastern Rock Darter
E. sellare	Maryland Darter
E. sequatchiense[b,c]	Sequatchie Blenny Darter
E. swannanoa	Swannanoa Darter
E. thalassinum	Seagreen Darter
E. zonale	Banded Darter
Subgenus *Fuscatelum*	
E. parvipinne	Goldstripe Darter
E. phytophilum	Rush Darter
Subgenus *Hololepis* *	
E. barratti[b]	Scalyhead Darter
E. collis	Carolina Darter
E. exile	Iowa Darter
E. gracile	Slough Darter
E. fusiforme	Swamp Darter
E. saludae	Saluda Darter
E. serrifer	Sawcheek Darter
E. zonifer	Backwater Darter
Subgenus *Ioa*	
E. vitreum	Glassy Darter
Subgenus *Litocara*	
E. nianguae	Niangua Darter
E. sagitta	Cumberland Arrow Darter
E. spilotum[b,c]	Kentucky Arrow Darter
Subgenus *Microperca*	
E. fonticola	Fountain Darter
E. microperca	Least Darter
E. proeliare	Cypress Darter
Subgenus *Oligocephalus**	
E. artesiae	Redspot Darter
E. asprigene	Mud Darter
E. cf. asprigene[a]	Gumbo Darter
E. australe	Conchos Darter
E. binotatum	Hanukkah Darter

TABLE 1. *Continued*

E. bison	Buffalo Darter
E. burri	Brook Darter
E. caeruleum	Rainbow Darter
E. cf. caeruleum[a]	Ozark Rainbow Darter
E. collettei	Creole Darter
E. cyanorum[b]	Blue Darter
E. ditrema	Coldwater Darter
E. fragi	Strawberry Darter
E. grahami	Rio Grande Darter
E. hopkinsi	Christmas Darter
E. kantuckeense	Higland Rim Darter
E. lawrencei	Headwater Darter
E. lepidum	Greenthroat Darter
E. lugoi	Tufa Darter
E. luteovinctum	Redband Darter
E. nuchale	Watercress Darter
E. paludosum[b]	Washita Darter
E. pottsii	Mexican Darter
E. pulchellum	Plains Darter
E. radiosum	Orangebelly Darter
E. segrex	Salado Darter
E. spectabile	Orangethroat Darter
E. cf. spectabile[a]	Ozark Darter
E. cf. spectabile[a]	Sheltowee Darter
E. cf. spectabile[a]	Wildcat Darter
E. cf. spectabile[a]	Ihiyo Darter
E. cf. spectabile[a]	Mamequit Darter
E. squamosum	Plateau Darter
E. swaini	Gulf Darter
E. tecumsehi	Shawnee Darter
E. uniporum	Current Darter
E. whipplei	Redfin Darter
Subgenus *Ozarka**	
E. autumnale	Autumn Darter
E. boschungi	Slackwater Darter
E. cragini	Arkansas Darter
E. mihileze	Sunburst Darter
E. pallididorsum	Paleback Darter
E. punctulatum	Stippled Darter
E. trisella	Trispot Darter
Subgenus *Poecilichthys*	
E. erizonum[b,c]	Current Saddled Darter
E. erythrozonum	Meramec Saddled Darter
E. euzonum	Arkansas Saddled Darter
E. kanawahe	Kanawha Darter
E. osburni	Candy Darter
E. tetrazonum	Missouri Saddled Darter
E. variatum	Variegate Darter
Subgenus *Psychromaster*	
E. tuscumbia	Tuscumbia Darter
Subgenus *Ulocentra**	
E. atripinne	Cumberland Sunbnose Darter
E. baileyi	Emerald Darter
E. barrenense	Splendid Darter
E. bellator	Warrior Darter
E. cf. bellator[a]	Sipsey Darter
E. cf. bellator[a]	Locust Fork Darter
E. brevirostrum	Holiday Darter
E. cervus	Chickasaw Darter
E. chermocki	Vermillion Darter
E. colorosum	Costal Darter
E. coosae	Coosa Darter
E. duryi	Blackside Snubnose Darter
E. etnieri	Cherry Darter
E. flavum	Saffron Darter
E. lachneri	Tombigbee Darter
E. planasaxatile	Duck Darter
E. pyrroghaster	Firebelly Darter
E. rafinesquei	Kentucky Darter
E. ramseyi	Alabama Darter
E. raneyi	Yazoo Darter
E. scotti	Cherokee Darter

Continued

TABLE 1. *Continued*

E. simoterum	Snubnose Darter
E. tallapoosae	Tallapoosa Darter
E. zonisitium	Bandfin Darter
E. cf. zonistium[a]	Blueface Darter
Subgenus *Vaillantia*	
E. chlorosoma	Bluntnose Darter
E. davisoni	Choctawhatchee Darter
Subgenus *Villora*	
E. edwini	Brown Darter

Notes: Genera and subgenera that were not monophyletic in the published phylogenetic analyses (see text) are marked with an asterisk. A revised classification of darters based on the phylogenetic analyses presented in this study is summarized in the Appendix and illustrated in Figure 4.

[a]Distinct species awaiting a formal description.

[b]Species previously treated as subspecies.

[c]Species previously treated as subspecies that are monophyletic in mtDNA gene trees.

expected to experience a rate of molecular evolution faster than that of nuclear protein-coding DNA but slower than that of the mitochondrial protein-coding genes.

The phylogenetic analyses presented in this paper use one of the largest and most completely sampled species-level nucleotide data sets of any comparable sized clade of animals. Our taxon sampling includes 245 of the 248 darter species recognized in this study (Table 1). The missing species are *Etheostoma sellare*, which is likely extinct (Neely et al. 2003), and two species endemic to Mexico, *E. pottsii* and *E. lugoi*, that are phylogenetically nested in a southwestern *Etheostoma* clade (Mayden et al. 2006; Lang 2007). We use the dense taxon sampling of darters to address five general questions relevant to the investigation of phylogenetic relationships for clades that have a fairly recent history of diversification. 1) Do trees estimated from gene regions with different molecular evolutionary rates provide resolution for differing regions of the darter phylogeny (e.g., deep vs. shallow nodes)? 2) What is the extent of mtDNA introgression as inferred from comparison among gene trees and relationships inferred from morphology? 3) How does the inferred molecular phylogeny of darters contrast with the currently accepted taxonomy, a taxonomy that serves as the basis for comparative studies? 4) Given the large number of darter species and the potential for the resolution of many nested clades in the resulting molecular phylogenies, what is the optimal way to use the phylogenies to create a clade-based classification of darters? 5) When using relaxed-clock methods, what is a viable strategy for estimating divergence times for a species-level phylogeny when the clade lacks a fossil record to provide absolute age calibrations?

MATERIALS AND METHODS

Recognition of Species Diversity and Taxon Sampling

There has been a dramatic increase in the number of recognized darter species since the listing of 129 species

in Page's monographic treatment of the clade; the increase to 248 species represents nearly a doubling of the number of recognized species over the past 25 years (Table 1). This increase in the recognized species diversity of darters is the result of the discovery of previously unknown or unrecognized species (e.g., Page et al. 2003; Page and Near 2007; Layman and Mayden 2009; Switzer and Wood 2009), resurrecting species from synonymy (e.g., Etnier and Starnes 1986; Near 2008), and recognition of species formerly ranked as subspecies (e.g., Etnier and Williams 1989; Piller et al. 2001; Robins and Page 2007).

We used two operational species concepts to assess the species diversity of darters. Ideally, darter species form monophyletic groups consistent with a history-based phylogenetic species concept (e.g., Baum and Donoghue 1995) and are diagnosable morphologically following a character-based apomorphy phylogenetic species concept (e.g., Nixon and Wheeler 1990). Included in our list of 248 darter species are those that are known to biologists and awaiting formal taxonomic description as well as 7 allopatrically distributed subspecies that exhibit morphological differences on the order of those observed between described species and which we recognize as species (e.g., Kuehne and Bailey 1961; Bailey and Richards 1963; Matthews and Gelwick 1988). The monophyly of three of these species has been confirmed in mtDNA gene trees (Table 1). However, we are withholding assessment of species diversity in darter lineages where sampled subspecies are not reciprocally monophyletic in gene trees and morphological delimitation is unclear, as is the case in the described subspecies of *Etheostoma olmstedi* (Cole 1967; Heckman et al. 2009), *E. caeruleum* (McCormick 1991; Ray et al. 2006), *E. flabellare* (McGeehan 1985; Blanton 2007), and *Percina evides* (Denoncourt 1969; Near et al. 2001). In addition, we treat *E. occidentale* and *E. orientale* as synonyms of *E. atripinne*, and *E. tennesseense* as a synonym of *E. simoterum* despite their recent description by Powers and Mayden (2007). Both mtDNA- and nuclear-inferred gene trees do not support monophyly of these species, and assessment of phenotypic traits demonstrates that these three species are not diagnosable with morphological apomorphies (Harrington and Near forthcoming).

Our phylogenetic analyses were based on DNA sequence data collected from one or more individuals of 245 darter species (see Table 1 for a list of species and Supplementary Table A1 for GenBank accession numbers; all supplementary material is available at http://www.systbio.oxfordjournals.org). Specimens were collected using a seine net or a backpack electroshocking apparatus, anesthetized using MS-222, and then either frozen whole in liquid nitrogen or a tissue biopsy was taken from the right pectoral fin and preserved in absolute ethanol. Voucher specimens were fixed in 3.7% formalin for approximately 10 days, washed in water for approximately 3 days, and transferred to 70% ethanol for long-term preservation. Specimens were deposited at three museum research

fish collections: the Yale Peabody Museum of Natural History (YPM), the David A. Etnier Ichthyology Collection at the University of Tennessee (UT), and the Illinois Natural History Survey (INHS). As shown in Supplementary Table A1, approximately 10% of the specimens included in this study were obtained from several other natural history museums including University of Alabama Ichthyology Collection (UAIC), the North Carolina State Museum (NCSM), Tulane University (TU), and the University of Kansas Natural History Museum (KU). The non-darter percid species *Perca flavescens* (Percinae) and *Sander vitreus* (Luciopercinae) were included as outgroups in all phylogenetic analyses.

All the 408 darter and non-darter percid specimens in our analyses were sequenced for the three genes included in our study, with the exception of one of the two sampled specimens of *Percina tanasi*, which was not sequenced for the nuclear *RAG1* gene. In order to provide external fossil age information and molecular evolutionary rates to calibrate the darter molecular phylogenies, a single specimen of each of 33 (of the 34) species of Centrarchidae was sequenced for *cytb* and *RAG1*. Gene sequences for *S7* were available for Centrarchidae on GenBank from a previous study (Near et al. 2004). Centrarchidae and Percidae are classified as subclades in the large acanthomorph teleost clade Perciformes (Smith and Craig 2007). Specimen information and GenBank accession numbers for all sequences are provided in Supplementary Table A1.

DNA Sequencing and Alignment

Standard phenol–chloroform extraction with ethanol precipitation protocols or Qiagen DNAeasy Tissue Extraction Kits (Qiagen, Valencia, CA) were used to isolate genomic DNA from tissues. The complete protein-coding cytochrome *b* (*cytb*) mitochondrial gene was amplified using polymerase chain reactions (PCRs), with primers and cycling conditions given in Near et al. (2000). The two nuclear genes (*S7* ribosomal protein intron 1 and *RAG1* exon 3) were amplified using PCR with primers and cycling conditions reported in the previous studies (Chow and Hazama 1998; Lopez et al. 2004). Amplified PCR products were cleaned using a Qiagen Qiaquick PCR Purification Kit or with enzymatic purification using exonuclease 1 and shrimp alkaline phosphatase that was incubated at $37°C$ for 15 min followed by $80°C$ to inactivate the enzymes.

Purified PCR products were used as templates for Big Dye (Applied Biosystems, Foster City, CA) cycle sequencing. Sequencing reactions were visualized on an ABI 3100 automated sequencer at the Molecular Systematics and Conservation Genetics Laboratory at Science Hill (Yale University, New Haven, CT). In most cases, the primers used for PCR were also used in the sequencing reactions, but additional sequencing primers presented in Bossu and Near (2009) were used in the *RAG1* sequencing reactions. The computer program Sequencher (GeneCodes, Ann Arbor, MI) was used to build contiguous sequences from the individual DNA

sequencing chromatograms. Because there were no insertions or deletions, the two protein-coding genes, *cytb* and *RAG1*, were aligned by eye and assessed with examination of the alignment of the translated amino acid sequences. The *S7* intron was aligned using the computer program MUSCLE version 3.6 with the default settings (Edgar 2004).

Model Selection, Data Partitioning, and Bayesian Phylogenetic Analyses

We specified 11 possible nucleotide data partitions among the three sequenced genes. The *S7* intron 1 was treated as a single partition in all analyses. The protein-coding genes were partitioned in one of three ways: 1) each codon position was assigned its own partition, 2) the first and second codon positions were combined into one partition and the third position assigned its own partition, or 3) all codon positions were combined into a single partition. The number of heterozygous nucleotide sites observed in the two nuclear genes (*S7* and *RAG1*) was minimal, and thus, the sites were treated as ambiguous data following Lemmon et al. (2009). Following Posada and Buckley (2004), we identified the optimal molecular evolutionary model for 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 two protein-coding genes was selected by calculating the Bayes factor from the difference of the log of the harmonic mean of the likelihood values sampled from the posterior distributions of the two compared Bayesian analyses (Newton and Raftery 1994; Nylander et al. 2004; Brandley et al. 2005). Despite concern that calculation of the Bayes factor from the harmonic mean of the likelihood scores can bias selection toward more parameter-rich models (Lartillot and Philippe 2006), Brown and Lemmon (2007) found that this method produces an acceptable false-positive rate.

All phylogenetic analyses were performed using the parallel version of the computer program MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003; Altekar et al. 2004) on a Linux cluster. The harmonic mean of the likelihood scores was determined using the *sump* command with the appropriate burn-in value. The criteria for choice among partitions given a calculated Bayes factor score were those outlined in Kass and Raftery (1995, table 5).

Four sets of MrBayes analyses were run: one for each of the three gene alignments (*cytb*, *S7*, and *RAG1*) and one for a data set where all three genes were concatenated. Species with heterospecific mitochondrial haplotypes, as determined in the previous studies and comparisons of mitochondrial and nuclear gene trees, were scored as missing data for the *cytb* gene in the three-gene concatenated data set. Posterior probabilities for the phylogenies and parameter values were estimated using Metropolis-coupled Markov chain Monte Carlo (MC3; Larget and Simon 1999; Huelsenbeck et al.

2001). Each MrBayes analysis was run three separate times for 6.0×10^7 generations with two simultaneous runs each with four chains (one cold and three heated chains with a heating parameter = 0.02 to ensure appropriate mixing). The cold chain was sampled once every 1000 generations. To ensure accurate branch length estimation in the partitioned data analyses, the "prset ratepr = variable" command was used to accommodate among–partition rate variation (Marshall et al. 2006). Although analyses of multiple-partition data sets have noted variation in the performance of different exponential priors on the branch length mean (McGuire et al. 2007), our preliminary analyses did not detect any noticeable differences in performance of the default exponential prior on branch length mean value of 10 versus mean values of 2, 20, 50, and 100.

The stationarity of the chains and convergence of the MC3 algorithm were assessed by plotting the likelihood score and all model parameter values against the generation number to determine when there was no increase relative to the generation number using the computer program Tracer version 1.4 (Drummond and Rambaut 2007). Convergence was also assessed by monitoring the potential scale reduction factors between the independent runs and by measuring the average standard deviation of the split frequencies between those runs; when this value was less than 0.005, it was assumed that the chains had reached stationarity. Finally, convergence was evaluated using the "compare" function in the online application AWTY (Nylander et al. 2008). In the end, the first 10% of the sampled generations were discarded as burn-in and the phylogenies were summarized in 50% majority-rule consensus trees.

Recent phylogenetic analyses of darter subclades have revealed instances of mtDNA introgression that range from a few sampled individuals exhibiting heterospecific mtDNA genomes to species with complete mtDNA genome replacement by heterospecific haplotypes (Bossu and Near 2009; Heckman et al. 2009; Keck and Near 2010). In comparing the phylogenies inferred from mtDNA with those inferred from the nuclear genes, we noted a number of topological differences that are likely the result of mtDNA introgression. The frequency of mtDNA introgression was noted, and mtDNA haplotypes that were identified as resulting from introgression were scored as missing data in the Bayesian phylogenetic analyses of combined mtDNA and nuclear gene data sets.

Detection and Diagnosis of Mitochondrial Introgression

We detected mtDNA introgression in darters with a direct assessment of incongruence between mitochondrial- and nuclear-inferred gene trees, as well as expectations from morphology (e.g., Bossu and Near 2009). Three different patterns of mtDNA introgression were diagnosed. *Proximal mtDNA transfer* involves the introgression of mtDNA among extant and sympatric species (Fig. 1). This pattern is detected by reference to a phylogeny that shows the phylogenetic nesting
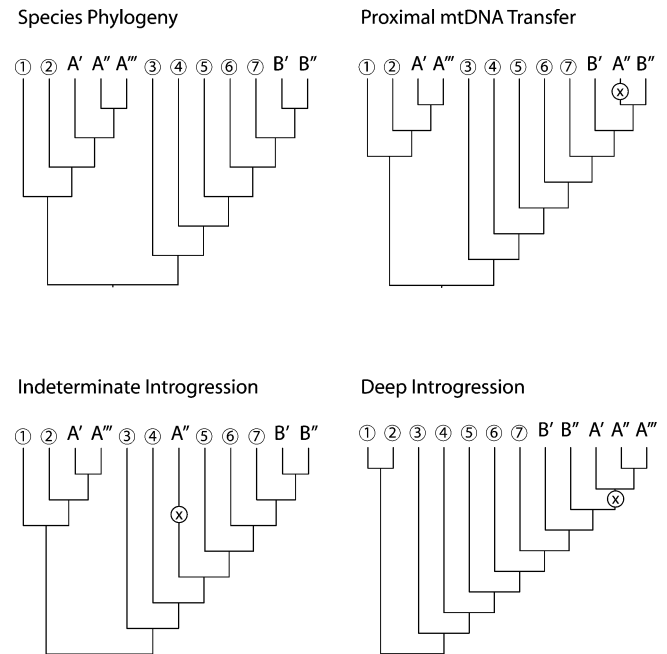

FIGURE 1. Phylogenetic trees illustrating three distinct patterns of mtDNA introgression observed in darters. The species phylogeny is contrasted with phylogenetic trees inferred from mtDNA resulting in proximal mtDNA transfer, indeterminate mtDNA introgression, and deep introgression. In the case of proximal mtDNA transfer and deep introgression, lineage B mtDNA is introgressed into lineage A. Introgression events are marked with an "x."

of haplotypes sampled from the recipient lineage in the donor lineage clade. These introgressed haplotypes may be fixed in the recipient lineage or show variation in the degree of introgression among geographic populations, and the introgressed haplotypes in the recipient lineage may be identical or very similar to those observed in the donor lineage. The second pattern of mtDNA introgression is *indeterminate mtDNA introgression*, which involves the introgression of mtDNA from a lineage that cannot be confidently identified because the introgressed haplotypes are distantly related to all other extant mtDNA lineages (Fig. 1). The third pattern of mtDNA is *deep introgression*, which is characterized by species diversification in the recipient lineage subsequent to the mtDNA transfer event (Fig. 1). The diversifying lineages contain the mtDNA genome of heterospecific origin. Therefore, the mtDNA gene will reflect the history of diversification within the clade, but will give a false signal of deeper phylogenetic affinity with the donor lineage.

