Phylogeography of Three Snubnose Darters (Percidae: Subgenus *Ulocentra*) Endemic to the Southeastern U.S. Coastal Plain

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The Yazoo Darter, *Etheostoma raneyi* (Percidae: subgenus *Ulocentra*), is a narrowly restricted endemic occurring in small tributaries in the Loessial Hills of the upper Yazoo River basin in northern Mississippi. The range of the species is shared between the Little Tallahatchie and adjacent upper Yocona rivers, but populations in the two rivers are separated by unsuitable habitat in the Mississippi Alluvial Plain. The Chickasaw Darter, *Etheostoma cervus*, and Firebelly Darter, *E. pyrrhogaster*, show analogous distributions in the Forked Deer and Obion rivers, respectively, of western Tennessee and Kentucky. Phylogenetic analyses of cyt *b* and control region mtDNA (1497 sites) data from *E. raneyi* ($n = 12$), *E. cervus* ($n = 4$), and *E. pyrrhogaster* ($n = 5$) recovered two clades of *E. raneyi* with high bootstrap and decay support that are congruent with localities of specimens from the Little Tallahatchie and Yocona drainages, respectively. Divergence between the clades of *E. raneyi* was 1.3% (SE = 0.3%). Within drainage divergence was 0.3% (SE = 0.1%) for the Little Tallahatchie clade and 0.1% (SE < 0.1%) for the Yocona clade. *Etheostoma cervus* and *E. pyrrhogaster* showed interspecific divergence of 1.3% (SE = 0.2%) and intraspecific divergence of 0.7% (SE = 0.2%) and 0.8% (SE = 0.2%), respectively. These results suggest isolation by vicariance as a mode of speciation in fishes restricted to the Upper Coastal Plain. Conservation action may be in order for *E. raneyi* as populations from the Little Tallahatchie and Yocona rivers should be treated as separate management units with the latter known from only five small streams, some of which are threatened by encroaching development.

SNUBNOSE darters of the subgenus *Ulocentra* (Percidae: genus *Etheostoma*), containing at least 21 species, are restricted primarily to streams of the southwestern Ohio and Mobile river basins (Etnier and Starnes, 1993; Warren et al., 2000; Porter et al., 2002; Boschung and Mayden, 2004). *Etheostoma raneyi* (Yazoo Darter), *E. pyrrhogaster* (Firebelly Darter), and *E. cervus* (Chickasaw Darter) are notable exceptions, being restricted to the Tallahatchie, Obion, and Forked Deer river drainages, respectively, which ultimately drain into the lower Mississippi River. Uniquely within *Ulocentra*, the entire range of each of these three species is restricted to small, moderate gradient, sand and fine gravel substrate tributaries draining the Loessial and Clay Hills of the eastern Mississippi Alluvial Valley (Bailey and Etnier, 1988; Suttkus et al., 1994; Keys et al., 1995; Powers and Mayden, 2003). This kind of habitat is found only in the upper reaches of each of the respective drainages in which the darters occur. In this region, the habitat suitable for *Ulocentra* is referred to as the Upper Coastal Plain (Etnier and Starnes, 1993). The swampy, lowlands of the Mississippi Alluvial Plain, through which each occupied river ultimately traverses, is inappropriate habitat for *Ulocentra*. Unlike the higher gradient, contiguous stream habitats of the Upper Coastal Plain, the extensive bottomlands in the lower reaches of these rivers isolate and restrict gene flow among populations of these darters.

We hypothesized that populations or sister species within different, isolated drainages would show genetic substructuring indicative of long-term isolation via vicariance. We tested this hypothesis by evaluating mitochondrial gene phylogeny and variation within *E. raneyi* and between the sister pair *E. pyrrhogaster* and *E. cervus* in relation to their respective geographic isolation by intervening inappropriate lowland habitat. In light of the phylogeographic results and recent field observations, we also summarize and discuss the conservation status of these species.

