CONSERVATION AND PHYLOGEOGRAPHY OF THE REDBAND DARTER, ETHEOSTOMA LUTEOVINCTUM (PERCIDAE)

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To the College of Graduate Studies:

We are submitting a thesis written by Matthew D. Wagner entitled "Conservation and Phylogeography of the Redband Darter, *Etheostoma luteovinctum*." We have examined the final copy of this thesis for form and content. We recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science in Biology.

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ABSTRACT

MATTHEW D. WAGNER. Conservation and Phylogeography of the Redband Darter, *Etheostoma luteovinctum* (Percidae) (under the direction of DR. REBECCA JOHANSEN.)

The Redband Darter, *Etheostoma luteovinctum*, is a benthic fish species distributed across the headwaters of the Caney Fork and Stones rivers (Cumberland River drainage) and the Duck and Elk rivers (Tennessee River drainage) of central Tennessee. Historically, the Redband Darter has been regarded as a species of special concern due to its small native range, but has been recently designated as stable. However, no recent assessment of the status of the species has been conducted Morphological and genetic variation have been noted in the species, but whether the variation indicates unrecognized species diversity remains unclear and no study has been published that documents the genetic diversity or phylogeographic relationships of extant populations

The objectives of this study were to 1) evaluate the current status of the species across its range to determine whether the recent stable designation is appropriate, 2) evaluate both phenotypic and genetic variation to identify any currently unrecognized taxonomic diversity, 3) provide the first hypothesis of the phylogeographic relationships of populations, 4) test previous hypotheses of stream capture in the focal region, and 5) test the utility of a relatively unexplored data type, amplified fragment length polymorphisms (AFLPs), in taxonomic and phylogeographic studies.

The current status of the Redband Darter was assessed by identifying and collecting all known historical localities (with a representative sub-sampling in the Duck R.) and collecting other nearby habitat-appropriate sites to document presence or absence

at each locality. Phenotypic variation was evaluated using standard meristic data and nuptial male color and pigmentation characteristics. Amplified fragment length polymorphisms (AFLPs) which have been used to assess genetic variation and phylogenetic relationships among closely related species of darters, but which have not been thoroughly explored in species-level taxonomic or phylogeographic studies, were generated using previously published primers to evaluate genetic variation across the range of the Redband Darter. These data were also used to estimate phylogeographic relationships and test previously proposed hypotheses of stream capture events in the study area.

Phylogentic analyses of the resulting 2601 AFLP fragments recovered two divergent and geographically defined clades; however, clades were not restricted to drainage or system boundaries and patterns suggest system and drainage transfers resulting from stream capture or movement through groundwater connections have played an important role in the history of the species. Significant genetic structure between the two clades also was observed based on populations-level analyses of Fst values generated from the AFLP data, further supporting the presence of two distinct genetic lineages. . Although some morphological traits varied between the two clades, members were not clearly diagnosable using morphology. Although taxonomic recognition is not proposed for the two identified clades, herein each clade should be recognized as an evolutionarily significant unit and regarded as such in future conservation efforts. This consideration is important given that the species was not found at 35.6% of historical localities, indicating the stable status of the species is no longer valid.

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CHAPTER I

INTRODUCTION

Darters are one of the most speciose groups of fishes in North America having their greatest diversity in the Southeastern United States. These small, benthic fishes commonly display sexual dimorphism, with males of many species becoming brilliantly colored in the breeding season (Page, 1983). Of the currently recognized species of darters, 44% are imperiled (Jelks et al., 2008). Tennessee is home to a rich diversity of darter species, including several endemic and imperiled species. Ranges of such species commonly are small and restricted to physiographic regions (Starnes and Etnier, 1986).

The Redband Darter, *Etheostoma luteovinctum* (Gilbert and Swain, 1887) is a Tennessee endemic, restricted to the Eastern Highland Rim and Inner and Outer Nashville Basin, known only from the Cumberland and Tennessee River drainages (Figure 1). Within the Cumberland River drainage, the species occurs in headwater streams of the Stones and Caney Fork River (CFR) systems, and several small direct Cumberland River tributaries (Sulphur, Eaton, and Spring Creeks). Within the Tennessee River drainage, *E. luteovinctum* is known from headwater streams of the Duck River system and a single locality in the Elk River system, Town Creek (Richland Creek-Elk R.).

This interesting inter-drainage distribution includes populations potentially isolated across drainage boundaries, as well as at smaller spatial scales. For example, the *Etheostoma squamiceps* species group (Page et al. 1992) and the Barcheek Darter species group (Page et al., 2003; Hollingsworth and Near, 2009) show examples of microendemism within drainages of the region. Others, like the *Etheostoma cinereum*

species complex, have distinct morphological and genetic lineages occurring in each of the drainages (Powers et al., 2004; Powers et al., 2012). The variation observed in other fishes that share this inter-drainage distribution suggests the potential for unrecognized diversity in *E. luteovinctum*.

In addition to its small range, *E. luteovinctum* is impacted by a variety of land use practices. The predominant land uses in the Eastern Highland Rim and the Outer Nashville Basin portions of its range are pasture and cropland, while the Inner Nashville Basin is dominated by land cleared for urban development, as well as pasture and cropland (Arnwine et al., 2003; Arnwine et al., 2005). These land uses are commonly associated with increased runoff that leads to increased turbidity and sedimentation in streams. This sediment load adds to the already rich nutrient loads, which are attributed to the high phosphorus content of the regional rocks and soils (Holland et al., 2003). The increased nutrient load commonly results in the growth of filamentous algae, aquatic mosses, and occasionally high densities of isopods. Increased turbidity and resulting sedimentation is particularly detrimental to fishes that use colorful mating displays to attract mates and use clean substrate to bury their eggs (Berkman and Rabeni, 1987; Rabeni and Smale, 1995). *Etheostoma luteovinctum* relies on visual cues for mate recognition in the spawning season. Most notably, the males of the species display large amounts of red-orange and blue-green pigmentation in mating displays to attract females. Increased turbidity can reduce visibility, which, in turn, can negatively affect mating opportunities. *Etheostoma luteovinctum* is also a known egg burier that uses the small amounts of clean, loose gravel substrate available in the primarily bedrock streams they occupy to spawn (Paxton, 1998). This increased sediment load can compress and

compact the available substrate potentially restricting the amount of substrate available for spawning (Berkman and Rabeni, 1987; Rabeni and Smale, 1995). The increased sediment load is also detrimental to the survival of benthic macroinvertebrates, which are a primary food source for fishes (Wood and Armitage, 1997).

Additionally, *E. luteovinctum* occurs in streams that frequently dry during low flow periods and droughts, potentially causing the extirpation of populations. Given that populations of *E. luteovinctum* are highly disjunct, recolonization of extirpated populations from adjacent source populations may be difficult. Furthermore, the ability to recolonize may be impeded by migrational barriers, such as larger streams or dams. Larger streams or rivers act as a potential migrational barriers for obligate headwater darters (Starnes and Etnier, 1986), as corridors with increased depth have been shown to limit darter movement (Hoger, 2012). The genetic structure among populations of other headwater darter species supports the theory that larger streams and rivers act as migrational barriers, thus restricting gene flow (Fluker et al., 2011). However, large rivers are not absolute migrational barriers for some darter species, such as *E. proeliare* (Lang and Echelle, 2011).

Dams, which are common in the region, have been shown to act as migrational barriers to other species of darters (Haponski et al., 2007; Beneteau et al., 2009) as well as other small stream fishes (Skalski et al., 2008). Other species including *Notropis rupestris*, *Hemitremia flammea*, *Fundulus julisia*, *Forbesichthys agassizii*, and *Etheostoma forbesi* which are all obligate headwater species and share a similar distribution with *E. luteovinctum*, are also current conservation concerns.

Currently, *E. luteovinctum* is designated by the state of Tennessee as "in need of management". Previously, Deacon et al. (1979) recognized *E. luteovinctum* as a species of special concern. However, Jelks et al. (2008) no longer regarded *E. luteovinctum* as a species of special concern due to an "improved status". Data to document the status improvement was not provided. Furthermore, Etnier and Starnes (1993) commented that although the fish has a restricted range, it is under no immediate threat because it is locally common. However, recent conclusions regarding the stability of *E. luteovinctum* were not based on a comprehensive status survey, as no such work has been conducted. Given the lack of a recent survey of *E. luteovinctum*, its limited range, and the known impacts to its habitat, which have led to prior conservation concerns, an updated survey is needed to adequately assess the current status of the species. Thus, one objective of this study was to survey historical localities to assess the current status of *E. luteovinctum* to test the current assumption that *E. luteovinctum* is a stable species.

Previous Studies of the Focal Species, *Etheostoma luteovinctum*:

The relationship of *Etheostoma luteovinctum* to other species of darters has varied depending on the data type used. Historically, *E. luteovinctum* was placed in the subgenus *Oligocephalus* (Bailey and Gosline, 1955; Page, 1981; Kuehne and Barbour, 1983; Bailey and Etnier, 1988; Shaw, 1996) based on morphology. The most recent study based on morphology (Shaw, 1996) found *E. luteovinctum* sister to *E. spectabile* and contained within the *E. caeruleum* group of *Oligocephalus*. The synapomorphies that characterize the subgenus *Oligocephalus* and that are shared with *E. luteovinctum* include: males with brilliant nuptial colors of reds, blues, and greens on the body, and presence of a blue, green, or dusky margin with a reddish or orange submarginal band on

the first dorsal fin (Page 1981; Bailey and Etnier 1988). Geographic variability in pigmentation has been used to describe multiple species within *Oligocephalus* (Ceas and Page, 1997; Ceas and Burr, 2002) and should be considered when analyzing potential diversity within *E. luteovinctum*.

One of the earliest studies using molecular markers, that included *E. luteovinctum*, examined a 500 base pair portion of the mitochondrial control region and recovered *E. luteovinctum* sister to *E. exile* and within *Oligocephalus* (Turner, 1997). In an analysis of the mitochondrial cytochrome *b* gene, Mendelson (2003) recovered *E. luteovinctum* in a clade containing *E.collettei*, *E. radiosum*, and the *E. caeruleum* group; while Near et al. (2011) using the same gene, but with different taxa included found *E. luteovinctum* sister to *E. exile*. With an analysis of the mitochondrial ND2 gene, Lang and Mayden (2007) found *E. luteovinctum* to be sister to *E. exile* and suggested they both be considered part of *Oligocephalus*. Contrary to the relationship to *E. exile* inferred from mitochondrial DNA, Lang and Mayden (2007) and Near et al. (2011) both found *E. luteovinctum* to be sister to *E. asprigene* using the first intron of the S7 nuclear ribosomal gene, but with low statistical support. Smith et al. (2011) using amplified fragment length polymorphisms generated a darter phylogeny and found *E. luteovinctum* to be sister to *E. exile* and contained within the subgenus *Oligocephalus*. In summary, most of these studies have concluded that *E. luteovinctum* is part of the subgenus *Oligocephalus* and most closely related to *E. exile*.

Variation within *E. luteovinctum* has been previously evaluated (Rogner, 1981) using both morphology and allozymes, but all extant populations were not included in this analysis. Based on allozymes, Rogner concluded that the populations in the Duck

River system and Hickory Creek (CFR) varied from the populations in the Stones River system. However based on an analysis of morphological variation he concluded that the populations in the Stones and Duck River systems were similar and differed from those in Hickory Creek (CFR). No taxonomic decisions were proposed. Lang (unpublished, pers. comm.) used the mitochondrial ND2 gene to examine genetic variation in *E. luteovinctum* and proposed four instances of genetic exchange among populations across systems (Figure 2) including: 1) Duck R. to Elk R., 2) Duck R. to Hickory Creek (CFR), 3) Stones R. to Barren Fork (CFR), and 4) Stones R. to Marshall Creek (CFR). Both Rogner (1981) and Lang invoked headwater transfer due to the karst topography of the region to explain these inter-system relationships. These studies provide a baseline of information on variation in *E. luteovinctum*, but have not thoroughly explored the potential diversity within the species. Thus, examination of representative populations from the entire range of the species for comparison of morphological and genetic variation, including use of other non-mitochondrial markers, is needed to further evaluate potentially unrecognized diversity. Understanding patterns of genetic variation is of particular interest, given the conservation concern for the species.

Genetic variation in darters has been historically assessed using allozymes (e.g. Wood and Mayden, 1997), mitochondrial markers (e.g. Turner, 1997; Song et al., 1998; Near et al., 2000; Near, 2002; Porter et al., 2002; Sloss et al., 2004; Near and Keck, 2005; Mayden et al., 2006), nuclear genes (e.g. Keck and Near 2008; Hollingsworth and Near 2009) or a combination of both nuclear genes and mitochondrial loci (e.g. Lang and Mayden 2007). Recently, the use of amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995), a multi-locus genetic dataset, have been used to generate phylogenies

and has become a popular alternative to mitochondrial genes for examining relationships of closely related species. This technique uses restriction endonucleases to cut genes from across the entire genome of an organism into fragments. A subset of the resulting fragments are then amplified using specific primer pairs and scored as either present or absent for each individual. The resulting presence/absence matrix is then used to reconstruct phylogenetic relationships.

Amplified fragment length polymorphisms have been used to investigate relationships in plants (Pelser et. al., 2003), arthropods (Mendelson and Shaw, 2005), and fishes (Allender et al., 2003; Sullivan et al., 2004). Recent studies have utilized AFLPs to examine interspecific darter relationships (Mendelson and Simons 2006, Mendelson and Wong 2010), as well as deeper relationships within darters (Smith et al., 2011). Only one study (Johnson, 2009) used AFLPs to examine variation in darters at the population level. Amplified fragment length polymorphisms have been used in phylogeographic studies of vertebrates (e.g. Wooten et al., 2010; Strickland, 2011), but no studies have used AFLPs to examine phylogeographic relationships of darters or to test biogeographic hypotheses. Such studies of darters have historically relied almost exclusively on mitochondrial DNA (Simons, 1989; Wiley and Hagen, 1997; Near et al., 2001; Ray et al., 2006). This has been largely due to few alternative genetic markers that provide resolution among populations at low levels of divergence (Avise, 1994; Faber and Stepien, 1997). Amplied fragment length polymorphisms provide a viable alternative that has not yet been utilized in phylogeographic studies of darters or other North American riverine fishes.

Objectives:

The lack of studies using AFLPs to look at darters at the intraspecific level validates that further research is needed to assess their utility as a marker for use in phylogeographic studies. This study aimed to (1) to fill gaps left by previous studies by generating an amplified fragment length polymorphism (AFLP) dataset, including populations that represented the entire range of the species, to compare observed patterns of genetic variation based on AFLPs to that previously proposed with mitochondrial DNA (Lang, pers. comm.) and allozymes (Rogner, 1981). (2) Use the AFLP data to reconstruct phylogeographic relationships and test previous cross-drainage transfer hypotheses, as to further evaluate of the utility of AFLPs in phylogeographic studies. (3) Assess variation in meristic characters and nuptial male pigmentation and color to evaluate how phenotypic variation relates to genetic variation observed from AFLPs. (4) Lastly, evaluate the current status of the species across its range to determine whether the recent stable designation is appropriate.

Hypotheses:

1. Null hypothesis: *Etheostoma luteovinctum* is a stable species that does not require additional conservation measures.

   Prediction: *Etheostoma luteovinctum* is not a stable species and a reduction in range will be observed as a result of historical and ongoing land use practices.

2. Null hypothesis: *Etheostoma luteovinctum* is a single clade of populations with no diagnostic morphological features.

   Prediction: Inferences from studies of mitochondrial DNA (Lang, pers. comm.) and allozymes (Rogner, 1981) indicate that there are genetic differences between

populations in the Cumberland and Tennessee River drainages. Therefore, it is predicted that these two drainages will be recovered as two clades in the phylogenetic analyses. It is predicted that there may be diagnostic morphological characters, such as previously unexamined pigment and color characters, at the stream, river, or drainage level as the complete range of the species has not been previously examined for differences.

3. Null hypothesis: Amplified fragment length polymorphisms will not provide sufficient resolution to examine phylogeographic relationships of *E. luteovinctum*.