Phylogenetic Classification of Darters

The classification of darters has historically been characterized by the recognition of genera and subgenera that were assumed to represent monophyletic groups of species (Page 1981, 1983; Bailey and Etnier 1988; Etnier and Starnes 1993; Boschung and Mayden 2004). Because we are able to present well-resolved and

strongly supported phylogenies based on mtDNA and nuclear genes that are, with a few exceptions, generally in agreement with one another, we developed a phylogeny-based classification of darters that follows the principles of phylogenetic nomenclature described in the *PhyloCode* (de Queiroz and Gauthier 1990, 1992, 1994; Cantino and de Queiroz 2009). We attempted to preserve the nomenclatural history of group names by retaining preexisting names for clades when they exist (e.g., Collette and Knapp 1966) and by providing new names for clades that have never had formal names applied. We based our decision to name a clade on both its diagnosability and the need to communicate the clade in comparative and taxonomic studies (e.g., Cantino et al. 2007). Thus, not all well-supported clades in the darter phylogenies were named. In addition, the clade names were given phylogenetic definitions that can only be applied in the context of a given phylogeny, and clades were identified by reference to a branch or a node in the phylogeny (de Queiroz and Gauthier 1990, 1992).

The clade names that we provide are unranked in that they do not refer to genera or subgenera. However, most of the clades that are named in our phylogenetic classification are nested within traditionally recognized genera and would therefore be available as genus-group (subgeneric) names under the ICZN according to Article 10.4 of that rank-based code (International Commission on Zoological Nomenclature 1999; Hillis and Wilcox 2005).

Divergence Time Estimation

There are considerable challenges to estimating divergence times using molecular data because of the near-ubiquitous presence of among-lineage molecular evolutionary rate heterogeneity and the need for external age calibration information (Sanderson 1998). Unfortunately, the fossil record for darters is poor and offers no information to provide minimal age constraints for nodes in molecular phylogenies (Ossian 1973; Smith 1981; Cavender 1986, 1998). Although the fossil record for non-darter percids (e.g., *Perca* and *Sander*) extends to the early Miocene, the morphological features preserved in these fossils do not permit the phylogenetic placement of the taxa relative to extant percid species (Svetovidov and Dorofeeva 1963; Cziczer et al. 2009; Murray et al. 2009). Previous analyses of molecular divergence times in darters have used external fossil calibrations from the percoid clade Centrarchidae (Near and Benard 2004; Near and Keck 2005; Carlson et al. 2009; Hollingsworth and Near 2009). Centrarchid fishes are classified in the same taxonomic order as darters (Nelson 2006) and have a rich fossil record that has been used to calibrate molecular phylogenies. There is also a substantial set of comparative molecular data sets available for this clade (Near et al. 2003, 2004; Near, Bolnick, et al. 2005).

Divergence times in darters were estimated using two calibration strategies that were derived from the centrarchid fossil calibrations. The first method used gene alignments that included all sampled darter species, 2 non-darter percid species, and 33 of the sampled species of Centrarchidae. The centrarchid fossil calibrations were used to estimate divergence times in relaxed-clock analyses of these large data matrices. The second approach used rates of molecular evolution estimated from the relaxed-clock analyses of Centrarchidae for each of the three genes. The Bayesian posterior distribution of estimated nucleotide substitution rates from the centrarchid relaxed-clock analyses was then used as a prior in relaxed-clock analyses of gene alignments that contained only the sampled darter and non-darter percid species (e.g., Saarma et al. 2007).

Divergence times were estimated using an uncorrelated lognormal (UCLN) model of molecular evolutionary rate heterogeneity implemented in the computer program BEAST v. 1.53 (Drummond et al. 2006; Drummond and Rambaut 2007). Data sets used in the MrBayes analyses were pruned to contain only one specimen per sampled species in the UCLN analyses. The optimal molecular evolutionary models were identified using AIC, partitioning schemes were assessed using Bayes factor comparisons, and the UCLN model was implemented in BEAST to estimate the posterior density of divergence times. A birth-death speciation prior was used for the branching rates in the phylogeny. Five sets of relaxed-clock analyses were performed. Divergence times were estimated using the centrarchid fossil calibrations and alignments of darters and centrarchids for each of the protein-coding *cytb* and *RAG1* genes. The centrarchid fossil calibrations were not used to estimate divergence times in darters for the *S7* intron 1 locus because substantial divergence between darters and centrarchids makes alignment of this locus problematic. The posterior densities of centrarchid molecular evolutionary rates estimated from alignments that contained only centrarchid species were used as priors in the analyses of darter alignments from each of the three genes (*cytb*, *S7*, and *RAG1*).

The calibration priors consisted of five centrarchid fossil ages that were identified as producing internally consistent age estimates using a fossil cross-validation analysis (Near, Bolnick, 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 2004b; Near, Bolnick, et al. 2005). Information on the age, taxonomic identity, and specifics of lower- and upper-bound ages used in the calibration priors are given in Near, Bolnick, 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 used in Near, Bolnick, et al. (2005).

The posterior molecular evolutionary rates estimated for each of the three genes using the centrarchid-only alignments were used as priors for the molecular

evolutionary rates in the analyses of the darter and non-darter percid alignments. The mean and standard deviation of the molecular evolutionary rate for each of the three genes were determined from the posterior distribution resulting from the fossil-calibrated UCLN analyses of the centrarchid gene alignments. The mean and standard deviation of the centrarchid molecular evolutionary rates were subsequently used to construct a normal prior on the molecular evolutionary rate in the UCLN analyses of divergence times using the darter and non-darter percid alignments.

Each of the six analyses was run three times, and each run consisted of 3.0×10^7 generations. The resulting trees and log files from each run were combined using the computer program LogCombiner v. 1.5.3 (http://beast.bio.ed.ac.uk/LogCombiner). Convergence of model parameter values and estimated node heights to their optimal posterior distributions was assessed by plotting the marginal posterior probabilities using the computer program Tracer v. 1.5 (http://beast.bio.ed.ac.uk/Tracer). The posterior probability density of the combined tree and log files was summarized using TreeAnnotator 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 on the chronograms 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 (i.e., without the DNA sequence data).

Analysis of Phylogenetic Resolution by Tree Depth

In order to determine whether genes that exhibited differing rates of nucleotide substitution provided resolved and significantly supported nodes at different time depths in the phylogeny, the posterior probability clade support values were plotted against the estimated age of the node. Only interspecific nodes in the gene trees were scored. The posterior probabilities of clade support were extracted from the MrBayes posterior tree files by adding all compatible groups to the standard 50% majority-rule consensus trees using the "allcompat" option in the MrBayes "sumt" command. Only nodes that were present in both the BEAST and the MrBayes sets of posterior trees were recorded. There was substantial congruence among the tree topologies inferred from the MrBayes and BEAST analyses; however, there were a few differences that involved the youngest clades in the darter phylogeny.

RESULTS

Model Selection, Data Partitioning, and Bayesian Phylogenetic Analyses

The optimal molecular evolutionary models selected using AIC were GTR+I+G for all partitions, except HKY+I+G was the optimal model for *RAG1* first and second codon positions combined, and *S7* intron 1 and GTR+G was the optimal model for *RAG1* first codon positions and *RAG1* second codon positions. For the two protein-coding genes (*cytb* and *RAG1*), partitioning among the three codon positions was favored with very high Bayes factor scores that were greater than 169. In the sequenced *cytb* genes, there were no sequencing ambiguities, frameshift mutations, or stop codons that would be indicative of nuclear-inserted mitochondrial pseudogenes (e.g., Bensasson et al. 2001). No anomalous mtDNA haplotypes were observed among the 158 species that were sampled with more than one individual. All data matrices and trees are available at TreeBASE (http://www.treebase.org; S11538).

Phylogenies estimated for each of the three gene regions were similar with regard to the monophyly of *Percina*, *Ammocrypta*, and a clade containing *Ammocrypta* and *Crystallaria* (Fig. 2). *Etheostoma* was not monophyletic in the *cytb* phylogeny because *E. cinereum* was in an unresolved clade with *Ammocrypta*, *Crystallaria*, and *Nothonotus*. This clade did not have significant Bayesian posterior support (Fig. 2). In the phylogenies inferred from the two nuclear genes, *E. cinereum* was nested well within *Etheostoma*, in a clade that contained the snubnose darters (subgenus *Ulocentra* or *Nanostoma*) and greenside darters (subgenus *Etheostoma s.s.*) (Table 1 and Fig. 2). Aside from *E. cinereum*, *Etheostoma* was monophyletic in the *cytb* phylogeny; however, this clade was not supported with a significant Bayesian posterior. In the *cytb* gene tree, the relationships among *Etheostoma*, *Percina*, *Nothonotus*, and the *Ammocrypta*–*Crystallaria* clade were not resolved with significantly supported Bayesian posterior probabilities (Fig. 2). In the *S7* phylogeny, *Nothonotus* was not monophyletic and was nested within *Etheostoma*; however, nodes resulting in *Nothonotus* paraphyly were not supported with significant Bayesian posterior probabilities (Fig. 2). The *RAG1* phylogeny resolved *Etheostoma* as a monophyletic group sister to *Nothonotus*, and both nodes were supported with significant Bayesian posteriors (Fig. 2). The 50% majority-rule phylogenies that summarize the Bayesian posterior trees estimated from each of the three gene regions are available as Supplementary Figures 1, 2, and 3.

In addition to *E. cinereum*, there were many other darter species that exhibited incongruence between the mitochondrial *cytb* gene tree and the phylogenies inferred from the two nuclear genes. Phylogenetic evidence suggests that all these species carry heterospecific mtDNA haplotypes as a result of either recent or ancient introgressive hybridization. Phylogenetic analyses of DNA sequences from mitochondrial and nuclear genes have previously shown evidence of mtDNA introgression in *E. fragi*, *E. uniporum*, *E. burri*, *E. podostemone*, *E. blennius*, *E. sequatchiense*, and *Nothonotus rufilineatus* (Piller et al. 2008; Bossu and Near 2009; Heckman et al. 2009; Keck and Near 2010). In a pattern similar to that reported in Williams et al. (2007), *Percina sipsi* was nested in the mitochondrial gene tree within a clade containing multiple sampled individuals of *P. sciera* (Supplementary Fig. 1). However, in our analysis, *P. sipsi* was not
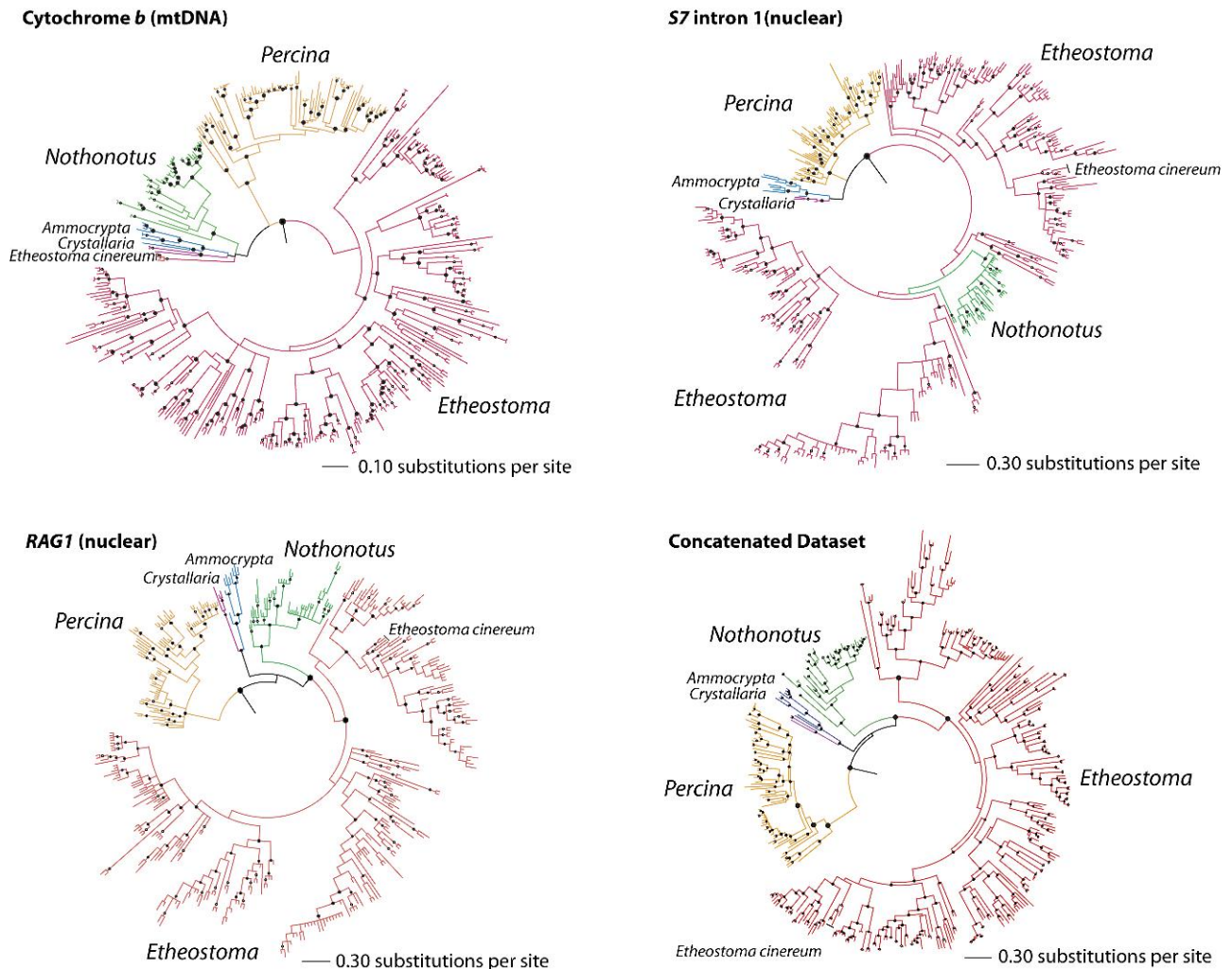
FIGURE 2. Phylogenies of darters inferred from a partitioned Bayesian analysis of the mitochondrial cytochrome *b* (*cytb*) gene, the nuclear-encoded *S7* ribosomal protein intron 1 (*S7*), a partitioned Bayesian analysis of the nuclear-encoded recombination activating gene 1 exon 3 (*RAG1*), and a concatenated data set consisting of the mitochondrial cytochrome *b* gene (*cytb*), the nuclear *S7* ribosomal protein intron 1 gene (*S7*), and the nuclear recombination activating gene 1 exon 3 (*RAG1*). Major clades are labeled and shaded by color. The position of *Etheostoma cinereum* in each phylogeny is labeled. Filled black circles mark interspecific clades supported with significant ($\geqslant 0.95$), and unfilled circles identify significantly supported intraspecfic clades.

nested within a clade of sampled *P. sciera* in the two nu-clear gene trees (Supplementary Figs. 2 and 3). Though *P. sciera* is much more widespread geographically than *P. sipsi*, the two species are sympatric in the Sipsey Fork (Lawrence and Winston counties, AL, USA), and the presence of *P. sciera*–like mtDNA haplotypes in *P. sipsi* is most likely due to mtDNA introgression.

The darter subgenera *Alvordius, Hadropterus, Eri-cosma, Belophlox, Hololepis, Ozarka, Boleichthys*, and *Oligo-cephalus*, as presented in Table 1, were not monophyletic in any of the three gene trees. *Boleosoma* was para-phyletic in the *cytb* gene tree because the monotypic subgenus *Ioa* was nested within the clade (Supplemen-tary Fig. 1). In the two nuclear gene phylogenies, *Boleo-soma* and the monotypic *Ioa* comprised monophyletic groups with a lack of resolution at the base of the clade (Supplementary Figs. 2 and 3). The subgenus *Catonotus*

was not monophyletic in the *cytb* and *S7* gene trees, as the spottail species group (e.g., *Etheostoma squamiceps*, Table 1) was not most closely related to a clade of all other *Catonotus* species (Supplementary Figs. 1 and 2). *Catonotus* was monophyletic and supported with a sig-nificant Bayesian posterior support value in the *RAG1* phylogeny (Supplementary Fig. 3).