MATERIALS AND METHODS

Darters were collected using a 3.3 m × 1.3 m seine and a Smith-Root model 12 backpack electrofisher. Whole specimens or tissue samples were either frozen or fixed in 95% ethanol. Sequences of the mitochondrially encoded cytochrome *b* (cyt *b*) and a 357 base-pair (bp) portion of the control region (CR) were obtained from *Etheostoma raneyi* ($n = 12$), *E. cervus* ($n = 4$), and *E. pyrrhogaster* ($n = 5$) from multiple localities in each major drainage within their respective geographic ranges (Fig. 1). Whole genomic DNA was extracted using phenol–chloroform methods following Hillis et al. (1996). The cyt *b* gene was amplified with 35 cycles of PCR using primers annealing to the flanking tRNA genes designed by Song et al. (1998). Denaturation, annealing, and extension temperatures and times were 95°C, 40 sec; 55°C, 60 sec; and 72°C, 90 sec, respectively. Amplified PCR products were purified by either gel extraction using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA) or by centrifugal filtration using Ultrafree-MC 30,000 NMWL filter units (Millipore Corp., Billerica, MA) following manufacturer's directions. Cycle sequencing reactions were read with an ABI 3100 automated sequencer. The same methods were used to obtain CR sequences except primers were from Porter et al. (2002) and annealing temperature was 56°C. Sequences were aligned by eye using Bio Edit (Hall, 1999). The cyt *b* and CR data were combined for analyses. Cytochrome *b* and CR sequences from *E. blennioides* ($n = 1$; subgenus *Etheostoma*), *E. simoterum* ($n = 1$), and *E. lachneri* ($n = 1$; both subgenus *Ulocentra*) were included for outgroup rooting.

Nucleotide frequencies and the transition/transversion ratio were calculated using MEGA2 (Kumar et al., 2001). Phylogenetic hypotheses were generated using NONA (Goloboff, 1999). Heuristic searches were conducted using only equally weighted unambiguous characters. Branches with lengths of zero were collapsed. Support for hypotheses

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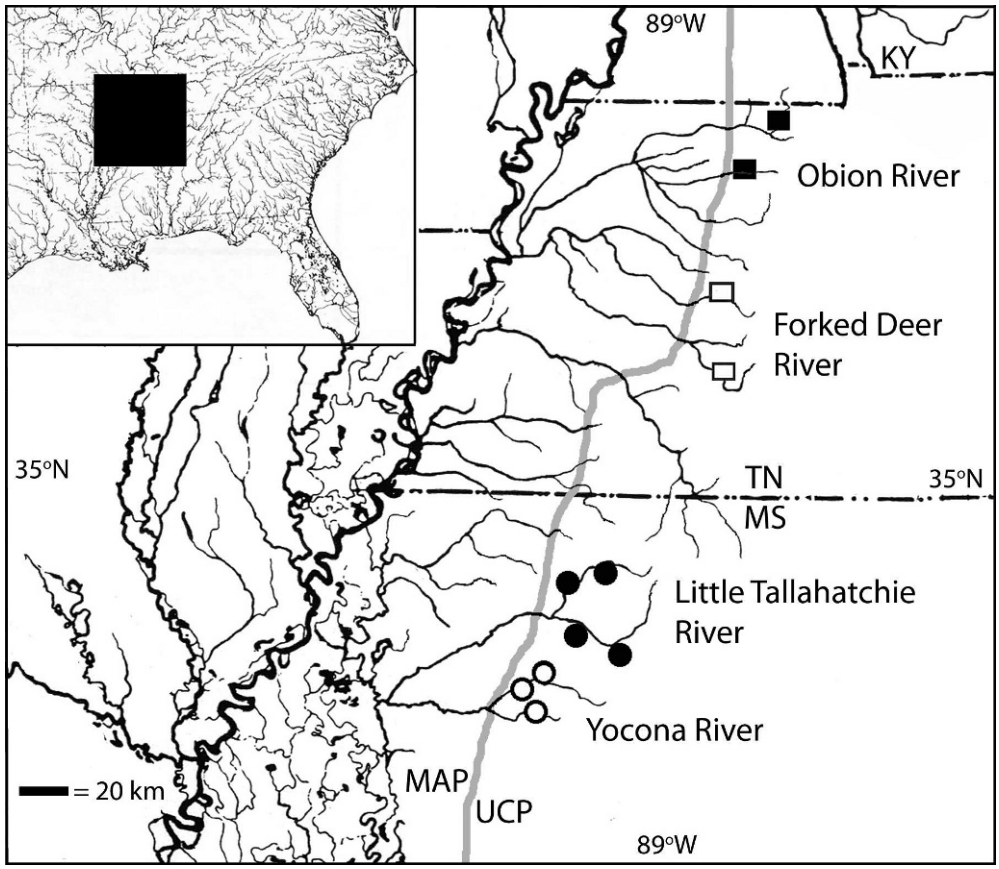