   Prediction: Amplified fragment length polymorphisms will provide sufficient resolution to examine phylogeographic relationships as they have in other non-fish groups focused at the intraspecific level.

4. Null hypothesis: Cross stream/system/drainage transfers have played no role in the history of *E. luteovinctum*.

   Prediction: Patterns of genetic variation observed in mitochondrial DNA (Lang, pers. comm.) and allozymes (Rogner, 1981) suggest transfers have played a role in the history of the species. Thus it is predicted that AFLP data will highlight the role of several cross stream/system/drainages transfers (Figure 2) that have shaped the current distribution and patterns of genetic diversity in *E. luteovinctum*.

Figure 1. Historical distribution of the Redband Darter, *Etheostoma luteovinctum*. Specific locality information is given in Appendix B.

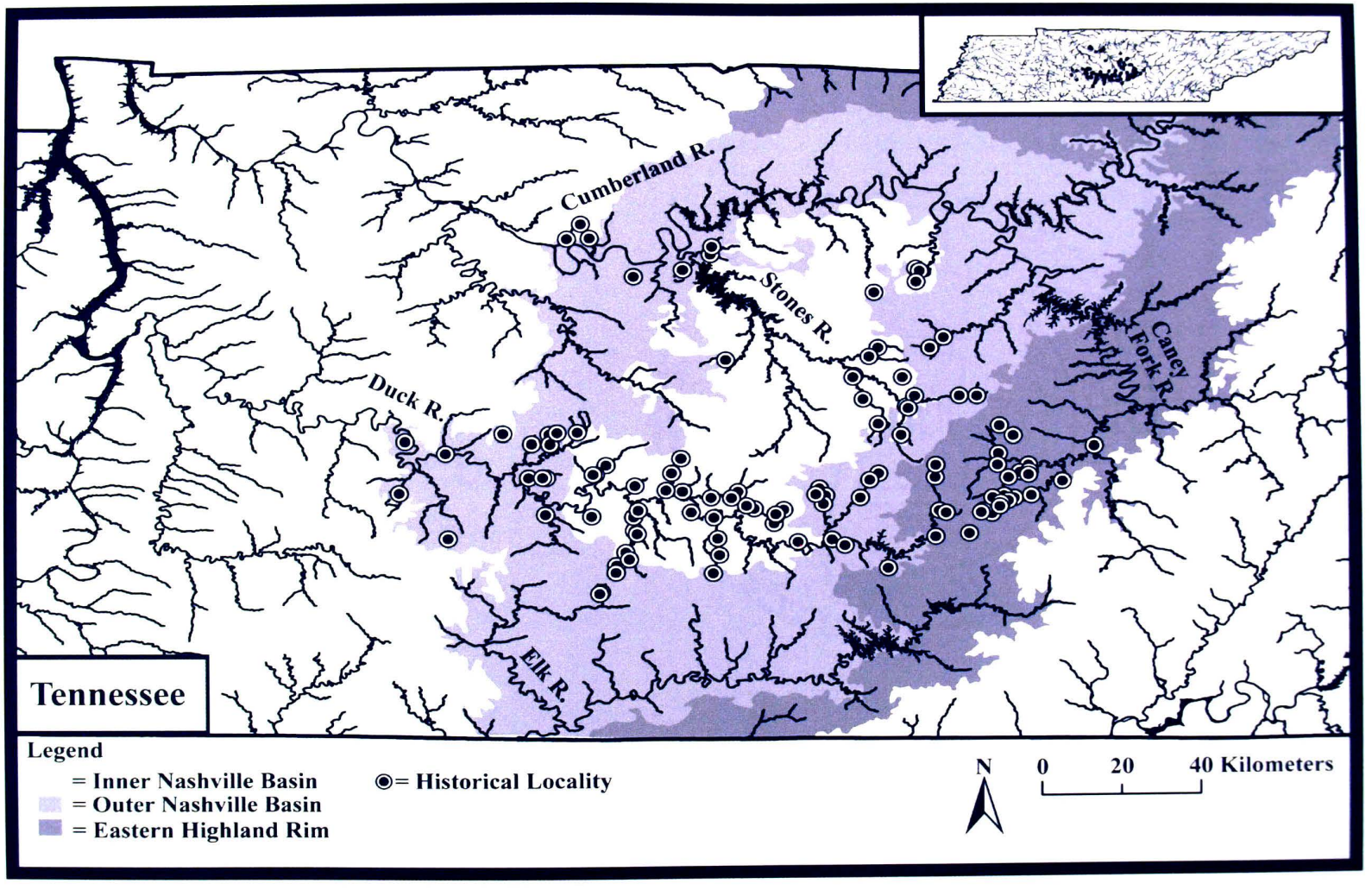
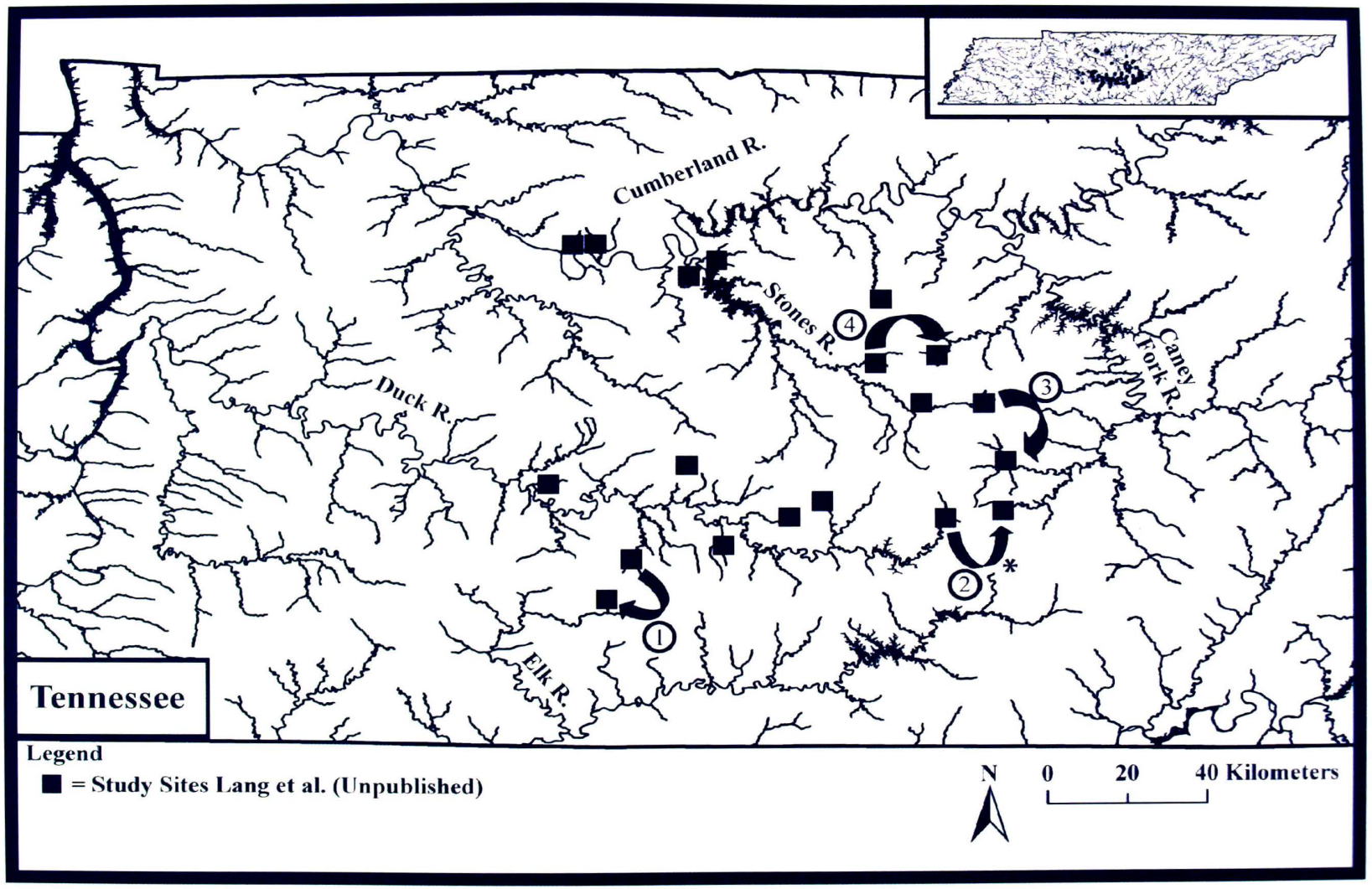

Cumberland R.
Stones R.
Caney Fork R.
Duck R.
Elk R.
Tennessee
Legend
= Inner Nashville Basin
= Outer Nashville Basin
= Eastern Highland Rim
= Historical Locality
N
0
20
40 Kilometers

Figure 2. Proposed instances of system transfers involving *E. luteovinctum* as recovered from analysis of the mitochondrial ND2 gene: 1) Duck R. to Elk R., 2) Duck R. to Hickory Creek (CFR), 3) Stones R. to Barren Fork (CFR), and 4) Stones R. to Marshall Creek (CFR). Modified from Lang et al. (unpublished, pers. comm.). *Proposed instance of system transfer involving *E. luteovinctum* as recovered from analysis of allozymes (Rogner, 1981).


Cumberland R.
Stones R.
Duck R.
Caney Fork R.
Elk R.
1
2
3
4
*
Tennessee
Legend
■ = Study Sites Lang et al. (Unpublished)
N
0
20
40 Kilometers

CHAPTER II

MATERIALS AND METHODS

Localities Examined and Collection Methods:

Historical localities for *Etheostoma luteovinctum* were obtained from various institutions and organizations (Appendix B lists museum accession numbers and locality information for all specimens and tissues examined). All historical localities obtained were geo-referenced using Geolocate (Rios and Bart, 2010) and a Tennessee Atlas and Gazetteer (DeLorme, 2007).

Specimens and tissues examined were collected using a 30 minute sampling period, a 2 meter x 4 meter (0.64 centimeters mesh) seine, and an backpack electrofisher or were borrowed from various institutions (See Appendix B). For tissues collected personally, either whole individuals or fin clips were placed in 15 mL conical vials of 95% Ethanol. Voucher specimens were retained for all tissues collected. All specimens collected for morphological analyses were euthanized with MS-222 and then were preserved in 10% formalin. Specimens were transferred to 70% ethanol for permanent storage in the David H. Snyder Museum of Zoology at Austin Peay State University.

Status Survey:

All identified historical localities in the Cumberland River drainage were sampled for *E. luteovinctum*, with the exception of Locke Branch (Caney Fork River system), which was inaccessible. Localities that were sampled in the Tennessee River drainage included a sub-set of localities encompassing the entire range of *E. luteovinctum* within the Duck River system and the individual locality within the Elk River system.

Localities not sampled were considered to have *E. luteovinctum* present as a conservative estimate of current range. A total of 21 additional habitat-appropriate localities were sampled across the known historical range to find potential new localities for the species. Appendix B provides specific locality information for all localities sampled. Results of the status survey were recorded in Microsoft Excel (Microsoft, 2010) and imported into ArcMap version 9.2 (ESRI, 2009) to generate distribution maps.

Amplified Fragment Length Polymorphisms:

DNA Extractions- A Qiagen DNEasy Blood and Tissue kit was used to extract whole genomic DNA from 97 individuals from 27 localities (Figure 3). Manufacturer's instructions were followed, except 80 µL of elution buffer was used to yield a greater concentration (minimum 200 ng/µL) of genomic DNA. The DNA was then quantified using a Nanodrop ND-1000 Spectrophotometer to ensure the concentrations were 200 ng/µL or greater. If the concentration was greater than 200 ng/µL, samples were diluted to 200 ng/µL using Qiagen Elution buffer.

Digestion-Ligation Reaction- A simultaneous digestion-ligation reaction using restriction enzymes EcoRI and PstI was conducted to digest genomic DNA (Figure 4, Part A) and ligate two double stranded adapters to the EcoRI and PstI cut sites of the digested DNA product (Figure 4, Part B; Vos et al., 1995; Mendelson and Shaw, 2005; Mendelson and Simons, 2006). Double stranded adapters, designed by Vos et al. (1995; Table 1), were prepared by combining 5.0 µL of the forward and reverse oligos (at 2.0 mM) and incubating at 85 °C for 15 minutes. The reaction was then left to cool slowly to room temperature and then diluted to a 100.0 uM (add 90 µL dH_2O) stock solution. The 100.0

uM stock was stored at -20 °C and a 5.0 uM working solution (190 µL dH_2O to 10 µL 100.0 uM stock) was mixed for the digestion/ligation reaction

Total reaction volume for each digestion-ligation reaction was 11.0 µL and included: 1.1 µL NaCl (0.5 M), 0.5 µL BSA (NEB 100X; 1.0 mg/mL), 1.1 µL 10X T4 Ligase Buffer, 1.0 µL Pst Adapter (50.0 uM), 1.0 µL EcoR1 Adapter (5.0 µM), 0.25 µL PstI enzyme (20,000 U/mL), 0.25 µL EcoR1 enzyme (20,000 U/ml), 0.33 µL T4 Ligase, and 5.5 µL DNA (200.0 ng/µL). All reagents were kept on ice until added to the digestion/ligation mixture. Reactions were spun briefly to mix and then kept on ice until placed in a thermocycler for 5 hours at 37 °C with the heated lid disabled. Resulting products were diluted (1:10; 99.0 µL dH_2O to 1.0 µL product) and stored at 4 °C. Products were visualized on an ethidium bromide stained 1.5% agarose gel run at 100 volts. Each well contained 5.0 µL of 1:10 dilution of the digestion-ligation product and 2.0 µL of Promega 6x Blue/Orange loading dye.

Pre-Selective PCR- Pre-selective PCR primers for the pre-selective PCR reactions matched the ligated adapters, but had an additional nucleotide (EcoRI: 5'-3'+A and PstI: 5'-3'+A; Table 1). The pre-selective PCR Master Mix included: 60.0 µL MgCl (50 mM), 200 µL dNTP (1.25 mM), 200 µL 10x PCR buffer, and 720 µL dH_2O. Reagents were mixed ahead of time and stored at 4 °C.

Each pre-selective PCR reaction included: 5.9 µL pre-selective PCR Master Mix, 1.0 µL PstA1+A primer (10 ng/µL), 1.0 µL EcoR1+A primer (10 ng/µL), 0.1 µL Taq (5 U/µL), and 2.0 µL of the 1:10 dilution of the digestion-ligation product. All components were kept on ice until ready for use.

Reactions were spun briefly to mix and then kept on ice until placed in a thermocycler at 95 °C with the heated lid enabled. Thermocycler conditions were as follows: initial denaturation of 95 °C for 1:30, followed by 21 cycles of 95 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min. Tubes were removed from the thermocycler and placed on ice. A pre-selective PCR stock solution was made by diluting products 1:10 (90 µL dH_2O to 10 µL pre-selective PCR product). In a new 200 uL PCR tube, 10 µL of the diluted pre-selective PCR product and 30 µL of ultrapure dH2O were mixed to create a 1:40 dilution to serve as the template for the selective PCR reactions.

Products from the 1:40 dilution were visualized on an ethidium bromide stained 1.5% agarose gel run at 100 volts. Each well contained 5 µL of the 1:40 diluted pre-selective PCR product and 2 µL of Promega 6x Blue/Orange loading dye.

Selective PCR- Primers for the selective PCR matched those of the pre-selective PCR, but with an additional two selective nucleotides added to the 3' end (Table 1.). The EcoRI pre-selective primer +GG and PstI pre-selective primer +AA/ +AG/ +GA/ +TT/ +CG were used for a total of 5 primer pair combinations including: (1) EcoRI+AGG and PSAI+AAA; (2) EcoRI+AGG and PSAI+AAG; (3) EcoRI+AGG and PSAI+AGA; (4) EcoRI+AGG and PSAI+ATT; (5) EcoRI+AGG and PSAI+ACG.

The pre-selective PCR master mix that was used in the pre-selective PCR reactions was also used in the selective PCR. All components were kept on ice until ready for use. Each selective PCR reaction included: 5.9 µL pre-selective PCR Master Mix, 1.0 µL PstA1+ANN primer (10 ng/µL), 1.0 µL EcoR1+AGG primer (10 ng/µL), 0.1 µL Taq (5 U/µL), and 2.0 µL of template (1:40 dilution of pre-selective PCR product).