The phylogenetic relationships of species tradition-ally classified in the subgenera *Oligocephalus, Ozarka*, and *Ulocentra/Nanostoma* were complex with respect to the relationships inferred from each of the three sam-pled genes. In the three gene trees, *Etheostoma hopkinsi* and *E. binotatum* were not closely related to any other species traditionally classified as *Oligocephalus*. On the other hand, *E. exile*, a species classified in the subgenus *Boleichthys* by Page (1981, 1983, 2000), was nested in a clade containing other *Oligocephalus* species and was

the sister species to *E. luteovinctum* in the *cytb* and *RAG1* gene trees (Supplementary Figs. 1 and 3). The phylogenetic placement of *E. exile* in each of the three gene trees was consistent with the previous phylogenetic analyses of the mtDNA-encoded *ND2* gene and the nuclear *S7* intron 1 (Lang and Mayden 2007) and was congruent with earlier taxonomic hypotheses based on external morphological characters (Bailey and Etnier 1988). Results from the Bayesian phylogenetic analyses of the two nuclear genes included a significantly supported clade that contained *E. exile* and all species of *Oligocephalus*, except *E. hopkinsi* and *E. bionotatum*. This new composition of *Oligocephalus* included all the species that comprised five distinct clades present in the *cytb* gene tree, the *E. exile–E. luteovinctum* sister species pair discussed above, the orangethroat darters (e.g., *E. spectabile, E. uniporum,* and *E. lawrencei*), the rainbow darters (e.g., *E. caeruleum, E. swaini,* and *E. asprigene*), the southwestern darter clade (e.g., *E. australe, E. lepidum,* and *E. grahami*), and the *E. whipplei* clade (e.g., *E. whipplei, E. artesiae,* and *E. radiosum*). The rainbow darters were paraphyletic in the *S7* and *RAG1* gene trees and the *E. exile–E. luteovinctum* sister species pair was not present in the *S7* gene tree.

The six species traditionally classified in the subgenus *Ozarka* were not monophyletic in any of the three gene trees (Williams and Robison 1980; Page 2000). The group was polyphyletic in the *cytb* Bayesian phylogeny; *Etheostoma trisella* was sister to a clade containing species classified in *Vaillantia* (*E. chlorosoma* and *E. davidsoni*) and *Doration* (e.g., *E. stigmaeum, E. meadiae,* and *E. akatulo*) but not supported with a significant Bayesian posterior (Supplementary Fig. 1). *Etheostoma pallididorsum, E. cragini, E. boschungi,* and *E. tuscumbia* were in a clade with the rainbow darters, the southwestern darter clade, the *E. exile–E. luteovinctum* clade, and the *E. whipplei* clade. As discussed above, the *E. punctulatum* species complex was in a clade containing the orangethroat darters and species traditionally classified in *Microperca* (Supplementary Fig. 1). Although the relationships among the *Ozarka* species were unresolved in the *RAG1* gene tree (Supplementary Fig. 3), *E. cragini, E. pallididorsum,* and the species of the *E. punctulatum* complex were resolved in a clade with significant Bayesian support in the *S7* gene tree (Supplementary Fig. 2). In the *RAG1* phylogeny, *E. trisella* was sister to a large clade of species that contained the snubnose darters, greenside darters, and *E. cinereum*. However, this relationship was not supported with a significant Bayesian posterior (Supplementary Fig. 3). In the *S7* gene tree, all the *Ozarka* species, except *E. trisella*, were in a clade with *E. tuscumbia* lacking a significant Bayesian posterior probability. The placement of *E. trisella* in the *S7* gene tree was unresolved; the species was in a polytomy along with the most recent common ancestor (MRCA) of all other sampled *Etheostoma* and *Nothonotus* species (Supplementary Fig. 2). *Psychromaster* is the more appropriate clade name for the species of *Ozarka* (sans *E. trisella*) because *E. tuscumbia* is nested in this lineage.

It appears that the polyphyly of *Oligocephalus* and *Psychromaster* is the result of at least two instances of mtDNA introgression as there are two distinct clades containing species of *Oligocephalus* and *Psychromaster* in the *cytb* gene tree, but *Oligocephalus* and *Psychromaster* are each monophyletic and distantly related in the nuclear gene trees. Based on our assessment, we identify the mtDNA in all species of *Oligocephalus*, except the orangethroat darters (e.g., *E. spectabile*), *E. punctulatum, E. autumnale,* and *E. mihileze* as containing mtDNA from a heterospecific origin.

In the *cytb* gene tree, the species of greenside darters traditionally classified in the subgenus *Etheostoma* and the snubnose darter species classified in the subgenera *Ulocentra* or *Nanostoma* formed a large monophyletic group supported with a significant posterior probability (Supplementary Fig. 1). Despite the monophyly of this group, none of the previously recognized subgenera *Etheostoma, Ulocentra,* and *Nanostoma* were monophyletic, and the paraphyly of these groups included several nodes with significant Bayesian posterior support (Supplementary Fig. 1). *Etheostoma zonale, E. lynceum,* and the other greenside darter species were nested in a clade containing most of the snubnose darter species. In addition, *E. baileyi* and *E. histrio* formed a clade that was not supported with a significant posterior.

The clade containing the snubnose and greenside darters was present and supported with a significant posterior in the *S7* gene tree; however, as discussed above, *E. cinereum* was nested in this clade and resolved as the sister lineage to one of three clades containing snubnose darter species (Supplementary Fig. 2). *Etheostoma baileyi* and *E. histrio* were nested as successive sister lineages at the base of the snubnose–greenside darter clade. The sister lineage of *E. histrio* was resolved as a polytomy that consisted of three significantly supported clades: the remaining greenside darter species, including *E. zonale* and *E. lynceum*, and two clades of all other snubnose darter species except *E. baileyi* (Supplementary Fig. 2). The phylogeny inferred from the *RAG1* locus was the most congruent with regard to the subgeneric taxonomy of greenside and snubnose darters. Greenside darters, snubnose darters, and *E. cinereum* were resolved in a clade with significant Bayesian posterior support (Supplementary Fig. 3). All the snubnose darter species and *E. baileyi* formed a monophyletic group, and all the greenside darter species, except *E. histrio*, formed a monophyletic group. Each of these two clades was supported with a significant Bayesian posterior probability.

All the specimens that were identified as containing heterospecific mtDNA haplotypes were removed from the data matrix and scored as missing data in the combined mtDNA and nuclear gene phylogenetic analyses. The resulting phylogeny contained significantly supported clades that corresponded to *Percina, Ammocrypta, Crystallaria, Nothonotus,* and *Etheostoma* (Fig. 3). There were two larger groups among these, *Ammocrypta–Crystallaria* and *Nothonotus–Etheostoma,*

FIGURE 3. Continued.

b)

● = 1.00 BPP
○ = 0.95-0.99 BPP

To Figure 3a

Etheostoma microperca
Etheostoma fonticola
Etheostoma proeliare
Etheostoma uniporum
Etheostoma fragi
Etheostoma pulchellum
Etheostoma squamosum
E. cf. *spectabile* (Sheltowee Darter)
E. cf. *spectabile* (Wildcat Darter)
E. cf. *spectabile* (Ozark Darter)
Etheostoma burri
Etheostoma spectabile
Etheostoma bison
E. cf. *spectabile* (Mamequit Darter)
Etheostoma kantuckeense
Etheostoma tecumsehi
Etheostoma kantuckeense
Etheostoma lawrencei
Etheostoma cf. *spectabile* (Ihiyo Darter)
Etheostoma lawrencei
Etheostoma artesiae
Etheostoma paludosum
Etheostoma cyanorum
Etheostoma radiosum
Etheostoma whipplei
Etheostoma whipplei
Etheostoma caeruleum
Etheostoma cf. *caeruleum*
Etheostoma swaini
Etheostoma collettei
Etheostoma swaini
Etheostoma ditrema
Etheostoma nuchale
Etheostoma swaini
Etheostoma asprigene
Etheostoma cf. *asprigene*
Etheostoma exile
Etheostoma luteovinctum
Etheostoma australe
Etheostoma lepidum
Etheostoma grahami
Etheostoma segrex

*

0.400

Etheostoma trisella
Etheostoma mariae
Etheostoma collis
Etheostoma saludae
Etheostoma parvipinne
Etheostoma phytophilum
Etheostoma barratti
Etheostoma fusiforme
Etheostoma serrifer
Etheostoma edwini
Etheostoma okaloosae
Etheostoma gracile
Etheostoma zonifer
Etheostoma binotatum
Etheostoma fricksium
Etheostoma hopkinsi
Etheostoma nianguae
Etheostoma sagitta
Etheostoma spilotum
Etheostoma kanawhae
Etheostoma osburni
Etheostoma tetrazonum
Etheostoma variatum
Etheostoma erythrozonum
Etheostoma erizonum
Etheostoma euzonum
Etheostoma chlorosoma
Etheostoma davisoni
Etheostoma akatulo
Etheostoma meadiae
Etheostoma stigmaeum
E. cf. *stigmaeum* (Highland Darter)
E. cf. *stigmaeum* (Bluegrass Darter)
Etheostoma jessiae
E. cf. *stigmaeum* (Clown Darter)
E. cf. *stigmaeum* (Longhunt Darter)

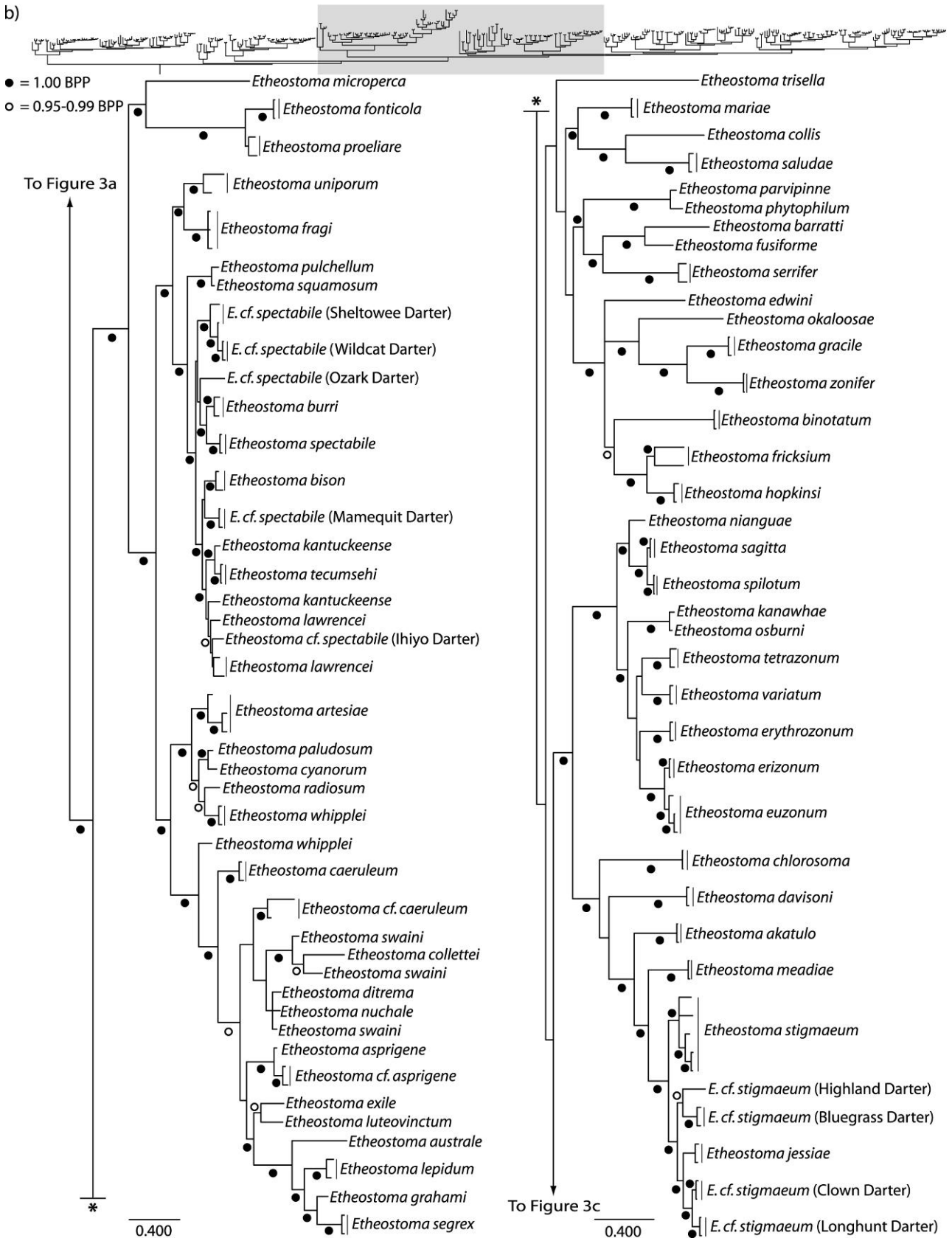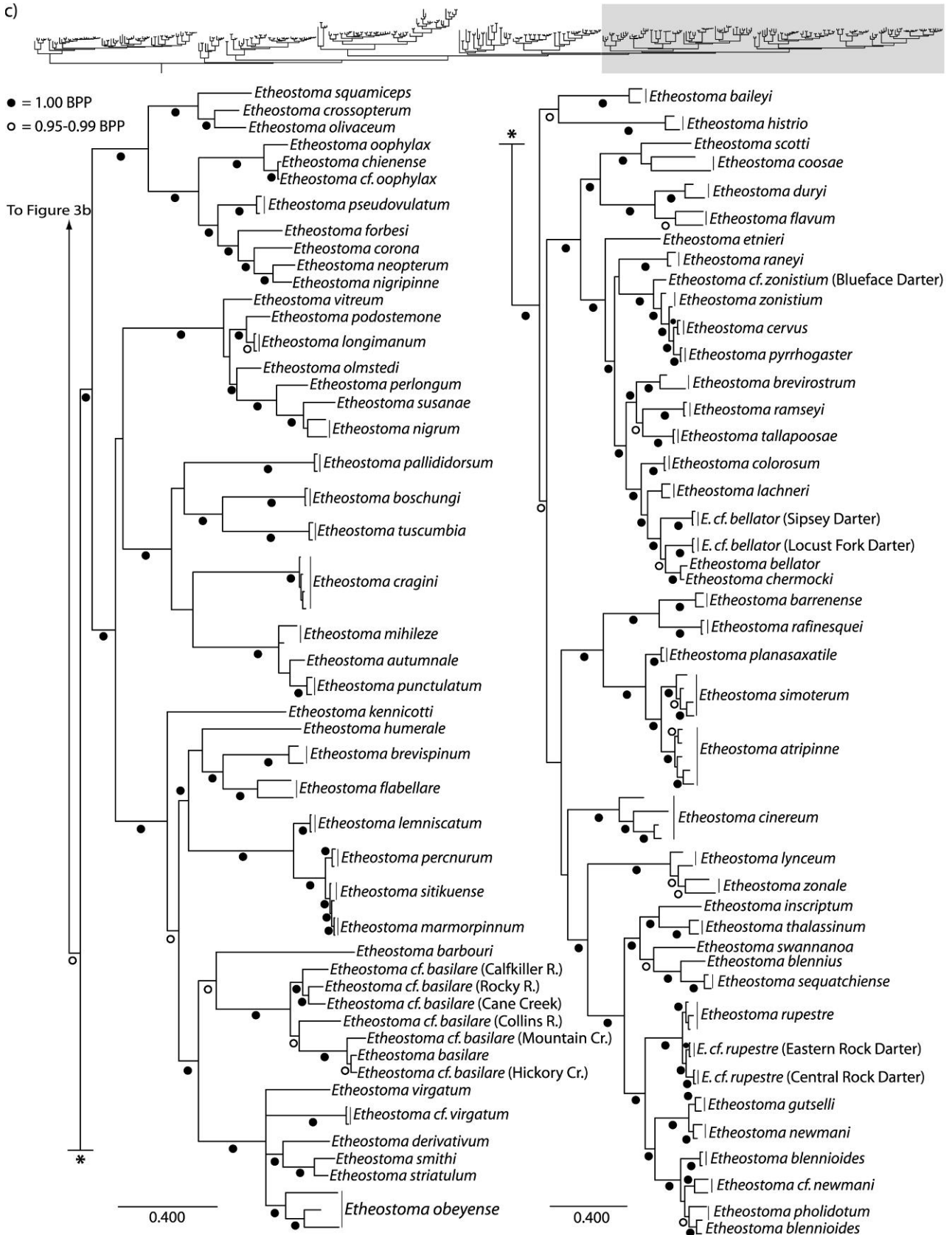To Figure 3c

0.400

FIGURE 3.   Continued.

c)

FIGURE 3.   Continued.

both of which were significantly supported as monophyletic. *Ammocrypta*, *Crystallaria*, *Nothonotus*, and *Etheostoma* were resolved as a clade that was sister to *Percina*, but this node was not supported with a significant Bayesian posterior (Fig. 3a).

The combined mtDNA and nuclear gene phylogeny was similar to the mtDNA *cytb* gene, particularly with respect to the paraphyly of *Catonotus* and the snubnose darters (Fig. 3a). Removal of mtDNA sequences with a heterospecific origin from the alignment resulted in the monophyly of both *Oligocephalus* and *Psychromaster* (Fig. 3b, c). *Etheostoma baileyi* and *E. histrio* formed a significantly supported clade that was sister to a clade containing *E. cinereum*, all other greenside darters, and all other snubnose darter species (Fig. 3c). *Etheostoma zonale* and *E. lynceum* were resolved with significant Bayesian posterior support as the sister lineage of a clade that contained all greenside darter species (sans *E. histrio*).

Phylogenetic Classification of Darters

A composite phylogeny based primarily on the analyses of the concatenated data sets was used to phylogenetically define 45 clade names for darters (Fig. 4 and Appendix). The proposed phylogenetic classification in Figure 4 represents the most current knowledge of the evolutionary relationships among darter species while also preserving previously used names when appropriate. Most of the names in the phylogenetic classification are converted clade names from ranked genus and subgenus names, and four of these converted clade names—*Austroperca*, *Astatichthys*, *Pileoma*, and *Richiella*—were treated as invalid synonyms in the most recent darter classifications. A total of 16 new clade names are proposed in the phylogenetic definitions (Fig. 4 and Appendix). Some of the well-supported nodes in the darter phylogeny are not named primarily as a result of incongruence between the mtDNA and nuclear gene trees (e.g., relationships within *Hadropterus*) or our desire to avoid the possibility of having to redefine clade names subsequent to future phylogenetic analyses.