Fig. 1. Map of eastern tributaries to Mississippi River in Tennessee and northern Mississippi with sampling localities for this study. Black boxes are localities for *Etheostoma pyrrhogaster*, white boxes are localities for *E. cervus*, black circles are localities for Little Tallahatchie River populations of *E. raneyi*, white circles are localities for Yocona River populations of *E. raneyi*. The gray line represents the approximate boundary between the Mississippi Alluvial Plain (MAP) and the Loessial and Clay Hills of the Upper Coastal Plain (UCP).

was evaluated by performing 1000 bootstrap replicates (Felsenstein, 1985) with NONA and decay analyses (Bremer, 1994) with Sepal (Salisbury, 2000). Trees were rooted with *E. blennioides*.

Genetic variation of *E. raneyi* from within and among the Yocona and Tallahatchie river drainages was examined by calculating pairwise distances using MEGA2 (Kumar et al., 2001). Within and among pairwise distances also were calculated for *E. cervus* and *E. pyrrhogaster* using the same methods.

RESULTS

Of the 1497 total characters examined, 347 characters were variable, with 225 being parsimony informative. Nucleotide frequencies were as follows: T = 0.305, C = 0.273, A = 0.250, G = 0.172. Transition/transversion ratio was 3.5:1 with 83.8% of all changes occurring at third positions. Eighteen equally parsimonious trees with a length of 524 steps (CI = 0.80, RI = 0.91) were recovered, and a strict consensus tree is presented (Fig. 2). A clade containing all *E. raneyi* was recovered as monophyletic in 100% of bootstrap replicates and had a decay value of 29. Two clades congruent with river drainage affinities of specimens of *E. raneyi* were recovered with high bootstrap and decay support: a Little Tallhatchie River clade and a Yocona River clade. The Little Tallahatchie clade was supported by 100% of bootstrap replicates and had a decay value of 4. The Yocona clade was recovered in 98% of bootstrap replicates and had a decay value of 7. Mitochondrial haplotypes of *Etheostoma cervus* were recovered as paraphyletic, forming a clade with *E. pyrrhogaster* with 100% bootstrap support and a decay value of 28. *Etheostoma pyrrhogaster* was recovered as monophyletic in 90% of bootstrap replicates and had a decay value of 3.

The sister-pair *E. cervus* and *E. pyrrhogaster* and the two drainage-based clades of *E. raneyi* showed higher among than within sequence divergences. Mean among drainage pairwise divergence for *E. raneyi* was 1.3% (SE = 0.3%). Mean pairwise sequence divergence for specimens from the Yocona River drainage was 0.1% (SE = 0%), and mean divergence from specimens from the Little Tallahatchie River drainage was 0.3% (SE = 0.1%). Mean pairwise divergence among *E. cervus* and *E. pyrrhogaster* was 1.3% (SE = 0.2%). Mean pairwise divergence for *E. cervus* was 0.7% (SE = 0.2%), and mean divergence for *E. pyrrhogaster* was 0.8% (SE = 0.2%).

DISCUSSION

We found genetic substructuring in *E. raneyi* that is congruent with the geographic isolation imposed on populations by the Little Tallahatchie and Yocona rivers. The Yocona River clade and Little Tallahatchie River clade were reciprocally monophyletic and each had high bootstrap and decay values. Mean pairwise sequence divergence among the two clades was greater than sequence divergence within each clade, all indicative of genetic divergence of