Reactions were spun briefly to mix and then kept on ice until placed in a thermocycler at 95 °C with the heated lid enabled. Thermocycler conditions were as follows: initial denaturation of 95 °C for 1:30, followed by 8 cycles of 95 °C for 30 s, 65 °C for 30 s (-1 °C per cycle), 72 °C for 1 min, followed by 22 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min.

Tubes were removed from the thermocycler and placed on ice. Products were visualized on an ethidium bromide stained 1.5% agarose gel run at 100 volts. Each well contained 5 µL of selective PCR product and 2 µL of Promega 6x Blue/Orange loading dye.

Fragment Analyses:

Selective PCR products were sent to the Interdisciplinary Center for Biotechnology Research at the University of Florida. Products were visualized on an ABI-3100 sequencer using the LIZ 600 size standard. Resulting fragments were analyzed using Genemarker v1.6 to score fragments as 1 (present), 0 (absent), or ? (ambiguous data, Genemarker was unable to make a presence/absence call) for each individual.

Smaller fragments have a higher probability of being homoplasious. For example, co-migrating bands from different genes are more likely to occur with smaller fragments than larger fragments (Koopman and Gort, 2004; Merchanda et al., 2004; Altoff et al., 2007). To reduce homoplasy only fragments of 125 base pairs or more were used in phylogenetic analyses and estimates of genetic diversity (Altoff et al., 2007). The maximum fragment length was initially set at 550 base pairs, 50 base pairs less than the LIZ-600 size standard as recommended by previous studies (Mendelson and Simons, 2006; Mendelson and Wong, 2010). Ultimately, the maximum fragment length was

further reduced to the maximum size where Genemarker made accurate calls, lack of ambiguous data in the fragment calls throughout all individuals analyzed, for each individual primer pair. Genemarker settings included: stutter peak filter off, minimum peak threshold of 50, smoothing on, local and global detection percentages 1%, minimum peak score to fail < 1 check < 1 pass (Holland et al., 2008).

The 1, 0, and ? data for all scored fragments was used to generate a data matrix in Microsoft Excel (Microsoft, 2010) for all specimens examined. This file was then converted into Nexus format for phylogenetic analysis in PAUP* 4.0b108 (Swofford, 2003) and additionally was formatted per programmer's instruction and analyzed by AFLP-SURV version 1.0 (Vekemens, 2002) to generate population genetic parameters.

Phylogeny Reconstruction:

Outgroup Selection- For outgroups, a tissue sample of *Etheostoma exile* was obtained from the Florida Museum of Natural History and a tissue sample of *Etheostoma caeruleum* was collected. Recent studies using AFLPs (Smith et al., 2011) and mitochondrial markers (Turner, 1997; Lang and Mayden, 2007; Near et al., 2011) recovered *E. exile* as sister to *E. luteovinctum*, justifying its use as an outgroup. *Etheostoma caeruleum* was selected as a secondary outgroup due to its more distant relationship to *E. luteovinctum* (Lang and Mayden, 2007).

Phylogeographic Relationships- Phylogeographic hypotheses were generated for the AFLP dataset using the Maximum Parsimony and Nei-Li distance criteria implemented in PAUP* 4.0b10 (Swofford, 2003). These methods have been used to resolve relationships among closely related species using AFLPs (Sullivan et al., 2004; Koopman, 2005; Koopman et al., 2008; Garcia-Pereira et al., 2010).

Maximum parsimony settings included using equal weights and a heuristic search with tree-bisection reconnection branch swapping. Starting trees were obtained via stepwise addition. Branches with lengths of zero were collapsed. For each node, support was assessed using 1000 bootstrap pseudoreplicates (Felsenstein, 1985).

Distance settings included using equal weights and a heuristic search with tree-bisection reconnections branch swapping. Starting trees were obtained via Neighbor-Joining. Branches with lengths of zero were collapsed. For each node, support was assessed using 1000 bootstrap pseudoreplicates (Felsenstein, 1985).

Population Genetic Variation:

AFLP-SURV version 1.0 (Vekemens, 2002) was used to generate population level genetic parameters, including a pairwise Fst distance matrix, that was used to generate an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) diagram in DAMBE 5.2.31 (Xia and Xie, 2001). For each group, support was assessed using 1000 random permutations and all other default settings. One of the limitations of AFLP-SURV is that the program can analyze only 576 AFLP loci at one time. For this reason, a separate analysis was run on scored AFLP loci from each selective primer pair. Each locality was considered as a separate population in these analyses. Localities with only one specimen (site 4, site 10, and site 22) were not included as AFLP-SURV requires more than one individual per population for analyses.

Morphological Variation:

Meristics- A total of 150 specimens was examined from 17 localities from the Tennessee and Cumberland River drainages (Figure 3; Table 2; see also Appendix B-Materials Examined for specific locality information). Meristic data were collected from specimens

greater than 35 mm standard length using standard methods (Page, 1983) for counts of infraorbital pores, suborbital pores, preopercularmandibular pores, supratemporal pores, cheek scales, nape-scale rows, pectoral-fin rays, lateral-line scales, pored lateral-line scales, ratio of pored lateral-line scales to lateral-scale rows, scales above the lateral-line, scales below the lateral-line, transverse scales, dorsal-fin spines, dorsal-fin rays, belly-scale rows, anal-fin rays, scales around the caudal peduncle, and caudal-fin rays. The total number of medial scale rows was counted laterally across the opercle. The total number of scales along the midline of the breast were counted from the pelvic girdle to the pectoral girdle.

Geographic variation in meristic variables was initially analyzed using frequency distributions of all characters examined to identify any modal variation in characters. Geographic variation was also analyzed using a principle components analysis (PCA) in SYSTAT version 8 (SPSS, 1999). Ten of the original 21 meristic variables examined were used in the analyses (Table 3). Factor scores for individuals were plotted using scatter plots in SYSTAT version 8 (SPSS, 1999). Meristic characters with high positive or negative component loadings ($\geq$ absolute value of 0.35) were considered to be variable and to have contributed to any separation among populations seen in the resulting scatterplots of PCA factor scores. To effectively examine meristic variation at the intradrainage and interdrainage levels, separate principle component analyses were made on groupings at the stream, system, drainage, or clade level.

Pigmentation and Color Variation- Specimens used for pigmentation and color comparisons were collected from mid-February to early April (2011 and 2012), during the breeding season of *E. luteovinctum*. On average, five live nuptial males per locality

were photographed using a Cannon 60D Digital SLR with a 60 mm macro lens in a glass photobox (see Appendix B-Materials Examined).

General pigmentation and color descriptions were made in the field, followed by detailed descriptions from photographs in the lab. All lateral counts and color descriptions were taken from the left side of the body. Counts of dorsal saddles, transverse red bars, transverse blue bars, and total transverse bars were made from photographs. For all color descriptions percentage of a body area covered by the color was also recorded. Descriptions of the lip color, teardrop presence, breast color, pectoral-fin color, pelvic-fin color, the proximal, medial, and distal band color in the first and second dorsal fins, anal-fin color, and upper and lower basicaudal spot color were made. Additionally, descriptions of the presence or absence of a red membrane posterior to the last spine of the first dorsal fin and the presence or absence of a red spot on the dorsum between the dorsal fins were recorded. All pigmentation and color characters were analyzed across sampled localities for geographic variation using frequency tables, while non-countable color and pigmentation characters were analyzed stream by stream and using descriptive comparisons.

Table 1. Adapter design for digestion/ligation reactions and primer design for PCR reactions (Vos et al. 1995). N2 and N3 represent selective bases that vary with primer pairs.

Adapter/Primer	Sequence
EcoRI:	5' – G A A T T – 3' 3' – C T T A A – 5'
EcoRI Adapters	Enzyme specific region
Forward Adapter:	5' – C T C G T A G A C T G C G T A C C – 3'
Reverse Adapter:	3' – C A T C T G A C G C A T G G T T A A – 5'
EcoRI Pre-Selctive PCR Primer:	5' – G A C T G C G T A C C A A T T C **A N_2 N_3** – 3'
EcoRI Selctive PCR Primer:	5' – G A C T G C G T A C C A A T T C **A N_2 N_3** – 3'
PstI:	5' – C T G C A – 3' 3' – G A C G T – 5'
PstI Adapters	Enzyme specific region
Forward Adapter:	5' – C T C G T A G A C T G C G T A C A T G C A – 3'
Reverse Adapter:	3' – C A T C T G A C G C A T G T – 5'
PstI Pre-Selctive PCR Primer:	5' – G A C T G C G T A C A T G C A G **A N_2 N_3** – 3'
PstI Selctive PCR Primer:	5' – G A C T G C G T A C A T G C A G **A N_2 N_3** – 3'

Table 2. Number of specimens examined from each creek, river system, and drainage for analysis of morphological variation.

Drainage/System/Creek	Site No.	No. of Specimens
Cumberland River Drainage		**99**
Cumberland River		**10**
Eaton Creek	S2	10
Stones River[1]		**52**
Dry Fork Creek-Stoners Creek	S4	10
Dry Fork Creek-East Fork Stones River	S7	9
Tributary to Cripple Creek	S9	8
Tributary to Shanborne Branch	S11	6
Tributary to Middle Fork Stones	S18	10
Tributary to West Fork Stones	S19	9
Caney Fork River[2]		**37**
Marshall Creek	S8	10
Caney Branch	S12	7
Mud Creek	S13	10
West Fork Hickory Creek	*	10
Tennessee River Drainage		**51**
Duck River		**39**
Bear Creek	S25	10
Collins Creek	S21	11
Grassy Branch	S24	7
McCormick Creek	S23	6
Welker Branch	S15	5
Elk River		**12**
Tributary to Town Creek	S27	12
Total Specimens		**150**

[1] In PCA graphs of meristic comparisons S18 and S19 individuals were labeled separately from other Stones River system individuals examined.

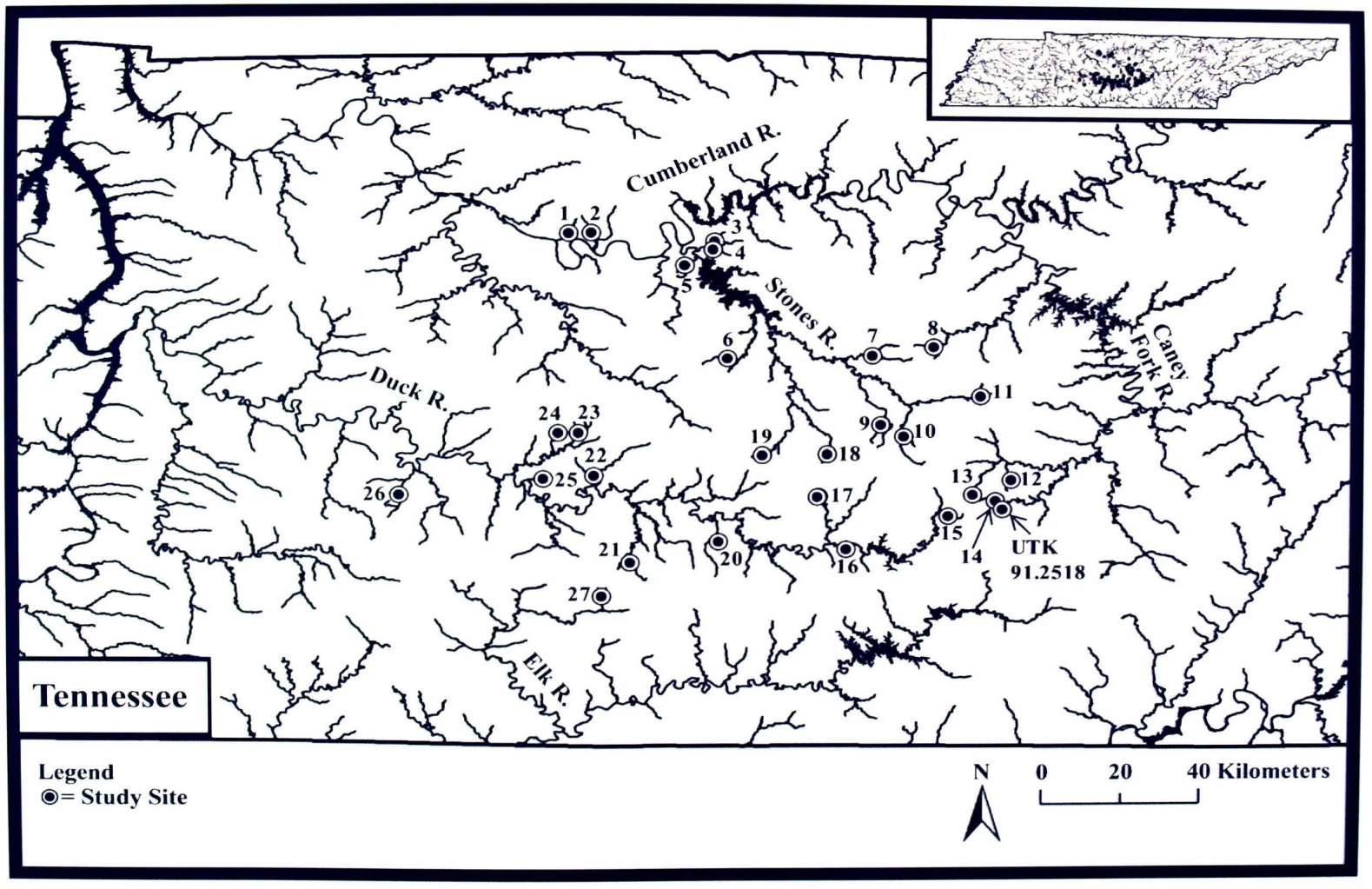
[2] In PCA graphs of meristic comparisons S8 individuals were labeled Marshall Creek-Lower Caney Fork River, S12 and S13 were labeled Barren Fork-Upper Caney Fork River, and * individuals were labeled West Fork Hickory Creek-Upper Caney Fork River.

*Borrowed collection UTK 91.2518 was used for meristic counts.

Table 3. Characters used to examine meristic variation among populations of Redband Darters. An * denotes variables used in the principle components analyses. L = left; R = right; A = anterior; P = posterior.

MERISTIC	ABBREVIATION
Infraorbital pores	IO
Suborbital pores (A, P)	SO
Preopercularmandibular pores	POM
Supratemporal pores (L, R)	ST
Cheek scale rows	CHEEK
Opercle scale rows *	OPERCLE
Nape scale rows	NAPE
Breast scale rows	BREAST
Pectoral rays	PRAYS
Lateral scale rows	LLINE
Pored lateral line scales *	PORED
Pored to unpored lateral line ratio *	LLRATIO
Scales below lateral line *	BELOW
Scales above lateral line *	ABOVE
Transverse scales *	TRANSVERSE
Dorsal spines *	D1
Dorsal rays	D2
Belly scale rows *	BELLY
Anal rays *	ARAYS
Scales around caudal peduncle *	CPED
Caudal rays	CRAYS

Figure 3. Study area illustrating localities sampled for analysis of genetic variation using amplified fragment length polymorphisms and analysis of morphological variation using color of nuptial males, pigmentation, and meristics. Specific locality information with corresponding site numbers is given in Appendix B.


Cumberland R.
Stones R.
Caney Fork R.
Duck R.
Elk R.
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Tennessee
Legend
◉= Study Site
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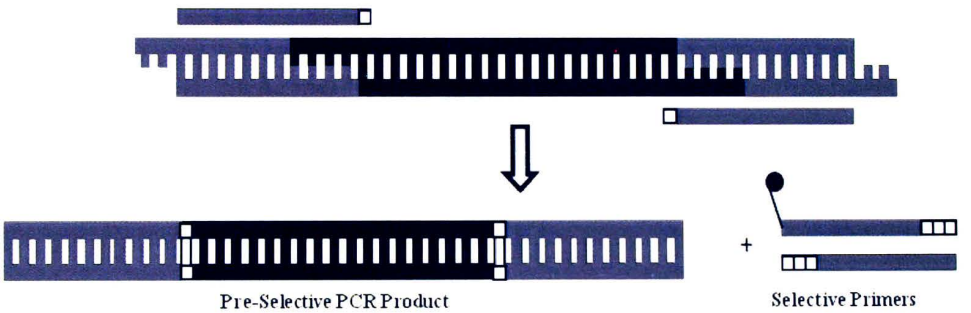
Figure 4. Flow chart diagram of amplified fragment length polymorphism procedures.