We recommend that *Ammocrypta*, *Crystallaria*, *Etheostoma*, *Nothonotus*, and *Percina* continue to be used as the primary clade names with species epithets. The other clade names highlight phylogenetically related groups of species and will find validity in discussions of character diversity, biogeography, and ecological differences. In addition, the phylogenetic classification and the clade names will provide a comparative basis with which to investigate the ecology and evolutionary biology of darters in a way that was not possible with

the previous darter classifications because the classifications did not consistently delimit monophyletic groups of species.

Divergence Time Estimates

The three Centrarchidae BEAST UCLN analyses that were run for each of the three genes resulted in very similar mean posterior divergence time estimates with broadly overlapping credible intervals, 95% HPD (HPD), for the MRCA of Centrarchidae and the MRCA of the subclades Centrarchinae, *Lepomis*, and *Micropterus* (Table 2). These posterior estimates of Centrarchidae divergence times were similar to previous estimates using different gene data sets and relaxed-clock methods (Near et al. 2003; Near, Bolnick, et al. 2005). There was significant among-lineage nucleotide substitution rate heterogeneity in all three genes, as measured by the coefficient of variation (*cytb* $\sigma = 0.362$, 95% HPD: [0.227, 0.506], *S7* intron 1 $\sigma = 0.269$, 95% HPD: [0.083, 0.467], *RAG1* $\sigma = 0.245$, 95% HPD: [5.1×10^{-3}, 0.511]). The BEAST UCLN runs on the empty alignments resulted in much older age estimates for Centrarchidae, indicating that the calibration priors used in the UCLN model did not have a strong effect on the posterior molecular age estimates.

The posterior estimate of the rate of molecular evolution for each of the three genes, as reported in the "meanRate" statistic in BEAST, differed substantially among genes, with the mtDNA-encoded *cytb* gene exhibiting the highest rate. The estimated pairwise rate of nucleotide substitution for the *cytb* gene was 1.80% per myr, a result similar to the 2.00% per myr often used as an external rate of mtDNA evolution in divergence time studies of vertebrates (e.g., Brown et al. 1979; Lovette 2004). The posterior estimated rate was approximately four times higher for the *S7* intron 1 than for the protein-coding *RAG1* gene (Table 2). The estimated pairwise rates for the nuclear genes were 0.34% per myr for the *S7* intron 1 and 0.09% per myr for the protein-coding *RAG1* gene.

For each of the *cytb* and *RAG1* gene alignments, two sets of BEAST UCLN analyses were run, each with a different calibration strategy. Analyses for both genes under both calibration strategies produced similar posterior age estimates for all the major darter clades (Table 3). The mean posterior age estimates for the MRCA of all darters, the Etheostomatinae, ranged between 30.7 and 38.4 Ma; however, the 95% HPD for each of the five estimates exhibited broad overlap (Table 3). Among the five major darter clades (Table 3), *Etheostoma* exhibited the oldest posterior age estimates, ranging

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FIGURE 3. Phylogeny of darters inferred from a partitioned Bayesian analysis of a combined data set consisting of the mitochondrial cytochrome *b* gene (*cytb*), the nuclear *S7* ribosomal protein intron 1 gene (*S7*), and the nuclear recombination activating gene 1 exon 3 (*RAG1*). The sequences for cytochrome *b* for species with heterospecific mitochondrial genomes were removed prior to the analysis. Filled black circles identify clades supported with a Bayesian posterior of 1.00, and unfilled circles identify clades with Bayesian posterior support that ranges between 0.95 and 0.99. The shaded portion of the phylogeny at the top of the figure indicates placement of clades in the darter phylogeny. Asterisks connect disconnected branches in the phylogeny. The phylogeny is presented in three parts, labeled (a), (b), and (c).

1. *Pileoma*
2. *Etnierperca*
3. *Imostoma*
4. *Cottogaster*
5. *Hadropterus*
6. *Chalinoperca*
7. *Alvordius*
8. *Swainia*
9. *Pagella*
10. *Etheostoma**
11. *Vexillapinna*
12. *Astatichthys*
13. *Austroperca*
14. *Gemmaperca*
15. *Poecilichthys*
16. *Maydenichthys*
17. *Vaillantia*
18. *Ulocentra*
19. *Neoetheostoma*
20. *Goneaperca*
21. *Catonotus*
22. *Oopareia*
23. *Psychromaster*
24. *Boleosoma*

Atlantis: roanoka, crassa, nevisense, peltata, evides, cf. evides, aurantiaca

1: austroperca, bimaculata, burtoni, cf. caprodes, caprodes, carbonaria, fulvitaenia, jenkinsi, kathae, macrolepida, rex, suttkusi

2: 3: antesella, shumardi, tanasi, uranidea, vigil; 4: aurora, brevicauda, copelandi

Percina*

5: apristis, aurolineata, crypta, lenticula, nigrofasciata, palmaris, cf. palmaris, sciera

6: kusha, cf. kusha, smithvanizi, sipsi, westfalli

7: gymnocephala, maculata, notogramma, pantherina

8: nasuta, cf. nasuta, oxyrhynchus, phoxocephala, squamata

9: macrocephala, williamsi, cymatotaenia, stictogaster

Odontopholis: beanii, cf. beanii, bifascia, clara, meridiana, pellucida, vivax

Ammocrypta*

Carnipellucida: asprella, cincotta

Crystallaria*

Nothonotus*: acuticeps, aquali, bellus, camurus, chlorobranchius, chuckwachatte, dennoncourti, douglasi, etowahae, jordani, juliae, maculatus, microlepidus, moorei, rubrus, rufilineatus, sanguifluus, cf. sanguifluus, tippecanoe, vulneratus, wapiti

Ceasia: bison, burri, fragi, kantuckeense, lawrencei, pulchellum, spectabile, squamosum, tecumsehi, uniporum, cf. spectabile A, cf. spectabile B, cf. spectabile C, cf. spectabile D, cf. spectabile E

Oligocephalus

11: aretesiae, cyanorum, paludosum, radiosum, whipplei

12: asprigene, cf. asprigene, caeruleum, cf. caeruleum, collettei, ditrema, nuchale, swaini

13: australe, grahami, lepidum, segrex

Boleichthys: exile, luteovinctum, fonticola, microperca, proeliare, collis

Microperca

10: saludae, mariae, fricksium, hopkinsi, binotatum, okaloosae, gracile, zonifer, edwini, trisella, barratti, fusiforme, serrifer, parvipinne, phytophilum

Hololepis

Fuscatelum

Litocara: nianguae, sagitta, spilotum

Iriperca: erizonum, erythrozonum, euzonum, kanawahe

15: osburni, tetrazonum, variatum

14: 17: chlorosoma, davisoni, akatulo, jessiae, meadiae, stigmaeum

16: cf. stigmaeum A, cf. stigmaeum B, cf. stigmaeum C, cf. stigmaeum D

Doration

Simoperca

18: baileyi, histrio, atripinne, barrenense, planasaxatilie, rafinesquei, simoterum, cinereum

19: blennioides, blennius, gutselli, inscriptum, lynceum, newmanii, cf. newmanii, pholidotum, rupestre, cf. rupestre A, cf. rupestre B, sequatchiense, swannanoa, thalassinum, zonale

Adonia: bellator, cf. bellator A, cf. bellator B, brevirostrum, cervus, chermocki, colorosum, coosae, duryi, etnieri, flavum, lachneri, pyrrhogaster, ramseyi, raneyi, scotti, tallapoosae, zonistium, cf. zonistium

Richiella: brevispinum, flabellare, humerale, kennicotti, lemniscatum, marmorpinnum, percnurum, sitikuense

21: barbouri, basilare, cf. basilare A, cf. basilare B, cf. basilare C, cf. basilare D, cf. basilare E, cf. basilare F

22: derivativum, obeyense, smithi, striatulum, virgatum, cf. virgatum

23: autumnale, boschungi, craigini, mihileze, pallididorsum, punctulatum, tuscumbia

24: longimanum, nigrum, olmstedi, perlongum, podostemone, susanae, vitreum

20: chienense, corona, crossopterum, forbesi, neopterum, nigripinne, olivaceum, oophylax, cf. oophylax, pseudovulatum, squamiceps
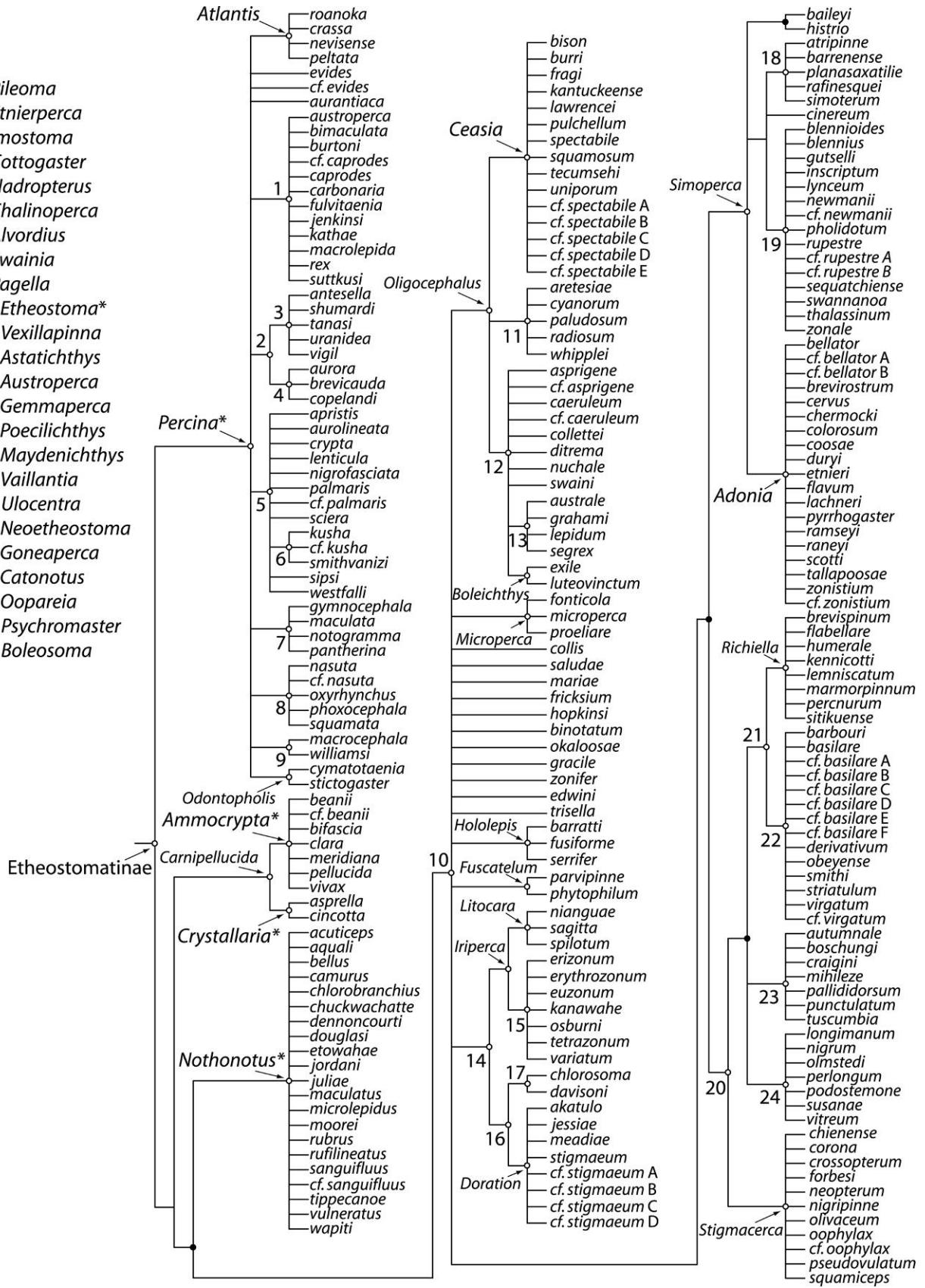
Stigmacerca

Etheostomatinae

TABLE 2.   Bayesian posterior age and molecular evolutionary rate estimates for Centrarchidae using three different genes

Gene	Centrarchidae MRCA [95% CI[b]] Ma	Centrarchinae MRCA [95% CI[b]] Ma	*Lepomis* MRCA [95% CI[b]] Ma	*Micropterus* MRCA [95% CI[b]] Ma	Estimated Rate[a] [95% CI[b]]
cytb	35.4 [27.1, 44.2]	27.5 [21.7, 34.1]	23.0 [16.5, 30.1]	8.9 [5.9, 12.3]	$8.99 \times 10^{-3} [7.46 \times 10^{-3}, 1.06 \times 10^{-2}]$
S7	39.2 [29.2, 50.3]	32.7 [23.7, 42.6]	21.1 [14.8, 28.0]	6.2 [3.4, 9.4]	$1.69 \times 10^{-3} [1.34 \times 10^{-3}, 2.08 \times 10^{-3}]$
RAG1	41.9 [29.3, 56.2]	30.9 [21.8, 41.3]	17.3 [11.1, 24.3]	6.2 [2.7, 10.2]	$4.37 \times 10^{-4} [3.24 \times 10^{-4}, 5.54 \times 10^{-4}]$

Notes: [a]Substitutions per site per million years.
[b]95% credibility interval.

between 26.1 and 33.5 Ma. The posterior mean age estimates for the younger clades, *Percina* and *Nothonotus*, were similar and ranged between approximately 18 and 21 Ma (Table 3). The topologies of the darter chronograms generated from the BEAST UCLN analyses of each of the three genes were similar to the consensus phylogenies resulting from the MrBayes analyses and did not differ with regard to major features identified in our discussions of darter phylogeny (Figs. 2 and 3). The 50% majority-rule consensus trees from each of the UCLN BEAST analyses are available in the Supplementary Materials.

Analysis of Phylogenetic Resolution by Tree Depth

The degree of phylogenetic resolution, as measured by the number of interspecific nodes with significant Bayesian posterior support, differed among the three gene trees. The Bayesian phylogeny inferred from the mitochondrial *cytb* gene contained 162 nodes with significant Bayesian posterior support (Fig. 2). The nuclear gene trees had fewer significantly supported nodes with 111 in the *S7* gene tree and 115 in the *RAG1* phylogeny (Fig. 2). The phylogeny resulting from the Bayesian analysis of the concatenated data set contained 210 nodes with significant posterior support (Fig. 3).

Plotting the clade posterior probability against the age estimate for each node in each of the three gene trees shows that *cytb* has a greater fraction of significantly supported nodes in the 0- to 10-Ma interval than observed in either of the two nuclear gene trees (Fig. 5). The plot for *cytb* illustrated the presence of poorly supported nodes in all three of the time intervals, but no nodes supported with a posterior probability less than 0.33 in any interval. In contrast, the plots for the *S7* and *RAG1* gene trees reflected an increase in proportion of nodes with low posterior support from old to younger nodes in the gene trees (Fig. 5); however, the *S7* and *RAG1* gene trees both show a high density of well-supported, relatively shallow nodes, so even if fewer of the deeper nodes are poorly supported, their capacity to resolve relatively young nodes was substan-

tial. The most poorly supported nodes in the *S7* and *RAG1* gene tree were among the youngest, whereas all the nodes older than 20 Ma were supported with a posterior probability greater than 0.75. The *RAG1* gene tree had a higher proportion of nodes supported in the intermediate (10–20 Ma) and oldest (20 Ma to root) age intervals than either the mitochondrial *cytb* or the nuclear *S7* gene trees (Fig. 5).

DISCUSSION

The high species diversity, ecological variation, and morphological disparity among major clades and patterns of allopatry among most groups of closely related species make darters an interesting system in which to investigate mechanisms of lineage diversification at a continental geographic scale (e.g., Page and Schemske 1978; Near and Benard 2004; Carlson et al. 2009). Despite the previous absence of large-scale darter phylogenies, evolutionary investigations of darters have provided insight into the patterns and tempo of allopatric speciation (Wiley and Mayden 1985; Mayden 1988; Near and Benard 2004), the geographic scale of allopatric diversification (Hollingsworth and Near 2009), the relative rates of prezygotic and postzygotic reproductive isolation (Mendelson 2003), the extent of mtDNA introgression among closely related vertebrate species (Ray et al. 2008; Bossu and Near 2009; Keck and Near 2010), and the relationship between functional morphological disparity and species co-occurrence (Carlson et al. 2009). All these studies used phylogenetic trees for their comparative analyses, but the taxonomic sampling of these phylogenies was small, targeting only select subsets of the entire species diversity of darters. Our phylogenetic data set for darters is the largest, near-complete taxonomic sample at the species level for any such species-rich animal clade of which we are aware. The phylogenetic trees resulting from our analyses of mtDNA and nuclear genes are well resolved and fairly consistent among the individual genes. The global phylogenetic hypotheses for darters presented here will serve as a basis for comparative analyses

←

FIGURE 4.   Phylogenetic classification of darters communicated using a composite phylogeny of darters based on the phylogenies inferred from mitochondrial and nuclear gene DNA sequences. The 45 darter clade names that are defined phylogenetically (see Appendix) are listed and marked with an unfilled circle. Filled circles mark clades that were well supported in the phylogenetic analyses but not named in this study. Clade names commonly associated with the rank of genus are marked with an asterisk.