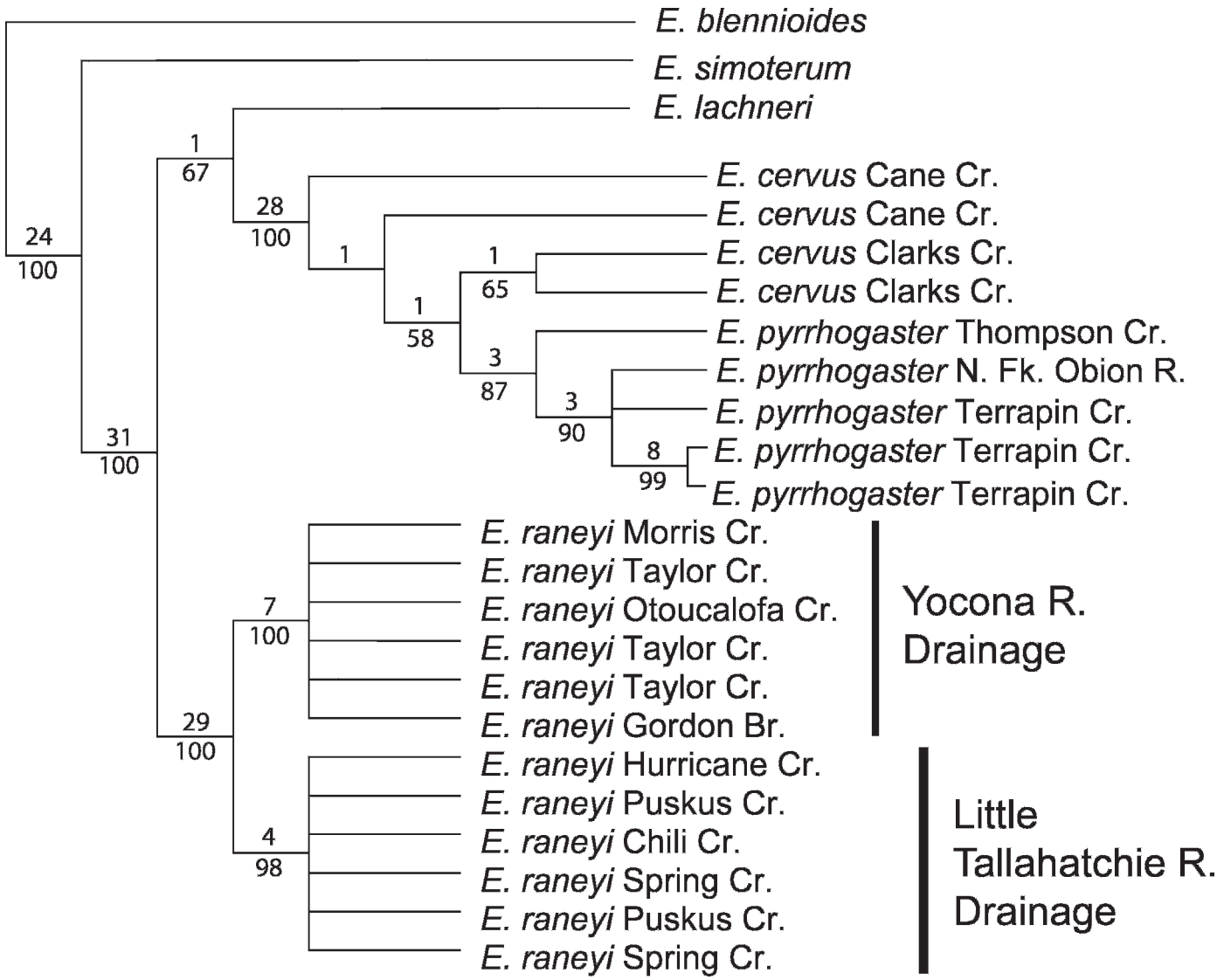

Fig. 2. Strict consensus of 18 equally parsimonious trees (524 steps, CI = 0.80, RI = 0.91) with bootstrap support for nodes greater than 50% listed below branches and decay support above branches.

mitochondrial loci. These phylogeographic patterns provide evidence for recognition of the clades as at least two distinct management units (MUs; Moritz, 2002). We emphasize that classification as management units is tentative and likely minimal from a conservation perspective. Sequence divergence in these two clades, although not exceptionally high at 1.3%, is as high as that observed among *E. cervus* and *E. pyrrhogaster* and comparable to values observed among other distinctive, derived darter species (George et al., 2006). Investigation of other mitochondrial or nuclear genes and phenotype divergence is needed to further clarify the status of clades of *E. raneyi* (e.g., evolutionarily significant units, distinct species, Mayden and Wood, 1995; Waples, 1995).