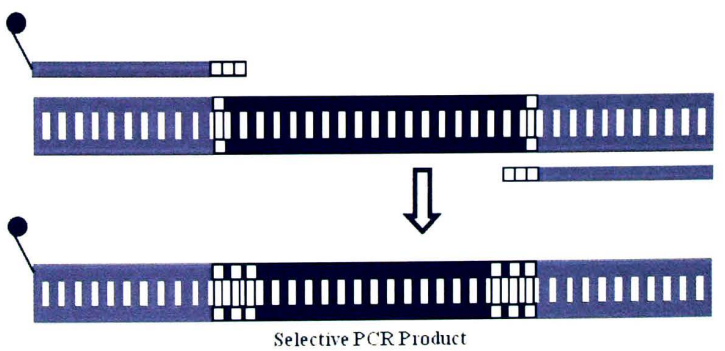
A) Digestion of genomic DNA with EcoRI and PstI resulting in restriction fragments of varying lengths

B) Ligation of EcoRI and PstI adapters to restriction fragments

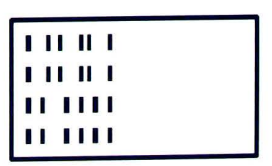
C) Pre-Selective PCR with digestion/ligation product and Pre-Selective primers (with an additional nucleotide on the 3' end)



D) Selective PCR with Pre-Selective PCR product and Selective PCR primers (with an additional three nucleotides on the 3' end and fluorescent label on EcoRI primer)



E) Use ABI-3100 capillary sequencer to visualize fragments



CHAPTER III

RESULTS

Status Survey Results:

A total of 101 historical localities were identified from museum collection records (Figure 1) of which 61 were re-sampled. Of those sites that were re-collected, *Etheostoma luteovinctum* was absent from 36 (35.6%; Figure 5). A large portion, 26 of 36 (72.2%), of these historical localities from which *E. luteovinctum* was absent were in the Cumberland River drainage. Given that there were originally only 40 historical localities known within the Cumberland River Drainage (Figures 5 and 6), the potential loss of over half of these is concerning.

Overall, *E. luteovinctum* was present at 25 of the 61 sampled historical localities and assumed to be present at the 40 (39 within the Duck River system and 1 within the Caney Fork River system) additional unsampled historical localities (Figure 6). In addition to the known historical localities, survey of other habitat appropriate sites resulted in identification of only four new localities (Figures 5 and 7) including: Mud Creek (Caney Fork River system), Weakley Creek (Duck River system), an unnamed tributary to the West Fork Stones River (Stones River system), and an unnamed tributary to the Middle Fork Stones River (Stones River system). The species was not found at the other 17 habitat appropriate localities sampled within the historical range of *E. luteovinctum* (Figure 5).

The total number of known localities for *E. luteovinctum* identified was 105 including the 101 historical localities and the four newly identified localities. However, *E. luteovinctum* was only present (including assumed present localities) at 65.7% (69 of

105) of these sites, indicating the current distribution of the species is restricted to only 69 extant or fewer locations within the Cumberland and Tennessee River drainages.

Phylogeographic Analyses:

Maximum Parsimony Analysis- A total of 2601 amplified fragment length polymorphism characters were generated for 95 individuals of *E. luteovinctum,* from 27 populations (Figure 3), and two outgroup taxa and used in the maximum parsimony analysis. Of these, 2029 of the fragments were parsimony-informative, 345 were parsimony-uninformative, and 227 were constant. All *E. luteovinctum* individuals formed a monophyletic group with 100% bootstrap support. *Etheostoma luteovinctum* was recovered in two well supported (91% and 72%) geographically definable and divergent clades, but clades were not consistent with drainage boundaries (Figures 8 and 9).

Clade A (Figure 8) included only populations from the Cumberland River drainage, including the direct Cumberland River tributaries (sites 1 and 2), Lower (sites 3-6) and East Fork (sites 7 and 9-11) Stones River, Lower Caney Fork River (site 8), and North Prong Barren Fork of the Caney Fork River (sites 12 and 13). Clade B (Figure 8) largely represented populations from the Tennessee River drainage including all of the Duck River (sites 15-17 and 20-26) and the Elk River (site 27), but also three populations from the Cumberland River drainage including the Middle Fork (site 18) and West Fork (Site 19) Stones River, and Hickory Creek of the Caney Fork River (site 14).

Relationships of individuals within each clade were largely unresolved. However, a sister relationship was recovered between a clade containing the Middle Fork (site 18) and West Fork (site 19) Stones Rivers and a clade containing individuals from Hickory Creek of the Caney Fork River (site 14). The clade containing the Middle Fork and West

Fork Stones River and Hickory Creek of the Caney Fork River was recovered in a well supported clade with two Duck River clades (sites 23 and 25), but relationships among these were unresolved.

The observed geographic relationships of the systems and drainages were consistent with system and drainage transfers. Relationships support possible transfers between the East Fork Stones River (sites 1-7, 9-11) and the Lower Caney Fork River (site 8), between the East Fork Stones River (sites 1-7, 9-11) and the Upper Caney Fork River (sites 12 and 13), between the Duck River (sites 15-17, 20-26) and the Elk River (site 27), between the Duck River (sites 15-17, 20-26) and the Middle Fork (site 18) and West Fork (site 19) Stones River, and between the Duck River (sites 15-17, 20-26) and Hickory Creek of the Caney Fork River (site 14).

Distance Analysis-The distance analysis also recovered a monophyletic Clade A (as observed in the Parsimony analysis) with 91% bootstrap support. However, Clade B, recovered by the parsimony analysis, was not recovered in the distance analysis. The Middle Fork (site 18) and West Fork (site 19) Stones River, Hickory Creek of the Caney Fork River (site 14), and site 23 and site 25 of the Duck River were recovered as a clade sister to a clade containing Clade A and the remainder of Clade B (tree not shown).

Population Genetic Analyses:

AFLP-SURV Analysis- AFLP-SURV version 1.0 (Vekemens, 2002) was used to generate separate UPGMA diagrams for each selective primer pair analyzed. All five analyses (Figures 10-14) recovered populations as more genetically differentiated than a random assemblage of populations (i.e. no panmixia), as determined by significant Fst values. Fst values for each primer pair analyses were: EcoRI+AGG and PSAI+AAA

(AAA), Fst = 0.2229 ($p<0.0001$); EcoRI+AGG and PSAI+AAG (AAG), Fst = 0.1554 ($p<0.0001$); EcoRI+AGG and PSAI+AGA (AGA), Fst = 0.1951 ($p<0.0001$); EcoRI+AGG and PSAI+ACG (ACG), Fst = 0.1590 ($p<0.0001$); EcoRI+AGG and PSAI+ATT (ATT), Fst = 0.1908 ($p<0.0001$).

All five analyses (Figures 10-15) recovered a cluster of populations corresponding to Clade A (Figure 5). Three of the five analyses recovered a cluster of populations corresponding to Clade B (Figure 5) observed in the maximum parsimony analysis (Figures 10, 12, and 14). Analyses of AAG and ATT did not recover an inclusive cluster corresponding to Clade B from the maximum parsimony analysis. Instead, AAG recovered Middle Fork (site 18) and West Fork (site 19) Stones Rivers, Hickory Creek of the Caney Fork River (site 14) and site 25 of the Duck River in a separate cluster (Figure 11), while ATT recovered Middle Fork (site 18) and West Fork (site 19) Stones Rivers in a separate cluster (Figure 13).

Results from the UPGMA of AAA loci (Figure 10) were most similar to the geographic relationships observed in the maximum parsimony analysis. The Elk River (site 27) clustered with the geographically proximate sampled Duck River site (site 21). The Lower Caney Fork River (site 8) clustered with the closest sampled East Fork Stones River site (site 7), rather than the other Caney Fork River populations. The Barren Fork of the Caney Fork River (sites 12 and 13) was genetically more similar to Lower (sites 3 and 5) and East Fork (sites 9 and 11) Stones River and direct Cumberland River tributary sites (site 1 and 2) than to the other Caney Fork River populations. The population from Hickory Creek of the Caney Fork River (site 14) clustered with populations from the Duck River rather than with other populations within the Caney Fork River. The Middle

Fork (site 18) and West Fork (site 19) Stones Rivers clustered with the Duck River (sites 23 and 25) and Hickory Creek of the Caney Fork River (site 14) populations, rather than with other Stones River populations.

The genetic similarity among these populations occurring across system and/or drainage divides (rather than with the populations or sites in their respective system or drainage) support transfers among systems and drainages. Transfers between the East Fork Stones River (sites 1-7, 9-11) and the Lower Caney Fork River (site 8), between the East Fork Stones River (sites 1-7, 9-11) and the Upper Caney Fork River (sites 12 and 13), between the Duck River (sites 15-17, 20-26) and the Elk River (site 27), between the Duck River (sites 15-17, 20-26) and the Middle Fork (site 18) and West Fork (site 19) Stones River, and between the Duck River (sites 15-17, 20-26) and Hickory Creek of the Caney Fork River (site 14). These results further support possible cross-system and drainage transfers described from the maximum parsimony results.

Morphological Analyses:

Meristics- Table 4-13 display ranges, means, modes, and standard deviation of all meristic characters examined except those that showed little to no variation including: opercle scale rows (range = 3-7, $\bar{x}$ = 4.79 ± 0.68, mode = 5), preopercularmandibular pores (range = 8-11, $\bar{x}$ = 9.89 ± 0.40, mode = 10), infraorbital pores (range = 6-10, $\bar{x}$ =7.75 ± 0.68, mode = 8), suborbital pores (range = 2-5, $\bar{x}$ = 4.02 ± 0.27, mode = 4), supratemporal pores (range = 1-5, $\bar{x}$ = 3.24 ± 0.28, mode = 3), pectoral-fin rays (range = 11-14, $\bar{x}$ = 12.67 ± 0.54, mode = 13), dorsal-fin spines (range = 8-11, $\bar{x}$ = 9.83 ± 0.56, mode = 10), dorsal-fin rays (range = 11-14, $\bar{x}$ = 12.33 ± 0.71, mode = 12), scales above

the lateral-line (range = 4-8, $\bar{x}$ = 5.84 ± 0.51, mode = 6), and caudal-fin rays (range = 11-17, $\bar{x}$ = 14.95 ± 0.65, mode = 15).

For characters that did show variation, variation in meristic counts within and among systems, drainages, and clades was examined. For most characters no diagnostic geographic variation was observed. Tables 4-13 summarize variation in meristic counts among populations both within and among systems. A summary of meristic differences between the Cumberland and Tennessee River drainages is presented in Table 14 and a summary of differences between Clade A and Clade B is presented in Table 15.

Variation in meristic counts between the Cumberland (CRD) and Tennessee (TRD) River drainages was noted in counts of cheek scale rows (Table 5) with the CRD having a mode of 1 ($\bar{x}$ = 4.58) versus the TRD with a mode of 5 ($\bar{x}$ = 2.98), breast scale rows (Table 6) with the CRD with modes of 5 and 10 ($\bar{x}$ = 6.48) versus the TRD with a mode of 0 ($\bar{x}$ = 3.16), pored lateral-line scales (Table 8) with the CRD with a mode of 34 ($\bar{x}$ = 33.73) versus the TRD with a mode of 38 ($\bar{x}$ = 36.41), and scales around the caudal peduncle (Table 13) with the CRD with a mode of 20 ($\bar{x}$ = 20.43) versus the TRD with a mode of 22 ($\bar{x}$ = 21.51).

Variation in meristic counts between Clade A and Clade B was noted in counts of breast scale rows (Table 6) with Clade A with a mode 10 ($\bar{x}$ = 7.04) versus Clade B with modes of 0 and 5 ($\bar{x}$ = 3.88), pored lateral-line scales (Table 8) with Clade A with a mode of 34 ($\bar{x}$ = 32.84) versus Clade B with a mode of 35 ($\bar{x}$ = 36.21), and scales around the caudal peduncle (Table 13) with Clade A with a mode 20 ($\bar{x}$ = 20.43) versus Clade B with modes of 21 and 22 ($\bar{x}$ = 21.13).

Multivariate Principle Component Analyses of Meristic Characters- Results from principle component analysis of 10 meristic characters (Table 3) for groups separated by river system showed moderate to high overlap of populations in the plot of principle component 1 vs. principle component 2 (Figure 15). Component loadings for the 10 meristic characters are listed in Table 16.

Results from principle component analysis of 10 meristic characters (Table 3) for Clade A and Clade B showed substantial overlap in the plot of principle component 1 vs. principle component 2 (Figure 16). Component loadings for the 10 meristic characters are listed in Table 16.

Many other principle component analyses were run using a variety of other groupings for populations and other morphological characters, but none of these analyses recovered separation among units examined.

Countable Color and Pigmentation Characters- Tables 17-20 display range, mean, mode, and standard deviation for all countable color and pigmentation characters (dorsal saddles, red transverse bars, blue transverse bars, and total transverse bars) examined. Little to no geographically meaningful variation was observed in counts of dorsal saddles (range = 3-7, $\bar{x}$ = 4.79 ± 0.58, mode = 5). For characters that did show variation, variation in counts within and among systems, drainages, and clades was examined.

Variation in counts between the Cumberland (CRD) and Tennessee (TRD) River drainages was noted in counts of red transverse bars (Table 18) with the CRD having a mode of 8 ($\bar{x}$ = 8.33) versus the TRD with a mode of 9 ($\bar{x}$ = 8.67), the CRD had a mode of 9 ($\bar{x}$ = 9.30) blue transverse bars (Table 19) versus t 10 ($\bar{x}$ = 9.67) in the TRD, and

with the CRD with a mode of 17 ($\bar{x}$ = 17.63) total transverse bars (Table 20), versus 19 ($\bar{x}$ = 18.33) in the TRD.

Variation in counts between Clade A and Clade B was noted in several color characters also. Clade A had a mode of 8 ($\bar{x}$ = 8.31) red transverse bars (Table 18) versus 9 ($\bar{x}$ = 8.63) in Clade B, Clade A had a mode of 9 ($\bar{x}$ = 9.27) blue transverse bars (Table 19) versus 10 ($\bar{x}$ = 9.63) in Clade B, and Clade A had a mode of 17 ($\bar{x}$ = 17.59) total transverse bars (Table 20) versus 19 ($\bar{x}$ = 18.26) in Clade B.

Plate 1 shows males displaying a higher number of red transverse bars, while Plate 2 (specimen A) shows a lower number of red transverse bars. Specimen B on Plate 2 shows a single case of extreme variation in transverse bars, where the transverse bars have actually blended into a single red band spanning the last third of the specimen.

Descriptive Color and Pigmentation Characters of Nuptial Males- The variation in color of the red and blue transverse bars was not geographically definable. The typical condition of red transverse bar color was noted as darker red on the dorsal half (where the red transverse bar overlapped with the dark lateral band) of specimens than the ventral half. The red transverse bars sometimes appeared as a lighter orange color and had a yellowish 1-2 scale width outline. The typical condition of blue transverse bar color was noted as a bright blue on the ventral portion and a darker blue-green on the dorsal half (where the red transverse bar overlapped with the dark lateral band and the dorsal saddle).

Variation in characters involving blue color was noted, but was not geographically definable. Noted variation in characters included: the percent of the pelvic fin and anal fin that had blue color (5-95%), the percent of blue color in the proximal

band of the second dorsal fins in membranes directly above dorsal saddles (0-100%), the percent of the base of the caudal peduncle that had blue color (0-100%), and presence (Plate 1, specimen A) or absence of a blue distal band on the caudal fin.

Variation in characters involving red color was noted, but was not geographically definable. Noted variation in characters included: presence (Plate 1, specimen A) or absence (Plate 2, specimen A and B) of a red membrane posterior to the last spine of the first-dorsal fin, presence (Plate 1, specimen B) or absence (Plate 2, specimen A and B) of a red spot on the dorsum between the dorsal fins, presence (Plate 1, specimen A) or absence of a red upper basicaudal spot, and presence (Plate 1, specimen A) or absence of a red lower basicaudal spot.