TABLE 3. Bayesian posterior age and molecular evolutionary rate estimates for darters using three different genes and two different sources of external age information: fossil calibration priors from Centrarchidae and external posterior rates of nucleotide evolution estimated from the BEAST analyses of Centrarchidae

Gene calibration	Etheostomatinae MRCA [95% CI[a]] Ma	Carnipellucida MRCA [95% CI[a]] Ma	Ammocrypta MRCA [95% CI[a]] Ma	Etheostoma MRCA [95% CI[a]] Ma	Nothonotus MRCA [95% CI[a]] Ma	Percina MRCA [95% CI[a]] Ma
cytb–Fossil[b]	30.7 [25.2, 36.5]	20.4 [15.6, 25.4]	14.0 [10.4, 17.8]	26.1 [21.3, 30.8]	17.6 [13.1, 22.4]	18.6 [14.5, 22.9]
cytb–Rate[c]	32.7 [25.4, 41.0]	21.5 [15.8, 27.9]	14.6 [10.3, 19.1]	28.7 [22.2, 35.8]	17.7 [12.7, 23.1]	19.4 [14.4, 24 .9]
RAG1–Fossil[b]	38.4 [26.5, 51.7]	24.0 [14.1, 34.8]	13.6 [7.0, 21.1]	30.2 [21.2, 40.5]	21.7 [12.6, 31.7]	20.9 [13.1, 29.6]
RAG1–Rate[c]	34.8 [23.1, 48.1]	21.9 [12.7, 32.2]	12.4 [6.6, 19.3]	27.1 [18.3, 37.2]	20.1 [11.4, 29.6]	18.9 [11.8, 27.4]
S7-Rate[c]	37.6 [26.9, 49.4]	21.1 [11.8, 31.3]	11.7 [6.6, 17.3]	33.5 [24.5, 44.0]	NA	17.8 [11.8, 24.7]

Notes: [a]95% credible interval.
[b]Calibrated with centrarchid fossils.
[c]Calibrated with molecular evolutionary rates estimated from fossil-calibrated centrarchid gene trees.

investigating patterns and mechanisms of lineage diversification and other types of large-scale comparative analysis, as well as provide an encouraging prospectus for the development of densely sampled species-level phylogenies inferred from protein-coding nuclear genes for vertebrate clades with a Cenozoic origin.

Sampling Strategies and Phylogenetic Resolution of a Species-Rich Clade

We were able to reconstruct the phylogenetic relationships of nearly all extant darter species, while avoiding inference pathologies stemming from incomplete taxon sampling (e.g., Heath et al. 2008). Complete taxon sampling, however, does not resolve issues relating to character sampling; more characters are generally required to resolve phylogenies that contain a moderate to high number of terminal taxa (e.g., Bremer et al. 1999).

Because the darter radiation was expected to contain a mixture of lineages with a fairly recent time since common ancestry and lineages with a much older common ancestor, we aimed to assess if a modest data set of mitochondrial- and nuclear-encoded gene sequences could result in well-resolved and congruent phylogenetic hypotheses for the clade. The analyses of the two sampled nuclear genes result in an appreciable degree of phylogenetic resolution among closely related species. However, our analyses do substantiate commonly held conceptions in molecular systematics that faster evolving genes provide phylogenetic resolution and clade support for younger nodes in the tree, whereas slower evolving genes are able to provide support for deeper nodes in the phylogeny (Fig. 5).

Phylogenies inferred using Bayesian inference from each gene resulted in a large number of significantly supported interspecific nodes, but not all genes
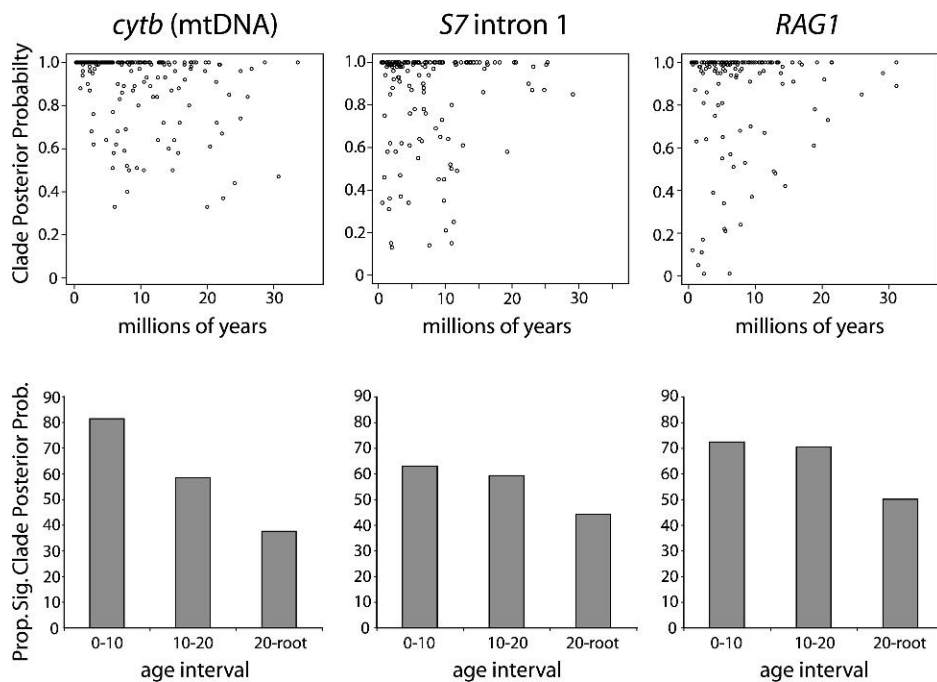

FIGURE 5. Plots showing the relationship between clade posterior probability and the estimated clade age and proportion of significantly supported clades among three age intervals for each of the three sampled genes, cytochrome b (cytb), S7 intron 1 (S7), and recombination activating gene 1 exon 3 (RAG1).

contributed equally to our understanding of the darter phylogeny (Fig. 2). Despite resolving a large fraction of the nodes in the phylogeny, the *S7* intron 1 gene tree contained relationships that were not congruent with those in the gene trees estimated from *cytb* and *RAG1*. The incongruence of the *S7* gene tree is illustrated by the incongruence among phylogenetic relationships of *Etheostoma* and *Nothonotus* that subtend some of the oldest nodes in the darter phylogeny (Fig. 2). Relative to *RAG1*, the *S7* gene tree had fewer nodes with significant posterior support in the 10- to 20-Ma age and the 20-Ma root node age intervals (Fig. 5). The proportion of 10–20 Ma age nodes supported with significant posteriors in the *S7* gene tree was similar to that observed in the mitochondrial *cytb* gene tree. In contrast, 10% more nodes in this age interval were supported in the *RAG1* gene tree. In addition, we had difficulty aligning the darter *S7* intron DNA sequences with those of non-percid teleosts (e.g., Centrarchidae), limiting our ability to include the external age information necessary to time-calibrate the *S7* gene tree.

mtDNA Introgression and Darter Phylogenetics

Our phylogenetic analyses of *cytb* and the two sampled nuclear genes indicate that 31 species, comprising more than 12% of all darter species (Tables 1 and 4), exhibit some degree of mitochondrial introgression, which ranges from complete replacement to an appreciable frequency of sampled individuals with heterospecific mtDNA genomes. As in the previous investigations of *Boleosoma* and *Nothonotus*, we found no introgression of the two examined autosomal (nuclear) loci in any of the species exhibiting mtDNA introgression (Heckman et al. 2009; Keck and Near 2010). Three different patterns of mtDNA transfer were observed in our phylogenetic trees. Among the 13 detected mtDNA introgression events in darters, 8 involved *proximal mtDNA transfer* (Table 4). This pattern is detected by reference to a phylogeny that shows the phylogenetic nesting of haplotypes sampled from the recipient lineage in the donor lineage clade. These introgressed haplotypes may be fixed in the recipient lineage or show variation in the degree of introgression among geographic populations, and the introgressed haplotypes in the recipient lineage may be identical or very similar to those observed in the donor lineage (Fig. 1).

There were three instances of *indeterminate mtDNA introgression* (Fig. 1 and Table 4), but the pattern is perhaps best exemplified by *E. fragi*, which is nested in the clade *Ceasia* based on the analyses of six nuclear genes and morphological characters (Ceas and Page 1997; Bossu and Near 2009). In the *cytb* gene tree, *E. fragi* is distantly related to *Ceasia* (Supplementary Fig. 1). The mtDNA haplotypes observed in *E. fragi* have an obscure phylogenetic placement among other lineages of *Etheostoma*, and the age of the node subtending this mtDNA lineage is much older than the MRCA of *Ceasia*. In a previous study, we had labeled the set of mtDNA

TABLE 4. Darter species identified as containing mitochondrial genomes of heterospecific origin and the observed phylogenetic introgression pattern

Species	Phylogenetic introgression pattern
Etheostoma artesiae	Proximal mtDNA transfer
Etheostoma burri	Proximal mtDNA transfer
Etheostoma olmstedi	Proximal mtDNA transfer
Etheostoma spectabile	Proximal mtDNA transfer
Etheostoma uniporum	Proximal mtDNA transfer
Nothonotus camurus	Proximal mtDNA transfer
Nothonotus rufilineatus	Proximal mtDNA transfer
Percina sipsi	Proximal mtDNA transfer
Etheostoma cinereum	Indeterminate introgression
Etheostoma fragi	Indeterminate introgression
Etheostoma podostemone	Indeterminate introgression
Etheostoma asprigene	Deep introgression
Etheostoma blennius	Deep introgression
Etheostoma caeruleum	Deep introgression
Etheostoma cf. *caeruleum*	Deep introgression
Etheostoma collettei	Deep introgression
Etheostoma cyanorum	Deep introgression
Etheostoma ditrema	Deep introgression
Etheostoma exile	Deep introgression
Etheostoma grahami	Deep introgression
Etheostoma lepidum	Deep introgression
Etheostoma luteovinctum	Deep introgression
Etheostoma nuchale	Deep introgression
Etheostoma paludosum	Deep introgression
Etheostoma punctulatum	Deep introgression
Etheostoma autumnale	Deep introgression
Etheostoma mihileze	Deep introgression
Etheostoma radiosum	Deep introgression
Etheostoma sequatchiense	Deep introgression
Etheostoma swaini	Deep introgression
Etheostoma whipplei	Deep introgression

haplotypes observed in *E. fragi* a mitochondrial fossil, meaning that the mtDNA donor lineage may have become extinct subsequent to transfer of the mtDNA into *E. fragi* (Bossu and Near 2009).

The other two species with heterospecific mtDNA labeled as examples of indeterminate mtDNA introgression may also be considered to harbor mitochondrial fossils (Table 4). For example, the phylogenetic relationships of *E. cinereum* have long intrigued darter systematists (Bailey and Gosline 1955; Page and Whitt 1973b; Page 1977; Bailey and Etnier 1988; Dimmick and Page 1992), and the phylogenetic analyses of mtDNA sequences have resulted in the placement of this species distantly related to other *Etheostoma* lineages and more closely related to *Carnipellucida*, *Percina*, or *Nothonotus* (Song et al. 1998; Sloss et al. 2004; Mayden et al. 2006; Lang and Mayden 2007). Our analyses of the two sampled nuclear genes place *E. cinereum* in the *Etheostoma* subclade *Simoperca*, a result consistent with the previous analyses of nuclear gene DNA sequences and allozymes (Wood and Mayden 1997; Lang and Mayden 2007; Bossu and Near 2009).

There were three instances of *deep introgression* observed in the darter phylogeny, two of which involved the clades *Oligocephalus* and *Psychromaster* (Fig. 1 and Table 4). When comparing the phylogeny of these lineages inferred from the two nuclear genes with the *cytb* gene tree, two separate introgression events

were detected. There was an mtDNA transfer event from a lineage related to the extant species of *Psychromaster* into the common ancestral lineage of *Vexillapinna*, *Boleichthys*, *Austroperca*, and *Astatichthys*. A second deep introgression event involved the transfer of mtDNA originating from an *Oligocephalus* lineage into the common ancestor of *E. punctulatum*, *E. autumnale*, and *E. mihileze*. In both cases, there has been species diversification in these lineages subsequent to the transfer and fixation of the heterospecific mtDNA; however, the phylogenetic affinities of these lineages in the mtDNA gene tree are dramatically obscured by the deep introgression.

Phylogeny and Classification of Darters

The phylogenetic resolution given by the three genes sampled in this study provided an excellent opportunity to revise the classification of darters in a phylogenetic context. The classification presented in Figure 4 and the Appendix represents the first attempt to provide a comprehensive taxonomy for darters that is based on the results of explicit phylogenetic analysis of comparative data. All previous delimitations of ranked taxonomic groups were based on morphological diagnoses that did not attempt to assess monophyly of the proposed groups (e.g., Bailey and Gosline 1955; Page 1974; 1981). The rich history of darter taxonomy is reflected in the scores of group names that have been introduced over the past two centuries (Collette and Knapp 1966; Collette 1967). Previous attempts to codify darter taxonomy have delimited groups of darter species using the available group names as ranked genera and subgenera (e.g., Jordan and Evermann 1896, p. 1015–1105; Bailey et al. 1954; Page 1974, 1981, 2000; Bailey and Etnier 1988).

The phylogenetic classification presented in Figure 4 and the Appendix communicates our best understanding of darter relationships and includes major alterations to the previous non-phylogenetic taxonomies for the clade. For example, there were 19 or 21 polytypic subgenera present in the most recently proposed darter classifications (Bailey and Etnier 1988; Page 2000). Our phylogenetic analyses indicate that 10 of these subgenera are not monophyletic (Table 1). Substantial non-monophyly of darter subgenera was also intimated in the previously published morphological and molecular phylogenetic analyses (e.g., Near 2002; Mayden et al. 2006; Ayache and Near 2009), but the extent of the failure of these taxonomies to reflect monophyletic lineages was not apparent until comparison with the comprehensive phylogenetic analyses presented in this investigation.

A previous phylogenetic analysis of darters using *cytb* gene sequences was reluctant to recognize the darter clade *Nothonotus* as distinct from *Etheostoma* (Mayden et al. 2006), as was proposed in a previous study (Near and Keck 2005), on the grounds that all darter lineages exclusive of *Percina* could form a clade named *Etheostoma*. Our phylogenetic analyses resolve *Percina* as the sister lineage of all other darters but consistently result in trees that place *Nothonotus* outside of a monophyletic *Etheostoma* and distinct from *Carnipellucida* (Figs. 2 and 3). Although our phylogenies are consistent with the clade-naming scenario presented in Mayden et al. (2006), we believe that the scenario has limited utility because it fails to communicate the wealth of knowledge now available about the phylogenetic relationships among *Carnipellucida*, *Nothonotus*, and *Etheostoma* (Fig. 4).

Reliance on a ranked classification of darters that emphasizes subgenera without assessing the monophyly of these groups has long been an impediment to comparative studies of darters. For example, Bart and Page (1992) analyzed a rich database of information on the size at maturity, fecundity, and egg size in 64 darter species to examine the effects of body size and phylogeny on variation in life history traits (e.g., Stearns 1984; Dunham and Miles 1985). Species data were pooled into clustered groups of genera, groups of subgenera, and subgenera for the analysis of covariance. However, 8 of the 15 polytypic subgenera sampled in the study of Bart and Page (1992) are not monophyletic in our phylogenetic analyses. Thus, the variance in life history variables explained by the taxonomic nesting in Bart and Page (1992) was calculated incorrectly, and as a result, the data need reevaluation in the context of our revised understanding of darter phylogeny.

Estimation of Divergence Times Without a Fossil Record: The Temporal Diversification of Darters

The primary challenges in estimating divergence times using molecular data are 1) the application of an appropriate model that accounts for molecular evolutionary rate heterogeneity across lineages and 2) accurate external information on absolute ages to serve as calibrations. The past decade has witnessed the development of several powerful strategies to correct for molecular evolutionary rate heterogeneity (e.g., Sanderson 1997, 2002; Thorne et al. 1998; Drummond et al. 2006), and, more recently, a thorough consideration of calibration methods has emerged (Near, Meylan, et al. 2005; Ho 2007; Ho and Phillips 2009).

Using external calibrations to estimate divergence times for clades lacking a fossil record can be complicated by the presence of substantial differences in molecular evolutionary rates; however, our analyses did not appear to be confounded by appreciable rate heterogeneity between darters and Centrarchidae. The posterior mean rate estimate for *cytb* and *RAG1* did not change substantially between BEAST runs that contained only centrarchid species (Table 2) and the runs that included all sampled centrarchid and darter species.

Our confidence in the posterior molecular age estimates for darters is buoyed by the consistency of the age estimates resulting from the analyses of different

genes under different calibration strategies (Table 3). Previous analyses have noted that mtDNA genes can overestimate molecular ages relative to those estimated from nuclear genes (Jansa et al. 2006; Brandley et al. 2011). This disparity in age estimates may be the result of saturated substitutions in mtDNA genes that leads to a severe over- or underestimation of the underlying rate of molecular evolution (Phillips 2009). Despite the likelihood that the *cytb* gene sampled for this study exhibits some degree of saturation at the deepest phylogenetic contrasts, there was no apparent trend in our results to indicate that the mtDNA *cytb* gene consistently resulted in age estimates that were older than ages estimated from the two nuclear genes (Table 3).

Comparison of posterior node ages estimated using external centrarchid fossil calibrations and those estimated using external rate calibrations reveals slight differences in the posterior age estimates between the two strategies. Although there were no significant differences in the posterior node heights estimated by the two calibration strategies, fossil-calibrated ages were on average younger for the *cytb* chronogram and older for the *RAG1* chronogram relative to those estimated using external rate calibrations (Table 3). This slight difference in node ages between the fossil and rate calibration analyses may be due to a difference in the width of the prior distribution of the rate calibrations, which are modeled on the posterior distributions of rate estimates from the centrarchid-only BEAST analyses (Table 2). The prior distribution of the mean rate of the *cytb* gene was much wider than that of the *RAG1* gene. In the rate calibration analysis, the sampling of rates within this prior distribution may be limited relative to the sampling of rates in the fossil-calibrated analysis for *RAG1*. For the *cytb* rate calibration analysis, the converse may be true: a wide posterior on rates implies a wider sampling of rate variation relative to the rates sampled in the fossil calibration analysis. The assessment of posterior ages resulting from fossil-based versus rate calibration should therefore ideally consider the density of fossil calibration and the disparity in the credible intervals of the individual genes in the analysis. However, our analyses of darter divergence times using external centrarchid calibrations demonstrate promising consistency in posterior ages estimated using fossil and rate calibrations.