Recent collections over the entire range of *E. raneyi* indicate the species is known from about 40 sites distributed throughout 21 tributary streams (Warren et al., 2000, 2002). Most of the occupied sites, however, lie in the Tallahatchie River drainage. In that drainage, 22 sites inhabited by *E. raneyi* are within or just downstream of lands in national forest ownership, ostensibly providing some assurance of maintenance of habitat integrity. The majority of sites in the Tallahatchie drainage yielded less than five individuals per 100 m of stream surveyed, but catches of 9–35 individuals per 100 m were not uncommon, particularly in spring-fed streams in the Tippah River, Big Spring Creek, and Cypress Creek watersheds. Populations in the Yocona River drainage are much more restricted, being confined to five small headwater tributaries, at least two of which are in the direct path of urban development associated with rapid growth of Oxford, Mississippi. Most Yocona populations appear to be small, but two sites in one tributary, just downstream of national forest lands, yielded two to nine individuals per 100 m. *Etheostoma raneyi* appears to be most secure in the Little Tallahatchie drainage, but the relatively small and fragmented range of the Yocona clade and imminent exposure to stream degradation from urbanization renders it vulnerable to severe decline if not extinction. We make the following conservation recommendations: populations of *E. raneyi* from the Yocona and Little Tallahatchie river drainages should be considered different MUs, and highest priority should be given to efforts to preserve each of them by state and federal management agencies; and each MU should be considered imperiled and stream surveys should be conducted in extant and historical localities to refine

assessments of the relative size of each population of *E. raneyi* throughout its range.

The paraphyly of haplotypes of *E. cervus* in relation to *E. pyrrhogaster* in our mtDNA analysis would prevent its recognition as a distinct species under strict interpretation of species concepts that rely on reciprocal monophyly as the primary criterion for recognition. However, the nodes rendering haplotypes of *E. cervus* as paraphyletic all have low decay indices and weak bootstrap support. Furthermore, at least some genetic models suggest the time to coalescence for species following a speciation event is approximately four times the number of generations as number of individuals making up the genetically effective population of the species (Avise, 1994). Although snubnose darters in the Clay and Loessial hills of the Mississippi Alluvial Valley apparently are not as abundant as some snubnose darter species in the Nashville Basin (5.38 individuals per m^2, Page and Mayden, 1981), a life history study revealed *E. pyrrhogaster* as one of the most abundant species in Terrapin Creek, Kentucky (Carney and Burr, 1989). Similarly, in collecting efforts by Powers and Mayden (2003), *E. pyrrhogaster* and *E. cervus* were repeatedly collected in series of 20 or more individuals of each species from less than 100 m of stream and less than one hour of collecting effort, which is indicative of relatively large population sizes for both species. Assuming large population sizes of these two species, many generations would be required following a speciation event for mtDNA of each to reach reciprocal monophyly.

Similarly, a phylogenetic analysis of mtDNA from snubnose darters recovered several species as paraphyletic and even polyphyletic (Porter et al., 2002). Within the *E. simoterum* complex, multiple species are diagnosable by meristic, morphometric, and pigmentation differences, but are non-monophyletic for mtDNA (Powers and Mayden, 2007). Another species non-monophyletic for mtDNA, *E. bellator* (Porter et al., 2002), appears to contain three distinct species diagnosable by fixed allozyme and morphological differences (Clabaugh et al., 1996) further illustrating that *Ulocentra* biodiversity is likely underestimated by the current taxonomy rather than overestimated. The presumably strong sexual selection in the sexually dimorphic *Etheostoma* apparently causes characters such as nuptial male pigmentation to evolve more rapidly than characters evolving largely by genetic drift such as mtDNA (Mendelson, 2003). Strict application of species concepts requiring monophyly for any particular gene would cause a major shift in the current paradigm of the biodiversity of *Ulocentra* and result in the synonymy of 11 of the currently recognized species. If placed in synonymy, hypotheses explaining the dramatic morphological differences, often in presumably sexually selected characters, among allopatrically distributed populations of the ''same species'' would also need to be developed as these differences are currently considered the results of evolutionary change following speciation events.

In agreement with others (Porter et al., 2002; Powers and Mayden, 2007), we conclude that the most parsimonious explanation for apparent incongruence among morphological and mtDNA data in *Ulocentra*, including *E. cervus* and *E. pyrrhogaster*, is that large populations following speciation events require many generations for mtDNA and likely other genetic markers to reach reciprocal monophyly. Our results for relationships of mtDNA haplotypes of *E. pyrrhogaster* and *E. cervus* are consistent with those of many other species of *Ulocentra* and do not refute the hypothesis that the Forked Deer and Obion River snubnose darters represent different evolutionary species isolated by a relatively recent speciation event. The greater pairwise divergence between species than within each species is congruent with differences between the species in meristic, morphometric, and nuptial male pigment characters (Powers and Mayden, 2003).