A single specimen from Bear Creek (Duck River system- Tennessee River drainage; S25) and one from Sulphur Creek (Cumberland River drainage; S1) displayed orange pigmentation in the membranes of their caudal fin, but this was not seen in any other individuals examined.

Little to no geographic variation was observed for lip color, breast color, pectoral-fin color, teardrop pigmentation, proximal, medial, and distal band color of the first-dorsal fin, and medial and distal band color of the second-dorsal fin.

Multiple female Redband Darters were found to display either red transverse bars (Plate 3, specimen A) or blue transverse bars (Plate 3, specimen B) throughout the range of the species, but no geographically definable variation was observed, as females were not consistently photographed at study sites.

Table 4. Frequency Distribution of Cheek Scale Rows

	No. Cheek Scale Rows											
Drainage/Clade/System	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**n**	**$\bar{x}$**	**SD**
Cumberland R. Drainage	**1**	**3**	**5**	**12**	**20**	**34**	**17**	**4**	**3**	**99**	**4.58**	**1.53**
Clade A												
Cumberland R.				1	2	5	2			10	4.80	0.92
Stones R.	1	1	—	4	7	8	9	3		33	4.73	1.61
Caney Fork R.		1	1	2	4	13	4	—	2	27	4.81	1.49
Clade B		1	4	5	7	8	2	1	1	29	4.10	1.59
Stones R.		1	3	4	5	4	1	1		19	3.79	1.51
Caney Fork R.			1	1	2	4	1	—	1	10	4.70	1.64
Tennessee R. Drainage	**2**	**13**	**10**	**6**	**5**	**10**	**4**	**1**		**51**	**2.98**	**1.88**
Clade B												
Duck R.	2	11	9	3	3	9	2			39	2.74	1.82
Elk R.		2	1	3	2	1	2	1		12	3.75	1.96
Clade A Total	**1**	**2**	**1**	**7**	**13**	**26**	**15**	**3**	**2**	**70**	**4.77**	**1.47**
Clade B Total	**2**	**14**	**14**	**11**	**12**	**18**	**6**	**2**	**1**	**80**	**3.39**	**1.85**

Table 5. Frequency Distribution of Nape Scale Rows

	No. Nape Scale Rows																	
Drainage/Clade/System	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**n**	**$\bar{x}$**	**SD**
Cumberland R. Drainage		**4**	**15**	**24**	**11**	**18**	**9**	**6**	**1**	**—**	**3**	**4**	**2**	**2**	**99**	**19.73**	**2.83**
Clade A																	
Cumberland R.		1	—	2	1	2	—	3	1						10	20.00	2.26
Stones R.			2	7	3	3	6	1	—	—	3	4	2	2	33	21.64	3.60
Caney Fork R.		1	8	8	2	5	2	1							27	18.44	1.55
Clade B																	
Stones R.		2	4	6	3	4									19	18.16	1.30
Caney Fork R.			1	1	2	4	1	1							10	19.60	1.43
Tennessee R. Drainage	**6**	**5**	**7**	**6**	**8**	**8**	**3**	**2**	**2**	**1**	**1**	**1**	**—**	**1**	**51**	**18.92**	**2.95**
Clade B																	
Duck R.	4	5	5	4	6	5	2	2	2	1	1	1	—	1	39	19.13	3.19
Elk R.	2	—	2	2	2	3	1								12	18.25	1.96
Clade A Total		**2**	**10**	**17**	**6**	**10**	**8**	**5**	**1**	**—**	**3**	**4**	**2**	**2**	**70**	**20.17**	**3.13**
Clade B Total	**6**	**7**	**12**	**13**	**13**	**16**	**4**	**3**	**2**	**1**	**1**	**1**	**—**	**1**	**80**	**18.83**	**2.51**

Table 6. Frequency Distribution of Breast Scale Rows

Drainage/Clade/System	No. Breast Scale Rows												n	$\bar{x}$	SD
	0	1	2	3	4	5	6	7	8	9	10	11			
Cumberland R. Drainage	**2**	**2**	**5**	**9**	**7**	**14**	**8**	**12**	**11**	**10**	**14**	**5**	**99**	**6.48**	**2.85**
Clade A															
Cumberland R.															
Eaton Cr.			1	2	1	1	1	—	—	2	1	1	10	6.20	3.29
Stones R.		1	3	4	—	2	2	3	3	5	8	2	33	7.06	3.12
Dry Fork Cr.-Stoners Cr.		1	3	4	—	1	—	1					10	3.10	1.73
Dry Fork Cr.-East Fork Stones						1	—	1	—	2	4	1	9	5.22	0.67
Trib. to Shanborne Branch							1	—	1	2	2		6	8.67	1.51
Trib. to Cripple Cr.							1	1	2	1	2	1	8	8.63	1.69
Caney Fork R.	1	—	—	1	1	5	1	2	7	2	5	2	27	7.33	2.66
Caney Branch					1	—	—	1	2	1	2		7	8.00	2.08
Marshall Cr.	1	—	—	1	—	5	1	1	—	—	1		10	5.10	2.56
Mud Cr.									5	1	2	2	10	8.89	1.29
Clade B															
Stones R.	1	1	1	1	4	5	4	1	1				19	4.53	1.98
Trib. to West Fork Stones			1	1	2	2	1	1	1				9	4.89	1.90
Trib. to Middle Fork Stones	1	1	—	—	2	3	3						10	4.20	2.10
Caney Fork R.															
West Fork Hickory Cr.					1	1	1	—	6	—	1		10	6.30	1.77
Tennessee R. Drainage	**14**	**7**	**6**	**1**	**6**	**6**	**2**	**4**	**2**	**1**	**2**		**51**	**3.16**	**3.02**
Clade B															
Duck R.	12	6	6	1	6	3	2	2	—	—	1		39	2.46	2.53
Grassy Branch	4	2	1										7	0.57	0.79
McCormick Cr.	5	—	—	—	1								6	0.67	1.63
Bear Cr.	3	3	2	—	—	1	1						10	1.80	2.10
Welker Branch		1	—	1	1	1	1						5	3.80	1.92
Collins Cr.			3		4	1	—	2	—	—	1		11	4.64	2.50
Elk R.															
Trib. to Town Cr.	2	1	—	—	—	3	—	2	2	1	1		12	5.42	3.45
Clade A Total	**1**	**1**	**4**	**7**	**2**	**8**	**4**	**5**	**10**	**9**	**14**	**5**	**70**	**7.04**	**2.96**
Clade B Total	**15**	**8**	**7**	**3**	**11**	**12**	**6**	**11**	**3**	**2**	**2**		**80**	**3.88**	**2.86**

Table 7. Frequency Distribution of Lateral Scale Rows

Drainage/Clade/System	No. Lateral Scale Rows																n	$\bar{x}$	SD
	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59			
Cumberland R. Drainage			**2**	**1**	**6**	**2**	**9**	**14**	**12**	**11**	**15**	**14**	**9**	**4**			**99**	**52.61**	**2.61**
Clade A																			
Cumberland R.			1	—	1	—	2	3	—	1	1	—	1				10	51.00	2.87
Stones R.					1	1	3	3	5	2	9	5	3	1			33	53.09	2.21
Caney Fork R.					3	—	2	5	6	5	1	3	1	1			27	52.15	2.30
Clade B																			
Stones R.								3	—	2	3	5	4	2			19	54.42	1.89
Caney Fork R.			1	1	1	1	2	—	1	1	1	1					10	50.40	3.03
Tennessee R. Drainage	**1**	—	—	—	—	**2**	**5**	**6**	**6**	**7**	**10**	**4**	**3**	**1**	**2**	**4**	**51**	**53.29**	**2.99**
Clade B																			
Duck R.	1	—	—	—	—	1	4	3	3	6	9	3	3	1	1	4	39	53.59	3.11
Elk R.						1	1	3	3	1	1	1	—	—	1		12	52.33	2.42
Clade A Total			**1**	**—**	**5**	**1**	**7**	**11**	**11**	**8**	**11**	**8**	**5**	**2**			**70**	**52.61**	**2.61**
Clade B Total	**1**	—	**1**	**1**	**1**	**3**	**7**	**9**	**7**	**10**	**14**	**10**	**7**	**3**	**2**	**4**	**80**	**53.20**	**2.98**

Table 8. Frequency Distribution of Pored Lateral-Line Scales

	No. Pored Lateral-Line Scales																											
Drainage/Clade/System	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**n**	**$\bar{x}$**	**SD**
Cumberland R. Drainage	**1**	**—**	**2**	**1**	**—**	**2**	**5**	**2**	**3**	**3**	**12**	**12**	**14**	**10**	**9**	**11**	**5**	**3**	**—**	**3**	**1**			**99**	**33.73**	**3.77**
Clade A																										
Cumberland R.							1	1	—	—	5	4	6	1	5	2	1	—	—	1				27	34.11	2.69
Stones R.	1	—	—	—	—	—	2	1	2	2	—	1	—	—	—	1								10	29.90	3.84
Caney Fork R.			2	1	—	1	2	—	—	1	5	4	7	5	—	3	2							33	32.70	3.72
Clade B																										
Stones R.											1	1	1	2	3	5	—	3	—	2	1			19	37.00	2.69
Caney Fork R.						1	—	—	1	—	1	2	—	2	1	—	2							10	33.70	3.47
Tennessee R. Drainage							**1**	**1**	**2**	**—**	**3**	**4**	**4**	**7**	**4**	**3**	**8**	**5**	**2**	**2**	**2**	**1**	**2**	**51**	**36.41**	**3.73**
Clade B																										
Duck R.							1	1	2	—	2	3	3	5	3	3	6	4	2	1	1	1	1	39	36.15	3.73
Elk R.											1	1	1	2	1	—	2	1	—	1	1	—	1	12	37.25	3.74
Clade A Total	**1**	**—**	**2**	**1**	**—**	**1**	**5**	**2**	**2**	**3**	**10**	**9**	**13**	**6**	**5**	**6**	**3**	**—**	**—**	**1**				**70**	**32.84**	**3.60**
Clade B Total						**1**	**1**	**1**	**3**	**—**	**5**	**7**	**5**	**11**	**8**	**8**	**10**	**8**	**2**	**4**	**3**	**1**	**2**	**80**	**36.21**	**3.57**

Table 9. Frequency Distribution of Scales Rows Below Lateral Line

	No. Scales Rows Below Lateral Line								
Drainage/Clade/System	**8**	**9**	**10**	**11**	**12**	**13**	**n**	**$\bar{x}$**	**SD**
Cumberland R. Drainage	**1**	**7**	**10**	**38**	**33**	**10**	**99**	**11.26**	**1.07**
Clade A									
Cumberland R.			1	8	1		10	11.00	0.47
Stones R.	1	6	6	15	3	2	33	10.58	1.17
Caney Fork R.		1	1	10	15		27	11.44	0.75
Clade B									
Stones R.			2	3	10	4	19	11.84	0.90
Caney Fork R.				2	4	4	10	12.20	0.79
Tennessee R. Drainage			**11**	**27**	**13**		**51**	**11.04**	**0.69**
Clade B									
Duck R.			8	21	10		39	11.05	0.69
Elk R.			3	6	3		12	11.00	0.74
Clade A Total	**1**	**7**	**8**	**33**	**19**	**2**	**70**	**10.97**	**1.02**
Clade B Total			**13**	**32**	**27**	**8**	**80**	**11.38**	**0.88**

Table 10. Frequency Distribution of Transverse Scales

Drainage/Clade/System	No. Transverse Scales							n	$\bar{x}$	SD
	14	15	16	17	18	19	20			
Cumberland R. Drainage	**1**	**5**	**6**	**8**	**35**	**33**	**11**	**99**	**18.16**	**1.31**
Clade A										
Cumberland R.			1	—	7	1	1	10	18.10	0.99
Stones R.	1	4	4	6	11	5	2	33	17.36	1.52
Caney Fork R.		1	1	—	11	14		27	18.33	0.96
Clade B										
Stones R.				2	4	9	4	19	18.79	0.92
Caney Fork R.					2	4	4	10	19.20	0.79
Tennessee R. Drainage		**1**	**3**	**17**	**17**	**13**		**51**	**17.75**	**0.98**
Clade B										
Duck R.		1	3	14	12	9		39	17.64	1.01
Elk R.				3	5	4		12	18.08	0.79
Clade A Total	**1**	**5**	**6**	**6**	**29**	**20**	**3**	**70**	**17.84**	**1.33**
Clade B Total		**1**	**3**	**19**	**23**	**26**	**8**	**80**	**18.18**	**1.10**

Table 11. Frequency Distribution of Belly Scale Rows

Drainage/Clade/System	No. Belly Scale Rows														n	$\bar{x}$	SD
	19	20	21	22	23	24	25	26	27	28	29	30	31	32			
Cumberland R. Drainage	**4**	**6**	**8**	**17**	**22**	**15**	**12**	**9**	**3**	**2**	**—**	**1**			**99**	**23.28**	**2.14**
Clade A																	
Cumberland R.		1	1	1	1	1	3	2							10	23.70	2.11
Stones R.			4	10	8	3	3	3	1	1					33	23.27	1.82
Caney Fork R.	3	1	1	4	7	5	3	3							27	22.96	2.05
Clade B																	
Stones R.	1	4	2	2	4	4	1	1							19	22.32	1.97
Caney Fork R.					2	2	2	—	2	1	—	1			10	25.60	2.32
Tennessee R. Drainage		**7**	**4**	**9**	**7**	**9**	**8**	**4**	**2**	**—**	**—**	**—**	**—**	**1**	**51**	**23.31**	**2.34**
Clade B															51	23.31	2.34
Duck R.		7	2	6	6	6	7	3	1	—	—	—	—	1	39	23.28	2.47
Elk R.			2	3	1	3	1	1	1						12	23.42	1.93
Clade A Total	**3**	**2**	**6**	**15**	**16**	**9**	**9**	**8**	**1**	**1**					**70**	**23.21**	**1.94**
Clade B Total	**1**	**11**	**6**	**11**	**13**	**15**	**11**	**5**	**4**	**1**	**—**	**1**	**—**	**1**	**80**	**23.36**	**2.42**

Table 12. Frequency Distribution of Anal-Fin Rays

Drainage/Clade/System	No. Anal-Fin Rays						n	$\bar{x}$	SD
	8	9	10	11	12	13			
Cumberland R. Drainage	**10**	**66**	**23**				**99**	**9.13**	**0.57**
Clade A									
Cumberland R.	2	7	1				10	8.90	0.57
Stones R.	3	20	10				33	9.21	0.60
Caney Fork R.	3	22	2				27	8.96	0.44
Clade B									
Stones R.	2	12	5				19	9.16	0.60
Caney Fork R.		5	5				10	9.50	0.53
Tennessee R. Drainage	**2**	**24**	**20**	**4**		**1**	**51**	**9.59**	**0.85**
Clade B									
Duck R.	2	24	12	—	—	1	39	9.36	0.81
Elk R.			8	4			12	10.33	0.49
Clade A Total	**8**	**49**	**13**				**70**	**9.07**	**0.55**
Clade B Total	**4**	**41**	**30**	**4**	—	**1**	**80**	**9.48**	**0.78**

Table 13. Frequency Distribution of Caudal Peduncle Scales

	No. Caudal Peduncle Scales													
Drainage/Clade/System	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**		**n**	**$\bar{x}$**	**SD**
Cumberland R. Drainage	**3**	**6**	**14**	**33**	**23**	**11**	**6**	**1**	**1**	**1**		**99**	**20.43**	**1.58**
Clade A														
Cumberland R.			2	2	2	2	2					10	21.00	1.49
Stones R.		4	6	14	6	1	—	—	1	1		33	20.15	1.70
Caney Fork R.	2	—	4	8	5	5	2	1				27	20.56	1.67
Clade B														
Stones R.	1	2	2	7	5	2						19	20.00	1.33
Caney Fork R.				2	5	1	2					10	21.30	1.06
Tennessee R. Drainage			**4**	**10**	**10**	**17**	**5**	**3**	**2**			**51**	**21.51**	**1.47**
Clade B														
Duck R.			3	8	6	13	5	3	1			39	21.56	1.48
Elk R.			1	2	4	4	—	—	1			12	21.33	1.50
Clade A Total	**2**	**4**	**12**	**24**	**13**	**8**	**4**	**1**	**1**	**1**		**70**	**20.43**	**1.66**
Clade B Total	**1**	**2**	**6**	**19**	**20**	**20**	**7**	**3**	**2**			**80**	**21.13**	**1.52**