The divergence times estimated for darters are notably similar to those estimated for other clades of North American freshwater fishes: Centrarchidae (Sunfish and Blackbasses) and the two most species-rich clades of Ictaluridae (Bullhead Catfishes and Madtoms), *Noturus*, and *Ameiurus* (Near, Bolnick, et al. 2005; Hardman M. and Hardman L.M. 2008). The mean posterior age estimates for the MRCA of all darters ranged from approximately 38.5–30.5 Ma (Table 3). These ages correspond to the Late Eocene and Early Oligocene and the Eocene–Oligocene boundary at 33.9 Ma (Luterbacher et al. 2004), a time characterized by dramatic global cool-

ing (Zachos et al. 2001; Woodburne 2004a). The global climate change associated with the Eocene–Oligocene transition was marked by noticeable trends of lineage extinction and origination in both the terrestrial and the marine fossil record across disparate clades in the Tree of Life (e.g., Thomas 1992; Graham 1999; Luterbacher et al. 2004). Thus, it appears that the descendants of several lineages, which now comprise a large component of the hyperdiverse freshwater fish fauna of eastern North America, originated near the Eocene–Oligocene transition approximately 34 Ma. The age estimates for darters and these other clades of North American fishes offer an insight into the timing of origination and diversification of the world's richest temperate freshwater fish fauna.

The crown clade *Etheostoma* dates to the earliest Oligocene, whereas the crown ages of other major darter clades (*Percina*, *Carnipellucida*, and *Nothonotus*) originated in the Late Oligocene and Miocene (Table 3). These age estimates demonstrate that the majority of reconstructed speciation events in the darter phylogeny date to the Miocene and Pliocene, indicating that most extant darter species and ecological communities of darter species have an origin within the last 15 myr. The dense species sampling in the darter chronograms will provide an informative basis for the investigation of long-standing hypotheses regarding the role of paleoclimate and paleogeography on the evolution and diversification of the species-rich North American freshwater fish fauna (e.g., Wiley and Mayden 1985; Mayden 1988; Near and Keck 2005).

Conclusions

As the architecture of the deepest parts of the Tree of Life become articulated through analyses of ever larger genomic-scale data sets, the challenges of resolving relationships among the most closely related branches and twigs remain. The history of investigating phylogenetic relationships among closely related animal species using molecular data is dominated by the use of mtDNA. Zoological systematists had been reluctant to explore the use of single-copy nuclear protein-coding genes for closely related lineages because mtDNA sequences consistently provided substantial phylogenetic resolution. Our investigation of darter phylogenetics based on the near-complete species sampling of a species-rich clade results in several important conclusions: 1) the sampling of a modest data set of nuclear genes results in appreciable phylogenetic resolution among closely related species; 2) mtDNA introgression in darters is extensive and complicated by the presence of at least three distinct phylogenetic patterns; 3) resolved phylogenetic hypotheses for darters allow the construction of an informative phylogeny-based classification; and 4) strategies of external calibration result in consistent relaxed-clock age estimates for clades across different genes and calibration methods. The results of our analyses provide critical insights in the pattern and timing

of darter diversification, but perhaps more importantly, these analyses offer a prospectus for the use of nuclear gene sequence data to resolve the most closely related portions of the Animal Tree of Life.

SUPPLEMENTARY MATERIAL

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

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APPENDIX

Phylogenetic Classification of Darters

Etheostomatinae Agassiz (1850, p. 298) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—The name Etheostomatinae has long been applied to the same set of clades. The name has varied in suffix with different applications to Linnaean ranks: Etheostomata (Agassiz 1850, p. 298), Etheostomidae (Jordan 1876, p. 215, 1877a, p. 5; Jordan et al. 1930, p. 282), Etheostomatini (Collette 1963, table 1), and Etheostomatinae (Jordan and Gilbert 1883, p. 486; Song et al. 1998, table 4). Definition (branch-modified node-based with two internal qualifiers).—The clade originating with the MRCA of *Etheostoma blennioides* Rafinesque and all extant organisms or species that share a more recent common ancestor with *E. blennioides* Rafinesque, *Percina bimaculata* Haldeman, and *Ammocrypta beanii* Jordan than with *Perca fluviatilis* Linnaeus or *Gymnocephalus cernuus* (Linnaeus) or *Percarina demidoffi* Nordmann or *Sander lucioperca* (Linnaeus) or *Zingel zingel* (Linnaeus) or *Romanichthys valsanicola* Dumitrescu, Banarescu, & Stoica. Reference phylogeny.—Wiley (1992, fig. 7) and Song et al. (1998, figs. 3 and 4). Composition.—Includes all species in *Percina*, *Carnipellucida*, *Nothonotus*, and *Etheostoma* (see below). Synapomorphies.—Species in the clade Etheostomatinae have the ceratohyals articulating via a band of cartilage, a dorsal flange on the maxilla, an enlarged coronomeckelian, loss of branchiostegal II, nonspatulate proximal ends of the branchiostegals, and the presence of four or fewer sensory foramina in the dentary (Simons 1991; Wiley 1992).

Percina Haldeman (1842, p. 330) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—*Percina* had long been applied to a subset of species of Etheostomatinae defined below as *Pileoma* (Jordan 1877a, p. 15). Most species of *Percina* were referred to as *Hadropterus* (Jordan and Evermann 1896, p. 1028) until Bailey et al. (1954, p. 140) reduced *Hadropterus* to the synonymy of *Percina*. Definition (node-based).—The least inclusive clade containing *Percina roanoka* (Jordan & Jenkins), *Percina macrocephala* (Cope), *P. bimaculata* Haldeman, *Percina aurantiaca* (Cope), and *Percina maculata* (Girard). Reference phylogeny.—Figures 2 and 3. Composition.—Includes *P. aurantiaca* (Cope), *P. evides* (Jordan & Copeland), populations of *P. evides* that are distributed west of the Wabash and Tennessee Rivers in Minnesota, Wisconsin, Iowa (extirpated), Illinois (extirpated), Missouri, and Arkansas that may represent an undescribed species (Denoncourt 1969; Near et al. 2001), and all species in *Atlantis*, *Pileoma*, *Etnierperca*, *Odontopholis*, *Pagella*, *Alvordius*, *Swainia*, and *Hadropterus* (see below). Synapomorphies.—Species of *Percina* are diagnosed from all other Etheostomatinae by the presence of modified scales on the breast and a unique electromorph of Ldh-B (Page and Whitt 1973a; Page 1974, 1983; Dimmick and Page 1992). Type species.—*Percina nebulosa* Haldeman (=*P. bimaculata* Haldeman).

Atlantis T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *P. roanoka* (Jordan & Jenkins), *Percina crassa* (Jordan & Brayton), and *Percina peltata* (Stauffer). Etymology.—Named for the Atlantic Ocean because all the species of *Atlantis* occupy river systems that drain into the Atlantic Ocean. Reference phylogeny.—Figure 3a. Composition.—Includes the species designated in the definition as well as *Percina nevisense* (Cope), and populations referred to as *P. peltata* in the upper Roanoke River system that may represent an undescribed species (Jenkins et al. 1972; Page 1983; Jenkins and Burkhead 1994; Schmidt and Daniels 2004). Synapomorphies.—No non-DNA synapomorphies have yet been discovered; however, species of *Atlantis* are distinguished by the presence of a mean number of lateral line scale rows less than 60 and a mean number of less than 10 modified scales along the midline of the belly (Page 1974, 1981). Type species.—*P. roanoka* (Jordan & Jenkins).

Pileoma DeKay (1842, p. 16) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic genus containing *Percina caprodes* (DeKay 1842) and subsequently synonymized with *Percina* (Jordan 1876, p. 224). Species in this clade had historically been classified in the subgenus *Percina* (e.g., Bailey and Gosline 1955; Page 1974). *Pileoma* is resurrected to avoid redundant group names. Definition (node-based).—The least inclusive clade containing *Percina jenkinsi* Thompson, *Percina rex* (Jordan & Evermann), *Percina burtoni* Fowler, and *P. caprodes* (Rafinesque). Reference phylogeny.—Figure 3a. Composition.—Includes the species designated in the definition and *Percina austroperca* Thompson, *Percina carbonaria* (Baird & Girard), *Percina fulvitaenia* Morris & Page, *Percina kathae* Thompson, *Percina macrolepida* Stevenson, *P. bimaculata* Haldeman, and *Percina suttkusi* Thompson, and populations referred to as *P. caprodes* in the upper Mississippi River System that may represent an undescribed species (Near 2008). Synapomorphies.—Species of *Pileoma* are diagnosed from all other species of *Percina* by the presence of a conical snout that comprises a modified ascending process of the premaxilla and a snout composed of soft connective tissue and extends beyond the upper jaw (Carlson and Wainwright 2010), and a large interorbital space on the top of the head (Page 1974). Type species.—*P. caprodes* (Rafinesque).

Etnierperca T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *P. tanasi* Etnier and *Percina copelandi* (Jordan). Etymology.—Named for David A. Etnier, ichthyologist, professor emeritus at the University of Tennessee, and our mentor who has made substantial contributions to the study of North American freshwater fishes. Reference phylogeny.—Figure 3a. Composition.—Includes all species of *Imostoma* and *Cottogaster*. Synapomorphies.—Species in the clade *Etnierperca* are diagnosed from all other species of *Percina* by a reduced or absent premaxillary frenum (Page 1974; Suttkus et al. 1994). Type species.—*P. tanasi* Etnier.

Imostoma Jordan (1877b, p. 49) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—*Imostoma* was described as a monotypic genus containing *Percina shumardi* (Jordan 1877b), subsequently applied as a subgenus, and expanded to include five species (Bailey and Gosline 1955; Etnier 1976; Williams and Etnier 1978; Suttkus 1985). Definition (node-based).—The least inclusive clade containing *Percina antesella* (Williams and Etnier 1978), *P. shumardi* (Girard), *Percina vigil* (Hay), and *Percina uranidea* (Jordan & Gilbert). Reference phylogeny.—Near (2002, figs. 3 and 6). Composition.—Includes the species designated in the definition as well as *P. tanasi* Etnier and populations referred to as *P. shumardi* that may represent one or more undescribed species (Boschung and Mayden 2004). Synapomorphies.—No non-DNA synapomorphies have yet been discovered; however, species of *Imostoma* have mostly scaleless bellies, the anal fin of adult males is greatly elongated, and the interorbital width is narrow (Page 1974). Type species.—*P. shumardi* (Girard).

Cottogaster Putnam (1863, p. 4) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—*Cottogaster* was long applied as a monotypic genus containing *P. copelandi* (Jordan 1882, p. 969) and later expanded to include *P. uranidea*, and *P. shumardi* (Jordan and Evermann 1896, p. 1044–1047). Later workers treated *Cottogaster* as a monotypic subgenus containing *P. copelandi* (Bailey and Gosline 1955, table 1; Page 1974, p. 84), which was expanded to three species with the descriptions of *P. aurora* and *P. brevicauda* (Suttkus et al. 1994). Definition (node-based).—The least inclusive clade containing *Percina aurora* Suttkus & Thompson, *Percina brevicauda* Suttkus & Bart, and *P. copelandi* (Jordan). Reference phylogeny.—Figure 3a. Composition.—Includes the species designated in the definition and populations currently referred to as *P. copelandi* in the Ozark and Ouachita Highlands that may represent an undescribed species (Suttkus et al. 1994). Synapomorphies.—No non-DNA synapomorphies have yet been discovered; however, species of *Cottogaster* are among the smallest sized *Percina* and have low meristic counts with the number of scale rows below the lateral line less than 7, scales above the lateral line less than 5, less than 10 spines in first dorsal fin, and modal number of vertebrae less than 40 (Page 1974, 1983; Suttkus et al. 1994). Type species.—*P. copelandi* (Jordan).

Pagella T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *P. macrocephala* (Cope) and *Percina williamsi* Page and Near. Etymology.—Named for Lawrence M. Page, ichthyologist at the Florida Museum of Natural History who has made substantial contributions to

the study of darters. Reference phylogeny.—Figure 3a. Composition.—Includes the species designated in the definition. Synapomorphies.—Species of *Pagella* are distinguished from all other species of *Percina* by the presence of a sickle-shaped suborbital bar that extends to the underside of the head and a black bar subtending a medial black spot on the base of the caudal fin (Page and Near 2007). Type species.—*P. macrocephala* (Cope).

Odontopholis Page (1974, p. 84) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—*Odontopholis* was applied as a subgenus containing *Percina cymatotaenia* and *P. stictogaster* (Page 1974, p. 84). Definition (node-based).—The least inclusive clade containing *P. cymatotaenia* (Gilbert & Meek) and *P. stictogaster* Burr & Page. Reference phylogeny.—Figure 3a. Composition.—Includes the species designated in the definition and populations currently referred to as *P. stictogaster* in the Green River System of Kentucky and Tennessee that may represent an undescribed species (Burr and Page 1993). Synapomorphies.—Species of *Odontopholis* are diagnosed from all other species of *Percina* by the absence of a row of modified scales along the midline of the belly, large black spots distributed on the ventral side of the body, a distinct uninterrupted light stripe that runs along the side of the body within a darkly pigmented lateral stripe, and breeding males possess a distinct caudal keel (Page 1974; Burr and Page 1993; Page and Sabaj 1994). Type species.—*P. cymatotaenia* (Gilbert & Meek).

Alvordius Girard (1859a, p. 68) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—*Alvordius* was applied as a genus to a group of five species: *P. maculata, P. nevisense, P. peltata, P. macrocephala*, and *P. phoxocephala* (Jordan 1877a, p. 14–15) but was later treated as a subgenus with the addition of *P. roanoka* and *P. vigil* (Jordan and Evermann 1896, p. 1030–1036). Bailey and Gosline (1955, table 1) treated *Alvordius* as a subgenus containing *P. maculata, P. notogramma, P. macrocephala, P. nevisense*, and *P. peltata*, which Page (1974, p. 83; 2000, table 12a) expanded to include *P. roanoka, P. gymnocephala, P. pantherina, P. smithvanizi*, and *P. kusha*. Subsequent phylogenetic analysis of mtDNA gene sequences did not support monophyly of this composition of *Alvordius* (Near 2002). Definition (node-based).—The least inclusive clade containing *P. maculata* (Girard) and *P. gymnocephala* Beckham. Reference phylogeny.—Figure 3a. Composition.—The species included in the definition as well as *Percina notogramma* (Raney & Hubbs) and *P. pantherina* (Moore & Reeves). Synapomorphies.—No non-DNA synapomorphies have yet been discovered; however, species of *Alvordius* are differentiated from other species of *Percina* by the presence of distinct lateral pigment consisting of round or oval blotches, a medially placed black caudal spot, body oval in cross section, the anterior portion of the first dorsal fin with uniform pigmentation on the lower half of the membranes, and the caudal fin posterior edge is nearly straight when extended (Raney and Hubbs 1948; Page 1974). Type species.—*P. maculata* (Girard).

Swainia Jordan and Evermann (1896, p. 1040) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic subgenus containing *Percina squamata* (Jordan and Evermann 1896, p. 1040) and later expanded to include *P. phoxocephala, P. nasuta*, and *P. oxyrhynchus* (Bailey and Gosline 1955, table 1). Definition (node-based).—The least inclusive clade containing *P. squamata* (Gilbert & Swain) and *P. phoxocephala* (Nelson). Reference phylogeny.—Figure 3a. Composition.—Includes the species designated in the definition, as well as *P. oxyrhynchus* (Hubbs & Raney), *P. nasuta* (Bailey), and populations of *P. cf. nasuta* in the Ouachita River system that represent an undescribed species (Robison and Buchanan 1988, p. 453–454). Synapomorphies.—Species of *Swainia* are diagnosed from all other species of *Percina* by the presence of elongate jaws and narrow heads (Carlson and Wainwright 2010). Type species.—*P. squamata* (Gilbert & Swain).

Hadropterus Agassiz (1854, p. 305) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—*Hadropterus* was applied as a genus containing *Percina nigrofasciata, P. aurantiaca*, and *Etheostoma variatum* (Jordan 1877a, p. 14). Eventually *E. variatum* was removed from *Hadropterus* and the genus was expanded to 11 species (Jordan and Evermann 1896, p. 1028–1039). Bailey (1951) applied *Hadropterus* as a genus to all species of *Percina* except those in *Pileoma*. Bailey and Gosline (1955, table 1) treated *Hadropterus* as a subgenus with *P. sciera* and *P. nigrofasciata*, and this group was expanded with the descriptions of *P. lenticula* and *P. aurolineata* (Richards and Knapp 1964; Suttkus and Ramsey 1967). Definition (node-based).—The least inclusive clade containing *Percina apristis* (Hubbs & Hubbs), *P. lenticula* (Richards & Knapp), *P. nigrofasciata* (Agassiz), *P. sciera* (Swain), *P. sipsi* Williams & Neely, *Percina crypta* Freeman, Freeman, and Burkhead, *Percina palmaris* (Bailey), and *P. kusha* Williams & Burkhead. Reference phylogeny.—Figure 3a. Composition.—Includes *Chalinoperca*, all the species designated in the definition, *P. aurolineata* (Suttkus & Ramsey), and *Percina westfalli* (Fowler). Synapomorphies.—No non-DNA synapomorphies have yet been discovered; however, species of *Hadropterus* typically exhibit 7–17 blotches of darker pigment along the side of the body that vary in shape from

round to vertical bars and by a vertical row of three dark spots at the base of the caudal fin (Richards and Knapp 1964; Page 1974; Williams et al. 2007). Type species.—*P. nigrofasciata* (Agassiz).

Chalinoperca T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *P. kusha* Williams & Burkhead 2007 and *P. smithvanizi* Williams & Walsh 2007. Etymology.—From the Greek words χαλινόσ meaning bridle and περκη meaning perch, in reference to the presence of a pre- and postorbital stripe that is continuous with the lateral stripe along the side of the body that resembles an equestrian bridle connected to reins (Williams et al. 2007). Reference phylogeny.—Figure 3a. Composition.—Includes the species designated in the definition as well as populations referred to as *P. kusha* from the Etowah River system that may represent an undescribed species. Synapomorphies.—Species of *Chalinoperca* are diagnosed by the pre- and postorbital pigmentation mentioned in the etymology and a series of connected dark blotches of pigment that form a lateral stripe along the side of the body. Type species.—*P. kusha* Williams & Burkhead.