The simplest explanation of the reduced gene flow between the MUs of *E. raneyi* and the relationships of *E. cervus* and *E. pyrrhogaster*, is a single vicariant event. These fishes are restricted to clear, moderate-gradient streams of the Upper Coastal Plain with sand and fine gravel substrate. The predominant geology of this region is loess overlying rocks dating to the Eocene. The downstream reaches of the Tallahatchie, Obion, and Forked Deer river drainages transect the Mississippi Alluvial Plain where streams are lower gradient, turbid, and have substrate primarily of fine sediments of late Pleistocene origin (Grub and Carillo, 1988). Pleistocene glaciation reduced atmospheric moisture which in turn reduced stream size and turbidity. The lowering of sea levels also increased stream gradient in the lower Mississippi and its tributaries possibly permitting gene flow among populations of species now confined to more upland streams (Robison, 1986). Presumably, *E. raneyi* and the ancestor to *E. cervus* and *E. pyrrhogaster* occupied these more upland Pleistocene streams. The advance and melting of Pleistocene glaciers and the resulting outflow of fine sediments is hypothesized as a vicariant event separating fishes in the Eastern and Central Highlands (Wiley and Mayden, 1985; Mayden, 1988a). This was also the hypothesized mode of speciation for the *Luxilus zonatus* species group in western tributaries to the Mississippi River (Mayden, 1988b). However, we are unaware of any hypotheses of vicariance for species restricted to the direct eastern tributaries of the Mississippi River. Increased turbidity and decreased gradient of streams of the Mississippi Alluvial Plain appears to have eliminated gene flow in upland species through the lower reaches of these direct tributaries to the Mississippi River, leaving *E. cervus*, *E. pyrrhogaster*, and both MUs of *E. raneyi* isolated in the permanent, mostly upland streams of the Loessial and Clay Hills.

MATERIAL EXAMINED

Sequences downloaded from GenBank are listed with only GenBank accession numbers. Voucher specimens collected by the authors were deposited in the University of Alabama Ichthyological Collection (UAIC) and are listed with drainage, accession number, and GenBank accession numbers as follows:

Etheostoma blennioides: (GenBank AF288426, AF404523).

Etheostoma cervus: TN, Henderson Co., Cane Creek (UAIC 13199.10, GenBank FJ423441–423442, $n = 2$), TN, Chester Co., Clarks Creek (UAIC 13569.12, GenBank FJ423443–423444, $n = 2$).

Etheostoma lachneri: (UAIC 13021.02, GenBank FJ423445).

Etheostoma pyrrhogaster: KY, Graves Co., Terrapin Creek (UAIC 10602.10, GenBank FJ423440, $n = 1$), TN, Henry Co., Terrapin Creek (UAIC 13214.14, GenBank FJ423438–423439, $n = 2$), TN, Weakley Co., Thompson Creek (UAIC 13570.05, GenBank FJ423437, $n = 1$), GenBank AF288438, AF404547.

Etheostoma raneyi: MS, Lafayette Co., Taylor Creek (UAIC 14931.01, GenBank FJ423426–FJ423428, $n = 3$), MS, Yalobusha Co., Gordon Branch (UAIC 14932.01, GenBank

FJ423432, $n = 1$), MS, Yalobusha Co., unnamed tributary to Otoucalofa Creek (UAIC 14933.01, GenBank FJ423433, $n = 1$), MS, Lafayette Co., Morris Creek (UAIC 14934.01, GenBank FJ423425, $n = 1$), MS, Benton Co., South Fork Chili Creek (UAIC 14935.01, GenBank FJ423421, $n = 1$), MS, Lafayette Co., Puskus Creek (UAIC 14936.01, GenBank FJ423430, FJ423434, $n = 2$), MS, Marshall Co., unnamed tributary to Spring Creek (UAIC 14937.01, GenBank FJ423429, FJ423435, $n = 2$), MS, Lafayette Co., Hurricane Creek (UAIC 14938.01, GenBank FJ423436, $n = 1$).

Etheostoma simoterum: (UAIC 12480.02, GenBank DQ089050, DQ089075).

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