Table 14. Summary of Differences in Meristic Characters Between the Cumberland and Tennessee River Drainages

Meristic Character	Drainage	Mode	Range	$\bar{x}$	SD
Cheek scale rows	Cumberland	1	0 - 8	4.58	1.53
	Tennessee	5	0 - 7	2.98	1.88
Nape scale rows	Cumberland	18	16 - 28	19.73	2.83
	Tennessee	19,20	15 - 28	18.92	2.95
Breast scale rows	Cumberland	5,10	0 - 11	6.48	2.85
	Tennessee	0	0 - 10	3.16	3.02
Transverse scales	Cumberland	18	14 - 20	18.16	1.31
	Tennessee	17,18	15 - 19	17.75	0.98
Scales below lateral line	Cumberland	11	8 - 13	11.26	1.07
	Tennessee	11	10 - 12	11.04	0.69
Lateral scale rows	Cumberland	54	46 - 57	52.61	2.61
	Tennessee	54	44 - 59	53.29	2.99
Pored lateral-line scales	Cumberland	34	22 - 42	33.73	3.77
	Tennessee	38	29 - 44	36.41	3.73
Belly scale rows	Cumberland	23	19 - 30	23.28	2.14
	Tennessee	22,24	20 - 32	23.31	2.34
Anal-fin rays	Cumberland	9	8 - 10	9.13	0.57
	Tennessee	9	8 - 13	9.59	0.85
Scales around caudal peduncle	Cumberland	20	17 - 26	20.43	1.58
	Tennessee	22	19 - 25	21.51	1.47

Table 15. Summary of Differences in Meristic Characters Between Clade A and Clade B

Meristic Character	Clade	Mode	Range	$\bar{x}$	SD
Cheek scale rows	Clade A	5	0 - 8	4.77	1.47
	Clade B	5	0 - 8	3.39	1.85
Nape scale rows	Clade A	18	16 - 28	20.17	3.13
	Clade B	20	15 - 28	18.83	2.51
Breast scale rows	Clade A	10	0 - 11	7.04	2.96
	Clade B	0,5	0 - 10	3.88	2.86
Transverse scales	Clade A	18	14 - 20	17.84	1.33
	Clade B	19	15 - 20	18.18	1.10
Scales below lateral line	Clade A	11	8 - 13	10.97	1.02
	Clade B	11	10 - 13	11.38	0.88
Lateral scale rows	Clade A	51,52,54	46 - 57	52.61	2.61
	Clade B	54	44 - 59	53.20	2.98
Pored lateral-line scales	Clade A	34	22 - 41	32.84	3.60
	Clade B	35	27 - 44	36.21	3.57
Belly scale rows	Clade A	22	19 - 28	23.21	1.94
	Clade B	24	19 - 32	23.36	2.42
Anal-fin rays	Clade A	9	8 - 10	9.07	0.55
	Clade B	9	8 - 13	9.48	0.78
Scales around caudal peduncle	Clade A	20	17 - 26	20.43	1.66
	Clade B	21,22	17 - 25	21.13	1.52

Table 16. Principle component loadings for 10 meristic variables for 37 Caney Fork River system specimens, 52 Stones River system specimens, 10 Cumberland River tributary specimens, 39 Duck River system specimens, and 12 Elk River system specimens.

	Component Loadings	
Variable	**PC 1**	**PC 2**
Opercle scale rows	0.54	0.14
Pored lateral line scales	0.66	0.65
Pored to unpored lateral line ratio	0.64	0.62
Scales below lateral line	0.57	-0.45
Scales above lateral line	0.56	-0.47
Transverse scales	0.69	-0.61
Dorsal spines	0.35	0.10
Belly scale rows	0.52	-0.21
Anal rays	0.40	0.26
Scales around caudal peduncle	0.42	0.04

Table 17. Frequency Distribution of Dorsal Saddles

Drainage/Clade/System	No. Dorsal Saddles 5	6	7	8	n	$\bar{x}$	SD
Cumberland R. Drainage	**1**	**3**	**44**	**12**	**60**	**7.12**	**0.56**
Clade A							
Cumberland R.		1	8	1	10	7.00	0.47
Stones R.		1	21	6	28	7.18	0.48
Caney Fork R.	1	1	7	4	13	7.08	0.86
Clade B							
Stones R.			8	1	9	7.11	0.33
Tennessee R. Drainage		**4**	**26**	**15**	**45**	**7.24**	**0.61**
Clade B							
Duck R.		3	23	14	40	7.28	0.60
Elk R.		1	3	1	5	7.00	0.71
Clade A Total	**1**	**3**	**36**	**11**	**51**	**7.12**	**0.59**
Clade B Total		**4**	**34**	**16**	**54**	**7.22**	**0.57**

Table 18. Frequency Distribution of Red Transverse Bars

Drainage/Clade/System	No. Red Transverse Bars					n	$\bar{x}$	SD
	7	8	9	10	11			
Cumberland R. Drainage	**8**	**28**	**21**	**2**	**1**	**60**	**8.33**	**0.82**
Clade A								
Cumberland R.	2	7	1			10	7.90	0.57
Stones R.	4	12	10	1	1	28	8.39	0.92
Caney Fork R.	1	6	5	1		13	8.46	0.78
Clade B								
Stones R.	1	3	5			9	8.44	0.73
Tennessee R. Drainage	**5**	**14**	**18**	**7**	**1**	**45**	**8.67**	**0.95**
Clade B								
Duck R.	3	13	16	7	1	40	8.75	0.93
Elk R.	2	1	2			5	8.00	1.00
Clade A Total	**7**	**25**	**16**	**2**	**1**	**51**	**8.31**	**0.84**
Clade B Total	**6**	**17**	**23**	**7**	**1**	**54**	**8.63**	**0.92**

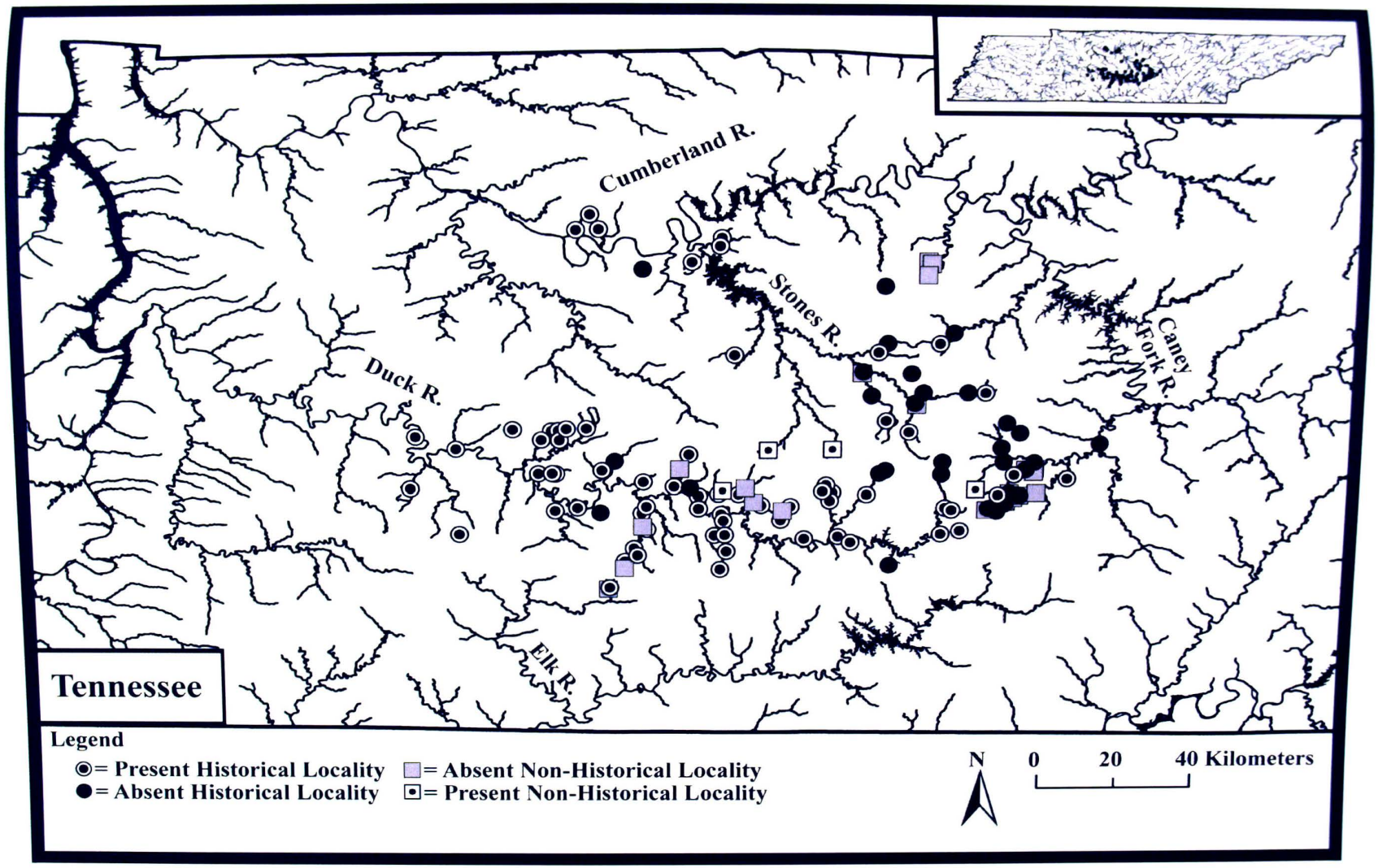
Table 19. Frequency Distribution of Blue Transverse Bars

Drainage/Clade/System	No. Blue Transverse Bars					n	$\bar{x}$	SD
	8	9	10	11	12			
Cumberland R. Drainage	**9**	**28**	**20**	**2**	**1**	**60**	**9.30**	**0.83**
Clade A								
Cumberland R.	2	7	1			10	8.90	0.57
Stones R.	5	12	9	1	1	28	9.32	0.94
Caney Fork R.	1	6	5	1		13	9.46	0.78
Clade B								
Stones R.	1	3	5			9	9.44	0.73
Tennessee R. Drainage	**5**	**14**	**18**	**7**	**1**	**45**	**9.67**	**0.95**
Clade B								
Duck R.	3	13	16	7	1	40	9.75	0.93
Elk R.	2	1	2			5	9.00	1.00
Clade A Total	**8**	**25**	**15**	**2**	**1**	**51**	**9.27**	**0.85**
Clade B Total	**6**	**17**	**23**	**7**	**1**	**54**	**9.63**	**0.92**

Table 20. Frequency Distribution of Total Transverse Bars

Drainage/Clade/System	No. Total Transverse Bars									n	$\bar{x}$	SD
	15	16	17	18	19	20	21	22	23			
Cumberland R. Drainage	**8**	**—**	**29**	**—**	**20**	**—**	**2**	**—**	**1**	**60**	**17.63**	**1.63**
Clade A												
Cumberland R.	2	—	7	—	1					10	16.80	1.14
Stones R.	4	—	13	—	9	—	1	—	1	28	17.71	1.82
Caney Fork R.	1	—	6	—	5	—	1			13	17.92	1.55
Clade B												
Stones R.	1	—	3	—	5					9	17.89	1.45
Tennessee R. Drainage	**5**	**—**	**14**	**—**	**18**	**—**	**7**	**—**	**1**	**45**	**18.33**	**1.91**
Clade B												
Duck R.	3	—	13	—	16	—	7	—	1	40	18.50	1.85
Elk R.	2	—	1	—	2					5	17.00	2.00
Clade A Total	**7**	**—**	**26**	**—**	**15**	**—**	**2**	**—**	**1**	**51**	**17.59**	**1.66**
Clade B Total	**6**	**—**	**17**	**—**	**23**	**—**	**7**	**—**	**1**	**54**	**18.26**	**1.83**

Figure 5. Results of the status survey of the Redband Darter, *Etheostoma luteovinctum*. Black circles represent historical localities where *E. luteovinctum* was not found. Circles with a black dot inside represent historical localities where *E. luteovinctum* was present or assumed to be present. White squares with a black dot inside represent habitat appropriate sites (non-historical) where *E. luteovinctum* was present. Grey squares represent habitat appropriate sites (non-historical) where *E. luteovinctum* was absent. Specific locality information is given in Appendix B.


Cumberland R.
Stones R.
Caney Fork R.
Duck R.
Elk R.
Tennessee
Legend
◉= Present Historical Locality
●= Absent Historical Locality
= Absent Non-Historical Locality
= Present Non-Historical Locality
N
0
20
40 Kilometers

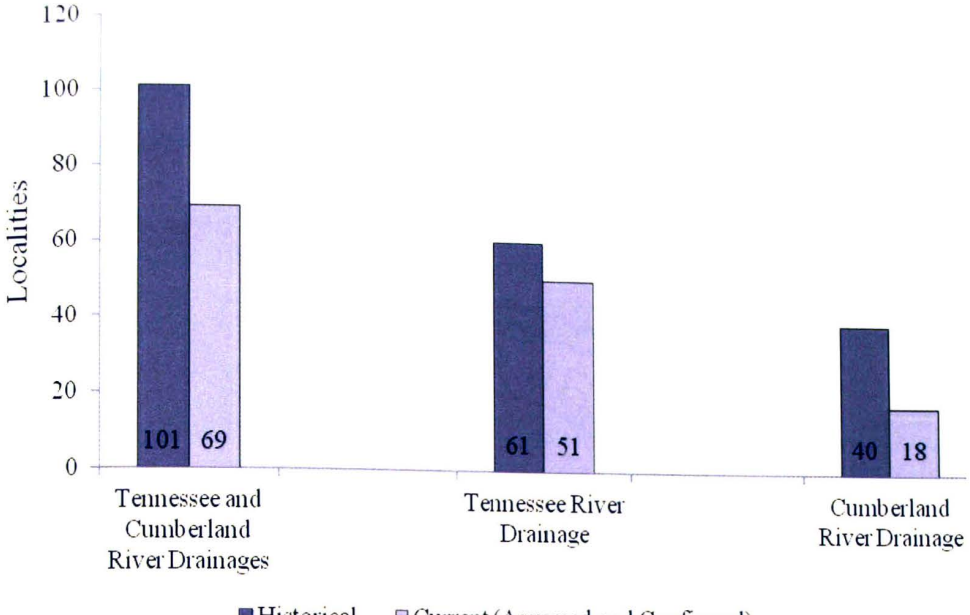


Figure 6. Graphical summary of status survey results comparing number of historical localities to number of current localities. Results include newly identified localities.

Figure 7. Current distribution of the Redband Darter, *Etheostoma luteovinctum*, including newly identified localities. Circles with black dots inside represent historical localities where *E. luteovinctum* was found or was assumed to be present. Squares with black dots inside represent newly identified localities. Specific locality information is given in Appendix B.