Carnipellucida B.P. Keck & T.J. Near, new clade name. Definition (node-based).—The least inclusive clade containing *Crystallaria asprella* (Jordan) and *Ammocrypta clara* Jordan & Meek. Etymology.—From the Latin words *carnis* meaning flesh and *pelluceo* meaning to be transparent. Reference phylogeny.—Figures 2 and 3a. Composition.—Includes all species in *Ammocrypta* and *Crystallaria* (see below). Synapomorphies.—Species of *Carnipellucida* are diagnosed from all other species of Etheostomatinae by the presence of a slender body (ratio of body depth to standard length ranges from 0.11 to 0.13), translucent flesh, and a single weak spine in the anal fin (Bailey et al. 1954; Page 1981). Type species.—*Ammocrypta clara* Jordan & Meek.

Ammocrypta Jordan (1877a, p. 5) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic genus containing *A. beanii* (Jordan 1877a p. 17) and later expanded to include *A. pellucida, A. clara*, and *A. vivax* (Jordan and Evermann 1896, p. 1061–1064). Williams (1975) recognized six species of *Ammocrypta* with the descriptions of *A. meridiana* and *A. bifascia*. Definition (node-based).—The least inclusive clade containing *A. beanii* Jordan, *A. clara* Jordan & Meek, and *Ammocrypta pellucida* (Agassiz). Reference phylogeny.—Figures 2 and 3a. Composition.—Includes the species designated in the definition as well as *A. bifascia* Williams, *A. meridiana* Williams, *A. vivax* Hay, and populations of *A. cf. beanii* in the Mobile Basin that may represent an undescribed species (Williams 1975; Wiley and Hagen 1997). Synapomorphies.—Species of *Ammocrypta* are diagnosed from all other species of *Carnipellucida* by the position of the first dorsal interneural spine between the neural spines of the 6th to 10th vertebrae, the descending process of the hyomandibula terminating at the end of the preopercular groove, a prominent spur on the hyomandibula, absence of a notch on the anterior margin of the preopercle, and a thin and concave mesethmoid (Williams 1975; Simons 1992). Type species.—*A. beanii* Jordan.

Crystallaria Jordan and Gilbert in Jordan (1885, p. 866) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic genus containing *C. asprella* that was later expanded with the description of *C. cincotta* (Jordan 1885; Welsh and Wood 2008). Definition (node-based).—The least inclusive clade containing *C. asprella* (Jordan) and *C. cincotta* Welsh & Wood. Reference phylogeny.—Figures 2 and 3. Composition.—Includes the species designated in the definition. Synapomorphies.—Species of *Crystallaria* are diagnosed from all other species of *Carnipellucida* by the presence of a bifurcate supraoccipital crest and 45–48 vertebrae (Bailey and Gosline 1955; Simons 1991). Type species.—*C. asprella* (Jordan).

Nothonotus Putnam (1863, p. 3) [B.P. Keck & T.J. Near], converted clade name. Comments on name.—Applied as a genus containing *Nothonotus maculatus, N. camurus, N. sanguifluus, N. vulneratus*, and *N. rufilineatus* (Jordan 1877a, p. 16). Was subsequently treated as a subgenus of *Etheostoma* (Jordan and Evermann 1896, p. 1076; Bailey and Gosline 1955, table 1; Page 1981, p. 35; Bailey and Etnier 1988, p. 24). Definition (node-based).—The least inclusive clade containing *Nothonotus acuticeps* (Bailey), *Nothonotus aquali* (Williams & Etnier), *Nothonotus jordani* (Gilbert), *Nothonotus juliae* (Meek), *N. maculatus* (Kirtland), *Nothonotus microlepidus* (Raney & Zorach), *Nothonotus moorei* (Raney & Suttkus), *N. rufilineatus* (Cope), and *Nothonotus tippecanoe* (Jordan & Evermann). Reference phylogeny.—Figures 2 and 3a. Composition.—Includes the species designated in the definition and *Nothonotus bellus* (Zorach), *N. camurus* (Cope), *Nothonotus chlorobranchius* (Zorach), *Nothonotus chuckwachatte* (Wood & Mayden), *Nothonotus denoncourti* (Stauffer & van Snick), *Nothonotus douglasi* (Wood & Mayden), *Nothonotus etowahae* (Wood & Mayden), *Nothonotus rubrus* (Raney & Suttkus), *N. sanguifluus* (Cope), *N. vulneratus* (Cope), *Nothonotus wapiti* (Etnier & Williams), and the population of *N. cf. sanguifluus* in the Caney Fork River, Tennessee, that represent an undescribed species. Synapomorphies.—No non-DNA synapomorphies have yet been

discovered; however, species of *Nothonotus* are characterized by a slab-sided body shape and darkened anterior interradial membranes of the first dorsal fin (Bailey and Etnier 1988; Keck and Near 2008). Type species.—*N. maculatus* (Kirtland).

Etheostoma Rafinesque (1819, p. 419) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Initially applied as a genus containing all species of Etheostomatinae recognized at the time, which consisted of *E. blennioides, E. flabellare, E. nigrum,* and *P. caprodes* (Rafinesque 1820, p. 35–38). Jordan (1877a, p. 16) limited *Etheostoma* to *E. flabellare, E. kennicotti,* and *E. squamiceps,* but later included all species of Etheostomatinae in this genus (Jordan 1888). In subsequent works, *Etheostoma* was a genus applied to all species of Etheostomatinae exclusive of *Percina, Ammocrypta,* and *Crystallaria,* and as a subgenus (Bailey et al. 1954; Bailey and Gosline 1955; Page 1981; Bailey and Etnier 1988; Lang and Mayden 2007). Definition (branch-modified node-based with two internal qualifiers).—The clade originating with the MRCA of *E. blennioides* Rafinesque and all extant organisms or species that share a more recent common ancestor with *E. blennioides* Rafinesque, *Etheostoma fusiforme* (Girard), and *E. lepidum* (Baird & Girard) than with *P. bimaculata* Haldeman, or *A. beanii* Jordan, or *N. maculatus* (Kirtland). Reference phylogeny.—Figures 2 and 3b,c. Composition.—Includes the species designated in the definition and *E. trisella* Bailey & Richards, *Etheostoma edwini* (Hubbs & Cannon), *Etheostoma fricksium* Hildebrand, *Etheostoma gracile* (Girard), *Etheostoma zonifer* (Hubbs & Cannon), *E. hopkinsi* (Fowler), *E. binotatum* Bailey & Richards, *Etheostoma mariae* (Fowler), *Etheostoma okaloosae* (Fowler), *Etheostoma collis* (Hubbs & Cannon), *Etheostoma saludae* (Hubbs & Cannon), *Microperca, Oligocephalus, Hololepis, Gemmaperca, Simoperca, Fuscatelum,* and *Goneaperca* (see below). Synapomorphies.—No non-DNA synapomorphies have yet been discovered. *Etheostoma* has proven difficult to diagnose morphologically. Past efforts at morphological diagnosis for the unnamed clade containing both *Etheostoma* and *Nothonotus* were limited to noting the absence of the characters in this clade that distinguish *Carnipellucida* and *Percina* (Bailey et al. 1954; Page 1981). Type species.—*E. blennioides* Rafinesque.

Microperca Putnam (1863, p. 4) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic genus containing *Etheostoma microperca* (Jordan 1877a, p. 17) and later expanded to include *E. proeliare* and *E. fonticola* (Jordan and Evermann 1896, p. 1103–1105). Definition (node-based).—The least inclusive clade containing *E. microperca* Jordan & Gilbert and *Etheostoma proeliare* (Hay). Reference phylogeny.—Figure 3b. Composition.—Includes the species designated in the definition as well as *Etheostoma fonticola* (Jordan & Gilbert) and populations referred to as *E. microperca* in the Ozark Highlands that may represent an undescribed species (Burr 1978; Buth et al. 1980). Synapomorphies.—Species of *Microperca* are the smallest species of Etheostomatinae with a maximum standard length less than 40 mm (Page and Burr 1979). Species of *Microperca* are further diagnosed from all other species of *Etheostoma* by the presence of an elongated and tuberculate pelvic fin in nuptial males (ratio of pelvic fin to standard length 0.25–0.31), and the preoperculomandibular canal has 6–8 pores (Page 1977; Burr 1978). Type species.—*E. microperca* Jordan & Gilbert.

Oligocephalus Girard (1859, p. 67) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a subgenus of *Etheostoma* (Jordan and Evermann 1896, p. 1083–1093). Subsequent delimitations of *Oligocephalus* included *E. lepidum, E. asprigene, E. australe, E. caeruleum, E. collettei, E. ditrema, E. exile, E. grahami, E. hopkinsi, E. luteovinctum, E. nuchale, E. okaloosae, E. pottsii, E. radiosum, E. spectabile, E. swaini,* and *E. whipplei* (Page 1981, p. 39; Bailey and Etnier 1988, p. 25). Definition (node-based).—The least inclusive clade containing *E. lepidum* (Baird & Girard), *E. whipplei* (Girard), *E. exile* (Girard), *E. caeruleum* Storer, and *E. spectabile* (Agassiz). Reference phylogeny.—Figure 3b. Composition.—Contains species of *Vexillapinna, Boleichthys, Austroperca, Astatichthys,* and *Ceasia* (see below). Synapomorphies.—No non-DNA synapomorphies have yet been discovered, but males of species of *Oligocephalus* exhibit brilliant nuptial colors of reds, blues, and greens on the body, and the first dorsal fin has a blue, green, or dusky margin with a reddish or orange submarginal band (Page 1981; Bailey and Etnier 1988). Type species.—*E. lepidum* (Baird & Girard).

Astatichthys Vaillant (1873, p. 106) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a genus containing *E. caeruleum, E. zonale,* and *E. pulchellum* (Vaillant 1873, p. 106–117), and subsequently synonymized with *Poecilichthys* and the subgenus *Oligocephalus* (Jordan 1876, p. 219; Jordan and Evermann 1896; Bailey and Richards 1963). Definition (node-based).—The least inclusive clade containing *E. caeruleum* Storer, *E. radiosum* (Hubbs & Black), *E. lepidum* (Baird & Girard), *E. exile* (Girard), *E. asprigene* (Forbes), and *E. collettei* Birdsong & Knapp. Reference phylogeny.—Figure 3b. Composition.—Includes all species designated in the definition, all species of *Vexillapinna, Boleichthys, Austroperca, E. swaini* (Jordan), *E. nuchale* Howell &

Caldwell, *E. ditrema* Ramsey & Suttkus, *Etheostoma thompsoni* Suttkus, Bart, & Etnier, populations of *E. cf. caeruleum* from the White and Little Red River drainages, populations of *E. cf. ditrema* from the central Coosa River system, populations of *E. cf. ditrema* from Coldwater Spring, populations of *E. cf. swaini* from the Cahaba River system, and populations of *E. cf. swaini* from the Black Warrior River system (Mayden et al. 2005; Ray et al. 2006). Synapomorphies.— No non-DNA synapomorphies have yet been discovered; however, males of *Astatichthys* species exhibit a color pattern of red-orange and blue or green on the lower body, red-orange and blue in the dorsal and anal fin of nuptial males, and have the deepest part of the body at the origin, or under the first dorsal fin (Ramsey and Suttkus 1965). Type species.—*E. caeruleum* Storer.

Vexillapinna B.P. Keck & T.J. Near, new clade name. Definition (node-based).—The least inclusive clade containing *E. whipplei* (Girard), *E. artesiae* (Hay), and *E. radiosum* (Hubbs & Black). Etymology.—From the Latin words *vexillium* meaning a standard or a flag and *pinna* meaning a wing, referring to the brilliantly colored fins of nuptial males. Reference phylogeny.—Figure 3b. Composition.—Includes the species designated in the definition as well as *Etheostoma cyanorum* (Moore & Rigney) and *Etheostoma paludosum* (Moore & Rigney). Synapomorphies.—Species of *Vexillapinna* are diagnosed from all other species of *Oligocephalus* by a slender body (body depth to standard length ratio less than 0.192) and a partly scaled operculum (Hubbs and Black 1941; Moore and Rigney 1952; Page 1981, 1983). Type species.—*E. whipplei* (Girard).

Boleichthys Girard (1859b, p. 103) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a genus containing *E. exile* and *E. fusiforme* (Jordan 1876, p. 220–221). Page synonymized the subgenera *Hololepis* and *Microperca* with *Boleichthys* (Page 1981, p. 40–41). Definition (node-based).— The least inclusive clade containing *E. exile* (Girard) and *Etheostoma leuteovinctum* Gilbert & Swain. Reference phylogeny.—Figure 3b. Composition.—Includes species designated in the definition. Synapomorphies.—Species of *Boleichthys* are diagnosed from other species of *Oligocephalus* by an average of more than 50 lateral line scales, a slender caudal peduncle (ratio of depth to standard length less than 0.095), the presence of a prominent and darkly pigmented suborbital bar, and a scaled breast (Page 1981, 1983; Bailey and Etnier 1988; Etnier and Starnes 1993). Type species.—*E. exile* (Girard).

Austroperca Hubbs (1936, p. 1) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—*Austroperca* is a replacement name for *Torrentaria* that was applied as a subgenus containing *E. australe* and *E. sagitta* (Jordan and Evermann 1896, 1080–1082). *Austroperca* was later reduced to *E. australe* (Bailey and Gosline 1955, table 1) and subsequently synonymized with *Oligocephalus* (Page 1981; Bailey and Etnier 1988). Definition (node-based).—The least inclusive clade containing *E. australe* Jordan, *E. grahami* (Girard), and *E. lepidum* (Baird & Girard). Composition.—Includes the species designated in the definition and *E. pottsii* (Girard), *E. lugoi* Norris & Minckley, and *Etheostoma segrex* Norris & Minckley. Reference phylogeny.—Lang and Mayden (2007, figs. 2 and 3). Synapomorphies.—Species of *Austroperca* are diagnosed from all other species of *Oligocephalus* by the loss or reduction of the basisphenoid and the presence of a distinctive bony spur on the posterolateral face of the hyomandibula (Norris and Minckley 1997; Norris 2001). Type species.—*E. australe* Jordan.

Ceasia T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *E. spectabile* (Agassiz) and *E. uniporum* Distler. Etymology.—Named for our colleague Patrick A. Ceas who has made substantial contributions to the study of darters, particularly species of *Ceasia*. Reference phylogeny.—Figure 3b. Composition.—Includes the species designated in the definition as well as *E. pulchellum* (Girard), *Etheostoma squamosum* Distler, *E. fragi* Distler, *E. burri* Ceas & Page, *Etheostoma tecumsehi* Ceas & Page, *Etheostoma kantuckeense* Ceas & Page, *Etheostoma bison* Ceas & Page, and *E. lawrencei* Ceas & Burr, and at least five undescribed species listed in Table 1 and Ceas and Burr (2002). Synapomorphies.—Species of *Ceasia* are diagnosed from other species of *Oligocephalus* by a blue colored anal fin that rarely has red pigment and the deepest part of the body anterior to or near the first dorsal fin origin (Distler 1968; Ceas and Page 1997; Ceas and Burr 2002). Type species.—*E. spectabile* (Agassiz).

Hololepis Agassiz in Putnam (1863, p. 4) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a genus containing *Etheostoma barratti* and *E. fusiforme* (Putnam 1863, p. 4) but synonymized with *Boleichthys* (Jordan 1876, p. 220). *Hololepis* was resurrected by Hubbs and Greene (1928, p. 374, 384) and expanded to include *E. serrifer, E. gracile, E. zonifer, E. saludae,* and *E. collis* (Hubbs and Cannon 1935; Collette 1962). Page (1981, p. 40–41) synonymized *Hololepis* and *Microperca* with *Boleichthys;* however, Bailey and Etnier (1988, 27–28) recognized *Hololepis* as delimited in earlier studies (e.g., Hubbs and Cannon 1935). Definition (node-based). —The least inclusive clade containing *E. barratti* (Holbrook), *E. fusiforme* (Girard), and *E. serrifer* (Hubbs & Cannon). Reference phylogeny.—Figure 3b. Composition. —Includes the species

designated in the definition and possibly three of the described subspecies of *E. barratti* and *E. fusiforme* that may represent separate species (Hubbs and Cannon 1935; Bailey 1950). Synapomorphies.—No non-DNA synapomorphies have yet been discovered, but species of *Hololepis* are distinguished from other species of *Etheostoma* by laterally flattened bodies with an upward arching incomplete lateral line (Hubbs and Cannon 1935; Collette 1962; Etnier and Starnes 1993). Type species.—*E. barratti* (Holbrook 1885).

Fuscatelum Page (1981, p. 36–37) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic subgenus containing *Etheostoma parvipinne*, which was subsequently was expanded to include *E. phytophilum* (Page 1981, p. 36–37; Bart and Taylor 1999). Definition (node-based).—The least inclusive clade containing *E. parvipinne* Gilbert & Swain and *E. phytophilum* Bart & Taylor. Reference phylogeny.—Figure 3b. Composition.—The species designated in the definition. Synapomorphies. —No non-DNA synapomorphies; however, species of *Fuscatelum* are distinguished from all other species of *Etheostoma* by the combination of a deep caudal peduncle (ratio to standard length greater than 0.110), absence of bright breeding colors, presence of a premaxillary frenum, and presence of tubercles on the anal fin of breeding males (Page 1981; Bart and Taylor 1999). Type species.—*E. parvipinne* Gilbert & Swain.

Gemmaperca B.P. Keck & T.J. Near, new clade name. Definition (node-based).—The least inclusive clade containing *E. chlorosoma* (Hay), *Etheostoma jessiae* (Jordan & Brayton), *Etheostoma sagitta* (Jordan & Swain), and *E. variatum* Kirtland. Etymology.—From the Latin word *gemma* meaning jewel and the Greek word περκη meaning perch in reference to the brightly colored males of most species in this clade. Reference phylogeny.—Figure 3b. Composition.—Contains all species of *Iriperca* and *Maydenichthys* (see below). Synapomorphies.—There are no non-DNA synapomorphies; however, pigmentation patterns include 4–9 dorsal saddles (Page 1981, 1983; Bailey and Etnier 1988). Type species.—*E. jessiae* (Jordan & Brayton).

Iriperca T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *Etheostoma nianguae* Gilbert & Meek, *E. sagitta* (Jordan & Swain), *Etheostoma variatum* Kirtland, *Etheostoma euzonum* (Hubbs & Black), *Etheostoma tetrazonum* (Hubbs & Black), and *Etheostoma osburni* (Hubbs & Trautman). Etymology.—From the Greek ιρισ meaning rainbow and περκη meaning perch in reference to the bright breeding colors of nuptial males. Reference phylogeny.—Figure 3b. Composition.—Includes all species in *Litocara* and *Poecilichthys* (see below). Synapomorphies. —No non-DNA synapomorphies; however, species of *Iriperca* are distinguished from other species of *Gemmaperca* by a relatively large body size and males with a combination of bright green, blue, and red breeding colors (Hubbs and Black 1940; Bailey 1948; Bailey and Etnier 1988). Type species.—*E. tetrazonum* (Hubbs & Black).

Litocara Bailey (1948, p. 79) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a subgenus containing *E. sagitta, E. spilotum*, and *E. nianguae* (Bailey 1948, p. 79). Definition (node-based).—The least inclusive clade containing *E. nianguae* Gilbert & Meek and. *sagitta* (Jordan & Swain). Reference phylogeny.—Figure 3b. Composition.—Includes the species contained in the definition and *E. spilotum* Gilbert. Synapomorphies.—Species of *Litocara* are diagnosed from all other species of *Iriperca* with the presence of two jet-black vertically aligned spots at the distal margin of the caudal peduncle that are often confluent and form a darkly pigmented short vertical bar (Bailey 1948; Kuehne and Bailey 1961; Page 1981; Bailey and Etnier 1988). Type species. —*E. sagitta* (Jordan & Swain).

Poecilichthys Agassiz (1854, p. 304) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a genus including *E. variatum, E. caeruleum, E. spectabile*, and *E. punctulatum* (Agassiz 1854, p. 3024). Later expanded to include *E. zonale* (Jordan 1876, p. 219–220), *E. lepidum*, and *E. grahami* (Jordan 1877a). Most recently applied as a subgenus containing most of the species listed in the definition (Page 2000; Switzer and Wood 2009). Definition (node-based).—The least inclusive clade containing *E. variatum* Kirtland, *E. euzonum* (Hubbs & Black), *E. tetrazonum* (Hubbs & Black), and *E. osburni* (Hubbs & Trautman). Reference phylogeny.—Figure 3b. Composition.—All the species designated in the definition as well as *Etheostoma erizonum* (Hubbs & Black), *Etheostoma erythrozonum* Switzer & Wood, and *Etheostoma kanawahe* (Raney). Synapomorphies.—Species of *Poecilichthys* are diagnosed from all other species of *Iriperca* by the presence of nuptial tubercles in both males and females, 4 or 5 primary saddles across the back, and dark and pale bands of pigment and color under a red marginal band in the first dorsal fin (Hubbs and Black 1940; Page and Cordes 1983; Bailey and Etnier 1988). Type species.—*E. variatum* Kirtland.

Maydenichthys T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *E. chlorosoma* (Hay), *Etheostoma davisoni* Hay, and *E. akatulo* Layman & Mayden. Etymology.—Named for

Richard L. Mayden, professor and ichthyologist at Saint Louis University. Reference phylogeny.—Figure 3b. Composition.—Includes all species in *Vaillantia* and *Doration* (see below). Synapomorphies.—Species of *Maydenichthys* are diagnosed from all other species of *Gemmaperca* by the absence of a premaxillary frenum (present in *E. jessiae*). Type species.—*E. akatulo* Layman & Mayden.

Vaillantia Jordan in Jordan and Brayton (1878, p. 89) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic genus containing *E. chlorosoma* (Jordan and Brayton 1878). Subsequently ranked as a subgenus and expanded to include *E. davisoni* (Page 1981, p. 35). Definition (node-based).—The least inclusive clade containing *E. chlorosoma* (Hay) and *E. davisoni* Hay. Reference phylogeny.—Mayden et al. (2006, fig. 5). Composition.—Includes the species designated in the definition and western populations referred to as *E. chlorosoma* (Brazos and Colorado Rivers) that may represent an undescribed species (Bart and Cashner 1986). Synapomorphies.—Species of *Vaillantia* are diagnosed from all other species of *Maydenichthys* by the presence of preorbital bars of pigment that extend around the snout giving the appearance of a black bridle (Page 1981). Type species.—*E. chlorosoma* (Hay).

Doration Jordan (1929, p. 156) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic genus containing *E. stigmaeum* (Jordan 1929, p. 156) and subsequently expanded to include *E. jessiae* (Cole 1967, p. 29). Definition (node-based).—The least inclusive clade that contains *E. stigmaeum* (Jordan), *E. akatulo* Layman & Mayden, and *E. meadiae* (Jordan & Evermann). Reference phylogeny.—Figure 3b. Composition.—Includes the species designated in the definition as well as *E. jessiae* (Jordan & Brayton) and at least three undescribed species presented in an unpublished PhD thesis (Layman 1994). Synapomorphies.—Species of *Doration* are diagnosed from all other species of *Maydenichthys* by the presence of a blue marginal band over a red band in the first dorsal fin (Page 1981; Bailey and Etnier 1988). Type species.—*E. stigmaeum* (Jordan).

Simoperca T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *E. baileyi* Page & Burr, *E. histrio* Jordan & Gilbert, *Etheostoma bellator* Suttkus & Bailey, *Etheostoma duryi* Henshall, *Etheostoma etnieri* Bouchard, *E. cinereum* Storer, *E. atripinne* (Jordan), *E. blennioides* Rafinesque, *E. zonale* (Cope), and *Etheostoma thalassinum* (Jordan & Brayton). Etymology.—From the Greek σιμοσ meaning snub-nosed and περκη meaning perch in reference to the blunt snout that characterizes species in this clade. Reference phylogeny.—Figure 3c. Composition.—Includes the species designated in the definition and all species in *Adonia, Ulocentra*, and *Neoetheostoma* (see below). Synapomorphies.—No non-DNA synapomorphies have yet been discovered; however, species of *Simoperca* are distinguished from all other species of *Etheostoma* by the presence of a blunt snout profile and a long urogential tube in females (Page 1981; Bailey and Etnier 1988). Type species.—*E. etnieri* Bouchard.

Adonia T.J. Near & B.P. Keck, new clade name. Definition (node-based): The least inclusive clade containing *E. bellator* Suttkus & Bailey, *Etheostoma coosae* (Fowler), *E. duryi* Henshall, and *Etheostoma etnieri* Bouchard. Etymology.—Named for Adonis, a man in Greek mythology who was characterized by unearthly beauty in reference to the elegant and beautiful male nuptial coloration exhibited by species of *Adonia*. Reference phylogeny.—Figure 3c. Composition.—Includes the species designated in the definition, as well as *Etheostoma brevirostrum* Suttkus & Etnier, *Etheostoma cervus* Powers & Mayden, *Etheostoma chermocki* Boschung, Mayden, & Tomelleri, *Etheostoma colorosum* Suttkus & Bailey, *Etheostoma flavum* Etnier & Bailey, *Etheostoma lachneri* Suttkus & Bailey, *Etheostoma pyrrhogaster* Bailey & Etnier, *Etheostoma ramseyi* Suttkus & Bailey, *Etheostoma raneyi* Suttkus & Bart, *Etheostoma scotti* Bauer, Etnier, & Burkhead, *Etheostoma tallapoosae* Suttkus & Etnier, *Etheostoma zonistium* Bailey & Etnier, populations referred to as *E. cf. zonistium* in the Black Warrior and upper Bear Creek river systems, and populations referred to as *E. cf. bellator* from Locust Fork and the Black Warrior system that each may represent undescribed species (Clabaugh et al. 1996; Boschung and Mayden 2004). Synapomorphies.—Species of *Adonia* are diagnosed from other species of *Simoperca* by the presence of one or a few long teeth on the vomer, and the premaxilla is free from the snout (Bailey and Etnier 1988). Type species.—*E. duryi* Henshall.

Ulocentra Jordan (1878, p. 223) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic genus containing *E. atripinne* (Jordan and Brayton 1878, p. 223) and subsequently expanded to include all "snubnose darters" that are classified here as *Ulocentra* and *Adonia* (Bailey and Gosline 1955; Bailey and Etnier 1988). Definition (node-based).—The least inclusive clade containing *E. atripinne* (Jordan) and *Etheostoma barrenense* Burr & Page. Reference phylogeny.—Figure 3c. Composition.—Includes the species designated in the definition and *Etheostoma planasaxatile* Powers & Mayden, *Etheostoma rafinesquei* Burr & Page, and *E. simoterum* (Cope). Synapomorphies.—Species of *Ulocentra* are diagnosed from other species of *Simoperca* by the

presence of a very narrow frenum and a lack of teeth on the vomer (Bailey and Etnier 1988). Type species.—*E. atripinne* (Jordan).

Neoetheostoma T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *E. blennioides* Rafinesque, *E. thalassinum* (Jordan & Brayton), and *E. zonale* (Cope). Etymology.—"Neo" meaning new and in combination with *Etheostoma* provides a name for the clade traditionally classified as subgenus *Etheostoma*. This new clade name is introduced to avoid redundancy of group names as *Etheostoma* is applied to a more inclusive clade (see above). Reference phylogeny.—Figure 3c. Composition.—Includes all species designated in the definition as well as *E. blennius* Gilbert & Swain, *Etheostoma gutselli* (Hildebrand), *Etheostoma inscriptum* (Jordan & Brayton), *E. lynceum* Hay, *Etheostoma newmanii* (Agassiz), *Etheostoma pholidotum* Miller, *Etheostoma rupestre* Gilbert and Swain, *E. sequatchiense* Burr, and *Etheostoma swannanoa* Jordan & Evermann. Synapomorphies.—No non-DNA synapomorphies have yet been discovered, but species of *Neoetheostoma* are distinguished from other species of *Simoperca* by a larger maximum body size, 4–7 dorsal saddles or blotches, and an extremely broad head (Richards 1966; Page 1981; Bailey and Etnier 1988). Type species.—*E. thalassinum* (Jordan & Brayton).

Goneaperca T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade that contains *Etheostoma basilare* Page, Hardman, & Near, *Etheostoma virgatum* (Jordan), *Etheostoma brevispinum* (Coker), *Etheostoma squamiceps* Jordan, *E. olmstedi* Storer, *E. punctulatum* (Agassiz), and *E. tuscumbia* Gilbert & Swain. Etymology. —From the Greek word γονευσ meaning parent and περκη meaning perch in reference to the male parental care behaviors of most species in this clade. Reference phylogeny.—Figure 3c. Content.—Includes all species of *Catonotus, Boleosoma, Stigmacerca,* and *Psychromaster* (see below). Synapomorphies.—No non-DNA synapomorphies have yet been discovered, but most species in the clade provide parental care for fertilized eggs, attach eggs to structures in the stream, or spawn in flooded fields and seepages. Type species.—*E. basilare* Page, Hardman, & Near.

Catonotus Agassiz (1854, p. 305) [T.J. Near & B.P. Keck], converted clade name. Comments on name.—Applied as a monotypic genus containing *E. flabellare* (Agassiz 1854, p. 305). Later applied as a subgenus and expanded to include *E. kennicotti, E. virgatum, E. obeyense,* and *E. squamiceps* (Bailey and Gosline 1955, table 1), and most recent applications of *Catonotus* as a subgenus include all species of *Richiella, Oopareia,* and *Stigmacerca* (see below) (Page 1981, 2000; Bailey and Etnier 1988). Definition (node-based).—The least inclusive clade containing *E. flabellare* Rafinesque and *E. virgatum* (Jordan). Reference phylogeny.—Figure 3c. Composition.—Includes all species of *Richiella* and *Oopareia* (see below). Synapomorphies.—Species of *Catonotus* can be diagnosed from all other species of *Goneaperca* by fleshy ridges on ventrolateral scales in breeding males, a widely interrupted infraorbital canal, two pores along the posterior segment of the infraorbital canal, absence of scales on the anterior of the body (e.g., cheek, opercle, and nape), and females remain inverted for an extended period when spawning (Page 1975; Braasch and Mayden 1985; Mayden 1985; Porterfield et al. 1999). Type species.—*E. flabellare* Rafinesque 1819.

Richiella Coker (1927, p. 18) [T.J. Near & B.P Keck], converted clade name. Comments on name.—Applied as a monotypic genus containing *E. brevispinum* and later synonymized with the subgenus *Catonotus* (Bailey and Gosline 1955; Kuehne and Small 1971). Definition (node-based).—The least inclusive clade containing *E. brevispinum* (Coker), *E. kennicotti* (Putnam), *flabellare* Rafinesque, and *Etheostoma percnurum* Jenkins. Reference phylogeny.—Porterfield et al. (1999, fig. 5). Composition.—Includes the species designated in the definition and *Etheostoma humerale* (Girard), *Etheostoma lemniscatum* Blanton, *Etheostoma sitikuense* Blanton, and *Etheostoma marmorpinnum* Blanton & Jenkins. Synapomorphies. —Species of *Richiella* are diagnosed from all other species of *Catonotus* by the presence of enlarged bright golden colored knobs on the first dorsal fin of both males and females (Braasch and Mayden 1985; Porterfield et al. 1999). Type species.—*E. brevispinum* (Coker).

Oopareia T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *E. virgatum* (Jordan 1880), *Etheostoma*

barbouri Kuehne and Small 1971, and *Etheostoma smithi* Page & Braasch 1976. Etymology.—From the Greek words ωον meaning egg and παρεια meaning cheek in reference to presence of a white bar on the cheeks that may serve as an egg mimicking structure (Page 2000). Reference phylogeny.—Figure 3c. Composition.—Includes the species designated in the definition and *E. obeyense* Kirsch 1892, *Etheostoma striatulum* Page and Braasch 1977, *Etheostoma derivativum* Page et al. 2003, *E. basilare* Page et al. 2003. Populations referred to as *E. virgatum* in the Buck Creek system of Kentucky, and populations referred to as *E. basilare* in the Collins River, Mountain Creek, Hickory Creek, Rocky River, Cane Creek, and Calfkiller River may each represent an undescribed species (Page and Braasch 1977; Page et al. 2003; Hollingsworth and Near 2009). Synapomorphies.—Species of *Oopareia* are diagnosed from all other species of *Catonotus* by the presence of an iridescent bar on the cheek, a preopercle with a crenulate edge, and red or orange coloration in the fins of breeding males (Braasch and Mayden 1985). Type species.—*E. virgatum* (Jordan).

Boleosoma DeKay (1842, p. 20) [B.P. Keck & T.J. Near], converted clade name. Comments on name.—Applied as a genus containing *E. olmstedi* and *E. nigrum* (Jordan 1876, p. 221–222, 1877a, p. 15). Later applied as a subgenus and expanded to include *E. perlongum, E. longimanum, E. podostemone,* and species of *Vaillantia* and *Doration* (Bailey and Gosline 1955). Cole (1967, p. 29) treated *Boleosoma* as a subgenus containing *E. nigrum, E. olmstedi, E. podostemone, E. longimanum,* and *E. perlongum.* Definition (node-based).—The least inclusive clade containing *E. nigrum* Rafinesque, *E. olmstedi* Storer, and *Etheostoma vitreum* (Cope). Reference phylogeny.—Figure 3c. Composition.—Includes the species listed in the definition as well as *E. longimanum* Jordan, *E. perlongum* (Hubbs & Raney), *E. podostemone* Jordan & Jenkins, *Etheostoma susanae* (Jordan & Swain), and populations referred to as *E. nigrum* in the upper Kentucky River and Atlantic Slope drainages and populations referred to as *E. olmstedi* from Atlantic Slope drainages from the Waccamaw River and south that may each represent undescribed species (Starnes W.C. and Starnes L.B. 1979; Heckman et al. 2009). Synapomorphies.—Species of *Boleosoma* are diagnosed from all other species of *Goneaperca* by the presence of a bifurcate genital papilla in females and the absence of a premaxillary frenum (Page 1981; Jenkins and Burkhead 1994, p. 852). Type species.—*E. olmstedi* Storer.

Psychromaster Jordan and Evermann (1896, p. 1099): 1099 [B.P. Keck & T.J. Near], converted clade name. Comments on name.—Applied as a monotypic genus containing *E. tuscumbia* (Jordan and Evermann 1896, p. 1099–1100). Definition (node-based).—The least inclusive clade containing, *E. pallididorsum* Distler & Metcalf, *E. punctulatum* (Agassiz), and *E. tuscumbia* Gilbert & Swain. Reference phylogeny.—Figure 3c. Composition.—Includes the species listed in the definition as well as *E. boschungi* Wall & Williams 1974, *E. cragini* Gilbert 1885, *E. mihileze* Mayden, and *E. autumnale* Mayden. Synapomorphies.—Species of *Psychromaster* are diagnosed from all other species of *Goneaperca* by the presence of 3–9 thick dark saddles on the dorsum (Williams and Robison 1980; Etnier and Starnes 1993). Type species.—*E. tuscumbia* Gilbert & Swain.

Stigmacerca T.J. Near & B.P. Keck, new clade name. Definition (node-based).—The least inclusive clade containing *E. squamiceps* Jordan, *Etheostoma pseudovulatum* Page & Ceas, and *Etheostoma nigripinne* Braasch & Mayden. Etymology.—From the Greek words στιγμα meaning a tattoo mark or brand and κερκοσ meaning tail in reference to the three distinct black spots at the base of the caudal fin in species of *Stigmacerca.* Reference phylogeny.—Figure 3c. Composition.—Includes the species in the definition as well as *Etheostoma crossopterum* Braasch & Mayden, *Etheostoma olivaceum* Braasch & Page, *Etheostoma chienense* Page & Ceas, *Etheostoma oophylax* Ceas & Page, *Etheostoma forbesi* Page & Ceas, *Etheostoma corona* Page & Ceas, *Etheostoma neopterum* Howell & Dingerkus, and populations of *E. cf. oophylax* in the Clarks River system of Kentucky and Tennessee. Synapomorphies.—Species of *Stigmacerca* are diagnosed from all other species of *Goneaperca* by the presence of a vertical row of three black spots on the base of the caudal fin, rays of the second dorsal fin in breeding males extend beyond the fin margin, spawning pairs of males and females have a briefly inverted spawning position, and females lay more than one egg at time (Page 1975; Braasch and Mayden 1985; Page et al. 1992). Type species: *E. squamiceps* Jordan.