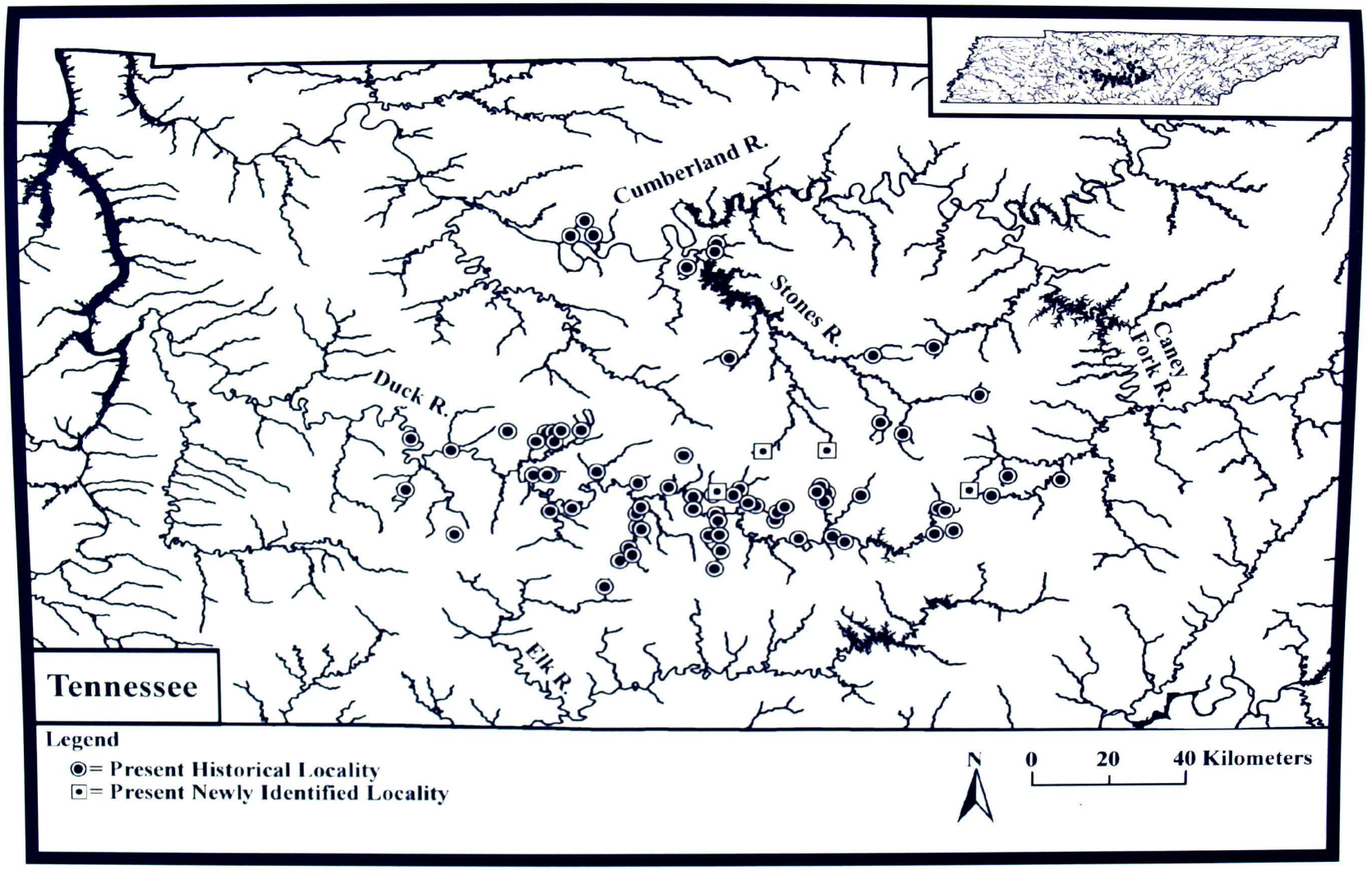

Cumberland R.
Stones R.
Caney Fork R.
Duck R.
Elk R.
Tennessee
Legend
◉= Present Historical Locality
⊡= Present Newly Identified Locality
N
0 20 40 Kilometers

Figure 8. Maximum-parsimony phylogram of *Etheostoma luteovinctum* based on 2601 amplified fragment length polymorphism characters. Values on nodes are bootstrap values from parsimony analyses followed by those from Nei-Lei distance analyses in parentheses. An asterisk (*) indicates a node was not recovered in the distance analysis. Individuals are numbered by site number, which corresponds to those in Figure 3. Numbers in parentheses following site numbers are the number of individuals. Specific locality information with corresponding site numbers is given in Appendix B.

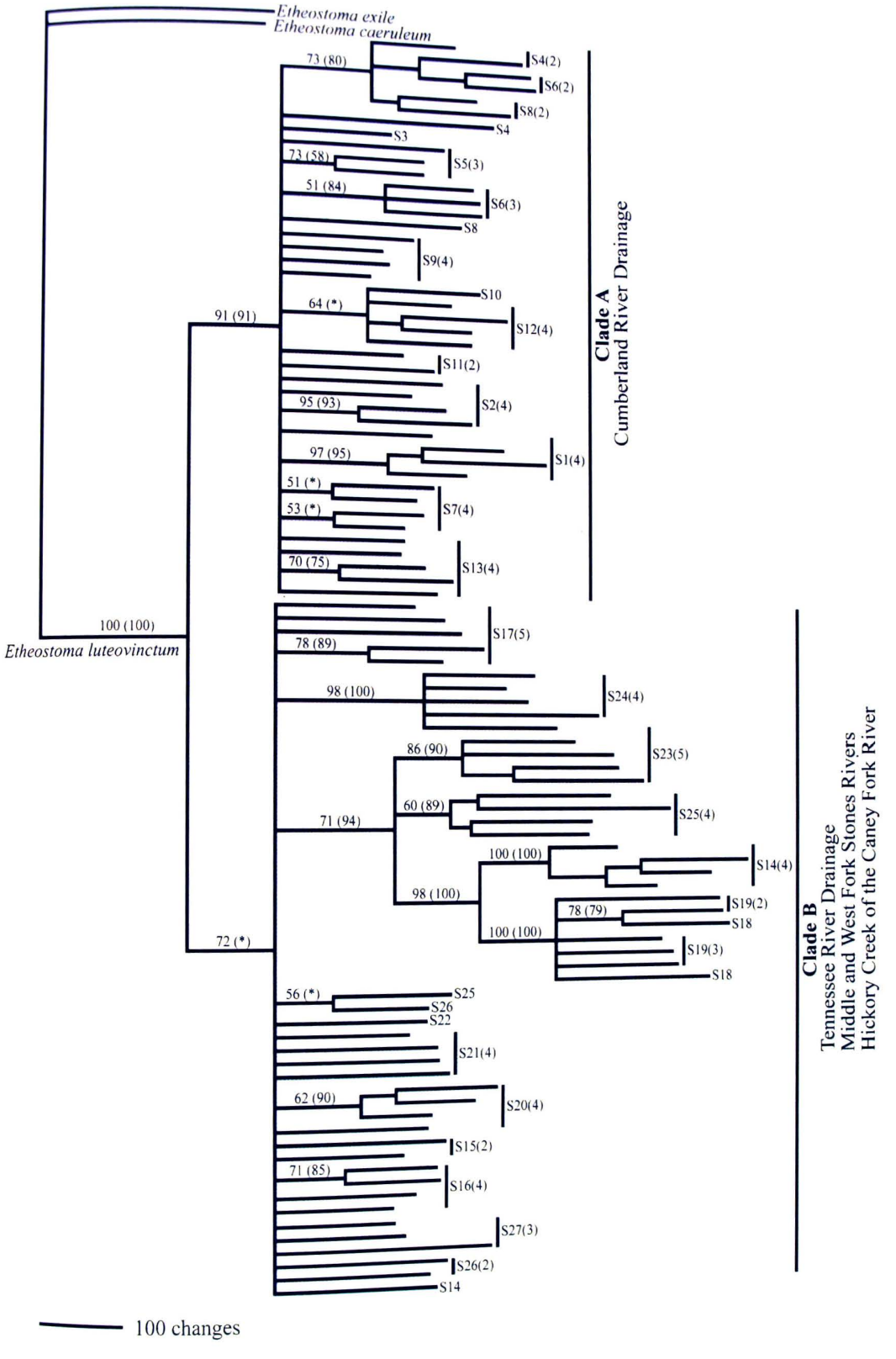

Etheostoma exile
Etheostoma caeruleum
73 (80)
S4(2)
S6(2)
S8(2)
S4
S3
73 (58)
S5(3)
51 (84)
S6(3)
S8
S9(4)
S10
64 (*)
91 (91)
S12(4)
S11(2)
95 (93)
S2(4)
97 (95)
S1(4)
51 (*)
S7(4)
53 (*)
70 (75)
S13(4)
Clade A
Cumberland River Drainage
100 (100)
Etheostoma luteovinctum
S17(5)
78 (89)
98 (100)
S24(4)
86 (90)
S23(5)
60 (89)
S25(4)
71 (94)
100 (100)
S14(4)
98 (100)
78 (79)
S19(2)
S18
100 (100)
S19(3)
S18
72 (*)
56 (*)
S25
S26
S22
S21(4)
62 (90)
S20(4)
S15(2)
71 (85)
S16(4)
S27(3)
S26(2)
S14
Clade B
Tennessee River Drainage
Middle and West Fork Stones Rivers
Hickory Creek of the Caney Fork River
100 changes

Figure 9. Geographic distribution of Clades A and B from the maximum parsimony analysis of 2601 amplified fragment length polymorphism loci. Clade A contained study sites 1-13 representing the East Fork Stones and Lower Stones River, Lower Caney Fork River, North Prong Barren Fork of the Caney Fork River, and Cumberland River tributaries. Clade B contained study sites 14-27 representing the Elk River, West and Middle Fork Stones River, Duck River, and Hickory Creek of the Caney Fork River. Specific locality information with corresponding site numbers is given in Appendix B.

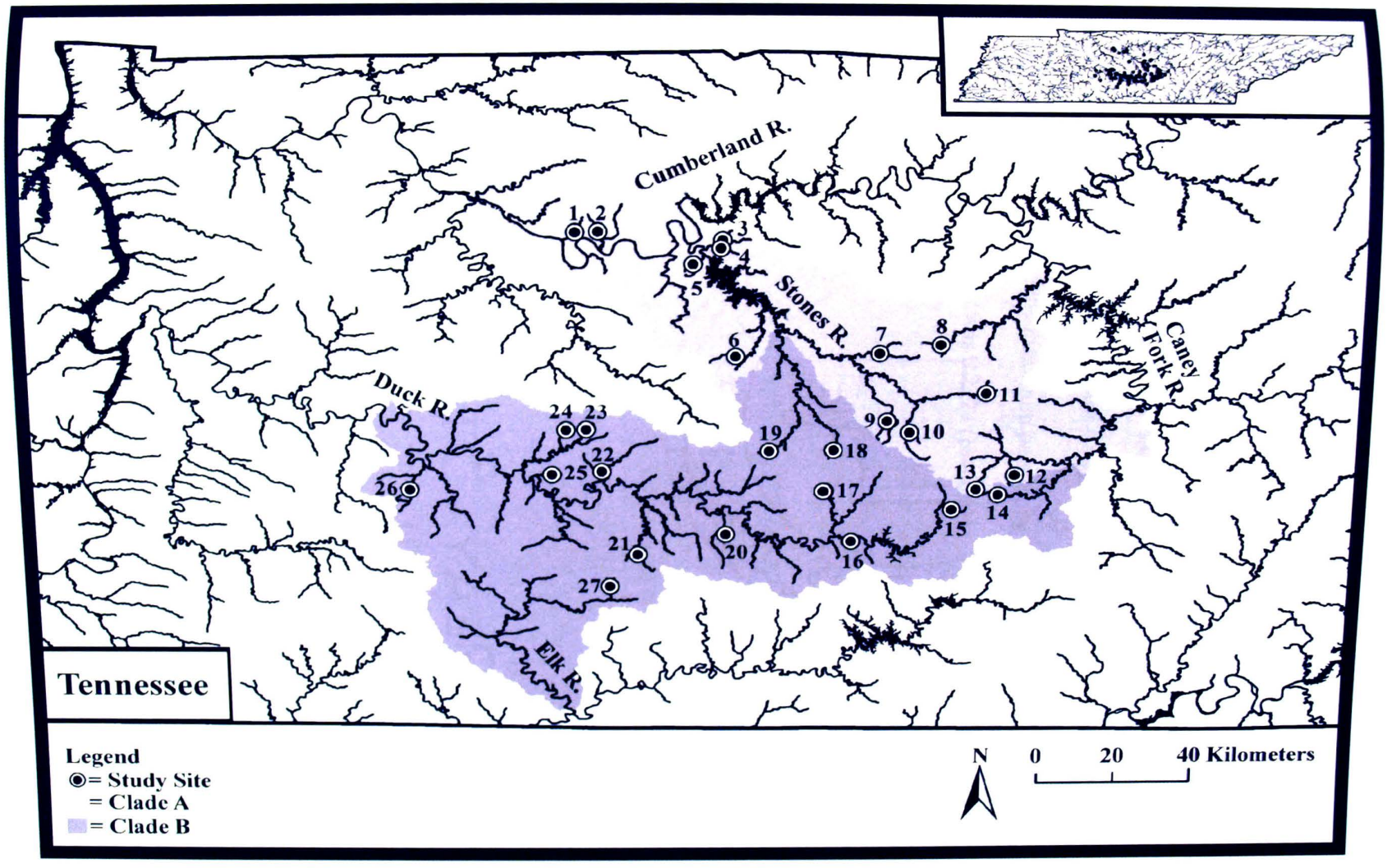

Cumberland R.
Stones R.
Caney Fork R.
Duck R.
Elk R.
Tennessee
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
Legend
= Study Site
= Clade A
= Clade B
N
0
20
40 Kilometers

Figure 10. Unweighted Pair Group Method with Arithmetic Mean diagram generated in DAMBE 5.2.31 (Xia and Xie, 2001) based on Fst values generated by AFLP-SURV version 1.0 (Vekemens, 2002) and 439 amplified fragment length polymorphism loci amplified with EcoRI+AGG and PSAI+AAA for *Etheostoma luteovinctum.* For each group, support was assessed using 1000 permutations and all other default settings were used. Clusters labeled 1 correspond to Clade A and clusters labeled 2 correspond to Clade B of Figure 8 as recovered in the maximum parsimony analysis. The overall Fst value for among population comparisons was 0.2229 ($p<0.0001$).

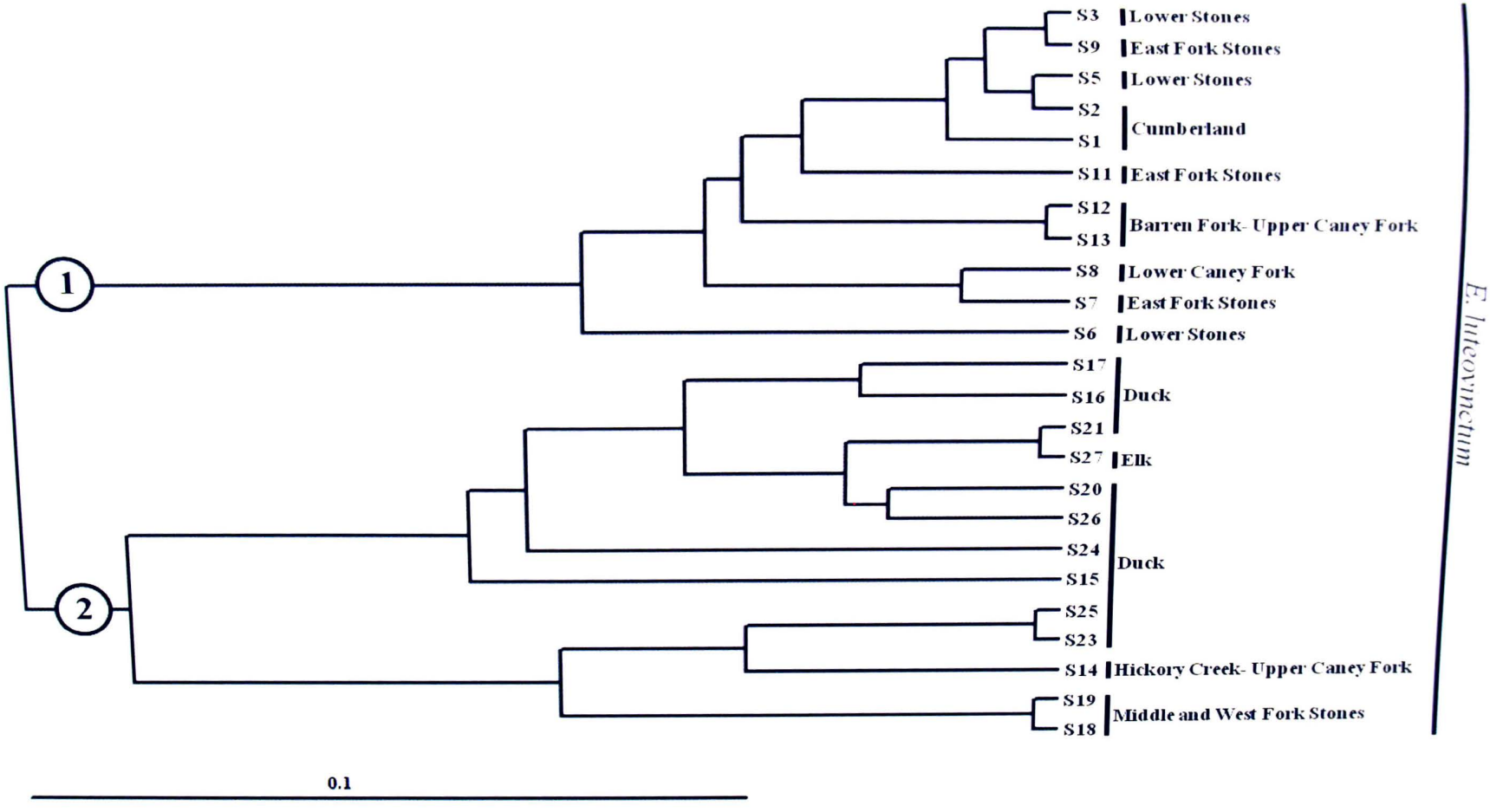

S3
S9
S5
S2
S1
S11
S12
S13
S8
S7
S6
S17
S16
S21
S27
S20
S26
S24
S15
S25
S23
S14
S19
S18
Lower Stones
East Fork Stones
Lower Stones
Cumberland
East Fork Stones
Barren Fork- Upper Caney Fork
Lower Caney Fork
East Fork Stones
Lower Stones
Duck
Elk
Duck
Hickory Creek- Upper Caney Fork
Middle and West Fork Stones
1
2
0.1
E. luteovinctum

Figure 11. Unweighted Pair Group Method with Arithmetic Mean diagram generated in DAMBE 5.2.31 (Xia and Xie, 2001) based on Fst values generated by AFLP-SURV version 1.0 (Vekemens, 2002) and 549 amplified fragment length polymorphism loci amplified with EcoRI+AGG and PSAI+AAG for *Etheostoma luteovinctum*. For each group, support was assessed using 1000 permutations and all other default settings were used. Clusters labeled 1 correspond to Clade A and clusters labeled 2 correspond to Clade B of Figure 8 as recovered in maximum parsimony analysis. The overall Fst value for among population comparisons was 0.1554 ($p<0.0001$).

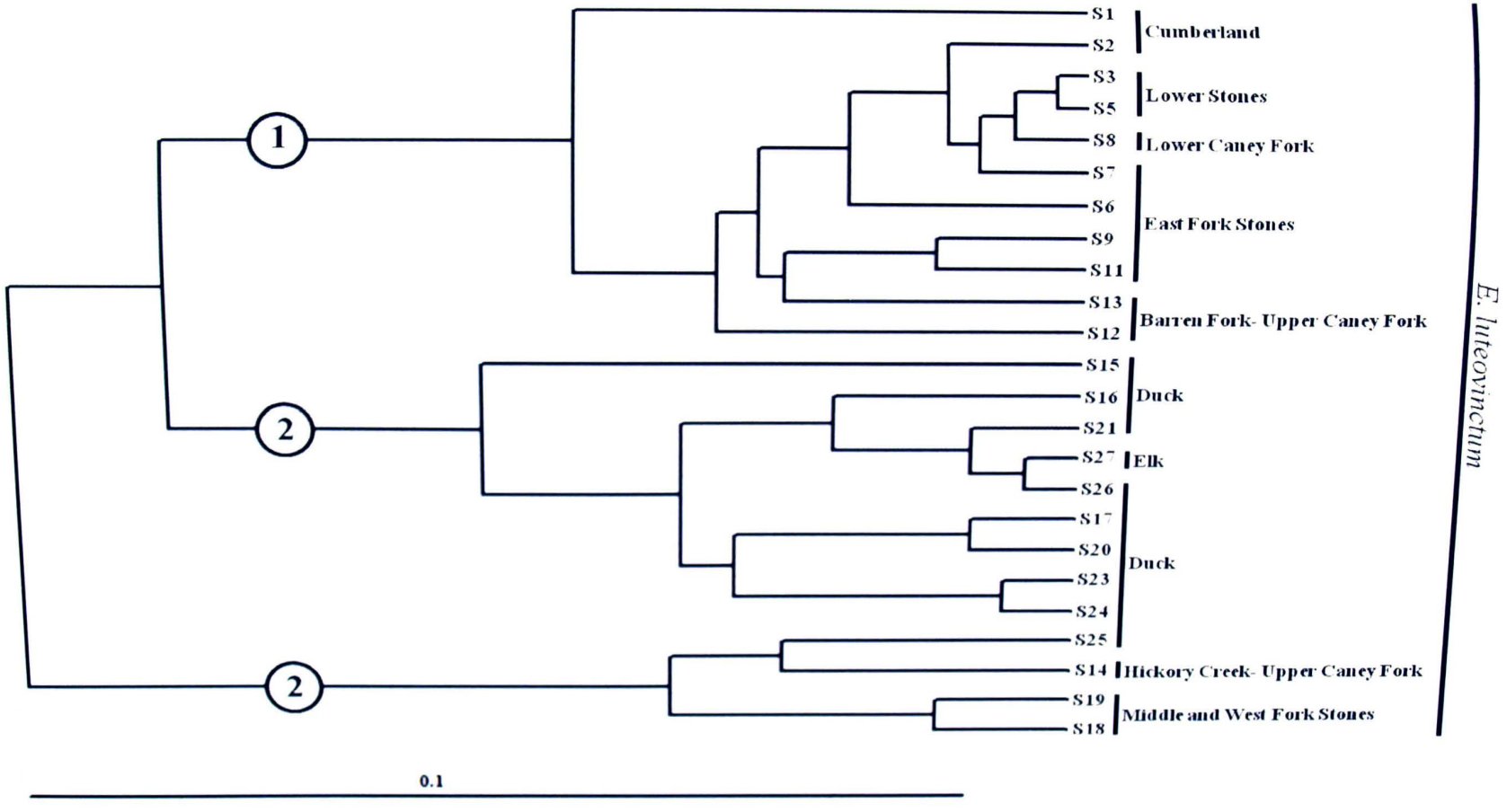

S1
S2
Cumberland
S3
S5
Lower Stones
S8
Lower Caney Fork
S7
S6
S9
S11
East Fork Stones
S13
S12
Barren Fork- Upper Caney Fork
S15
S16
S21
Duck
S27
Elk
S26
S17
S20
S23
S24
Duck
S25
S14
Hickory Creek- Upper Caney Fork
S19
S18
Middle and West Fork Stones
1
2
2
0.1
E. luteovinctum

Figure 12. Unweighted Pair Group Method with Arithmetic Mean diagram generated in DAMBE 5.2.31 (Xia and Xie, 2001) based on Fst values generated by AFLP-SURV version 1.0 (Vekemens, 2002) and 569 amplified fragment length polymorphism loci amplified with EcoRI+AGG and PSAI+ACG for *Etheostoma luteovinctum*. For each group, support was assessed using 1000 permutations and all other default settings were used. Clusters labeled 1 correspond to Clade A and clusters labeled 2 correspond to Clade B of Figure 8 as recovered in maximum parsimony analysis. The overall Fst value for among population comparisons was 0.1590 ($p<0.0001$).

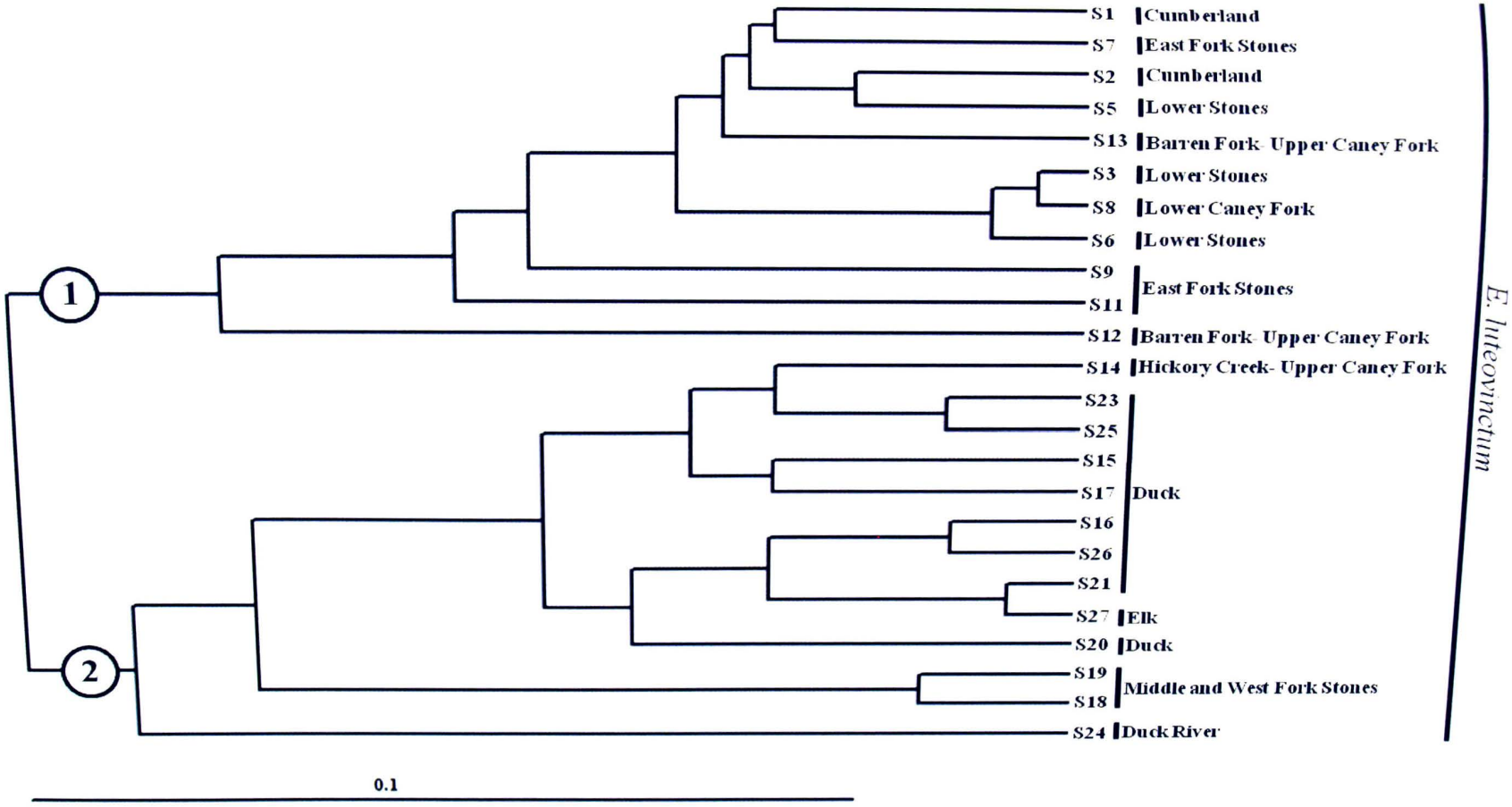

S1 Cumberland
S7 East Fork Stones
S2 Cumberland
S5 Lower Stones
S13 Barren Fork- Upper Caney Fork
S3 Lower Stones
S8 Lower Caney Fork
S6 Lower Stones
S9
S11 East Fork Stones
S12 Barren Fork- Upper Caney Fork
S14 Hickory Creek- Upper Caney Fork
S23
S25
S15
S17 Duck
S16
S26
S21
S27 Elk
S20 Duck
S19
S18 Middle and West Fork Stones
S24 Duck River
1
2
0.1
E. luteovinctum

Figure 13. Unweighted Pair Group Method with Arithmetic Mean diagram generated in DAMBE 5.2.31 (Xia and Xie, 2001) based on Fst values generated by AFLP-SURV version 1.0 (Vekemens, 2002) and 515 amplified fragment length polymorphism loci amplified with EcoRI+AGG and PSAI+AGA for *Etheostoma luteovinctum*. For each group, support was assessed using 1000 permutations and all other default settings were used. Clusters labeled 1 correspond to Clade A and clusters labeled 2 correspond to Clade B of Figure 8 as recovered in the maximum parsimony analysis. The overall Fst value for among population comparisons was 0.1951 ($p<0.0001$).

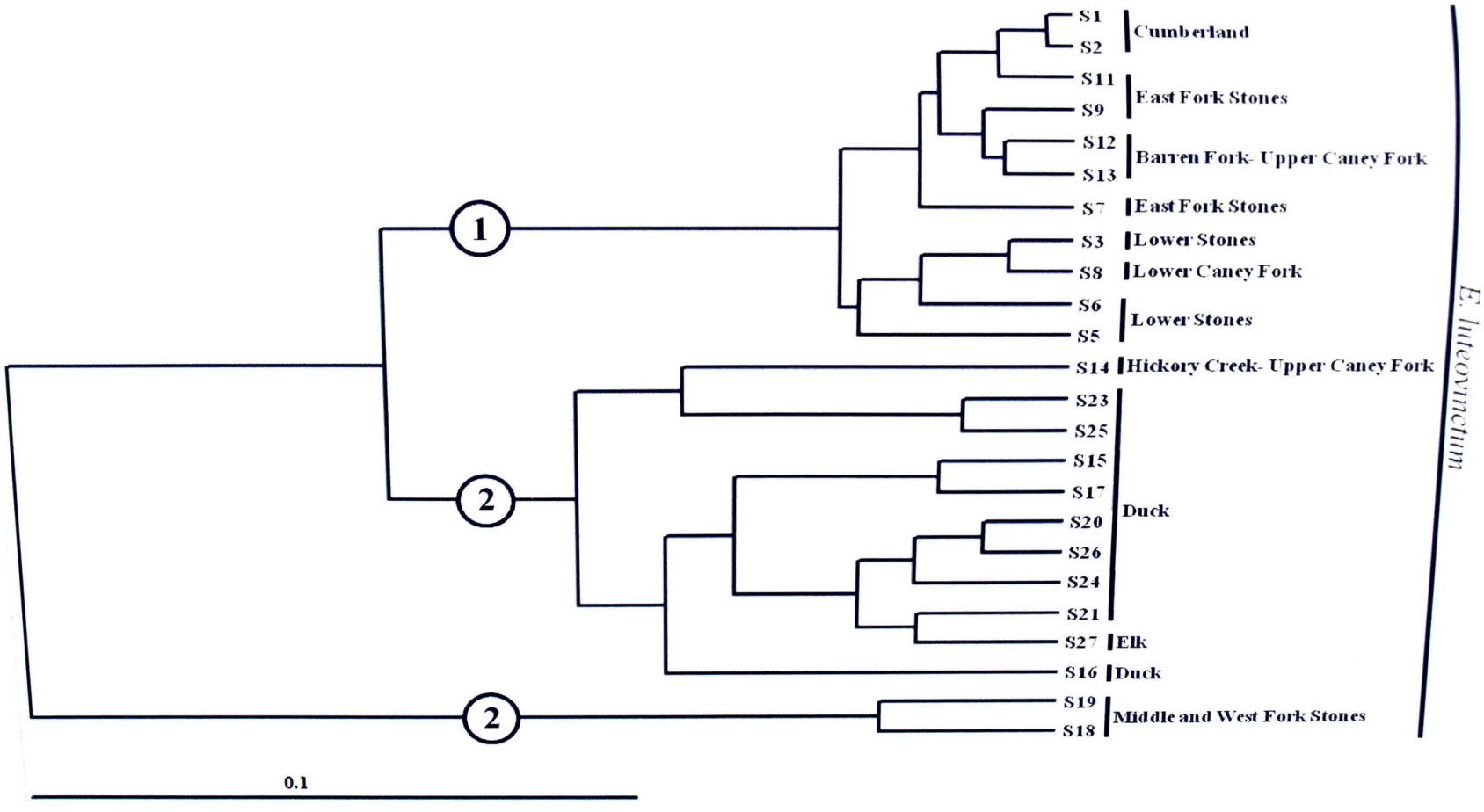
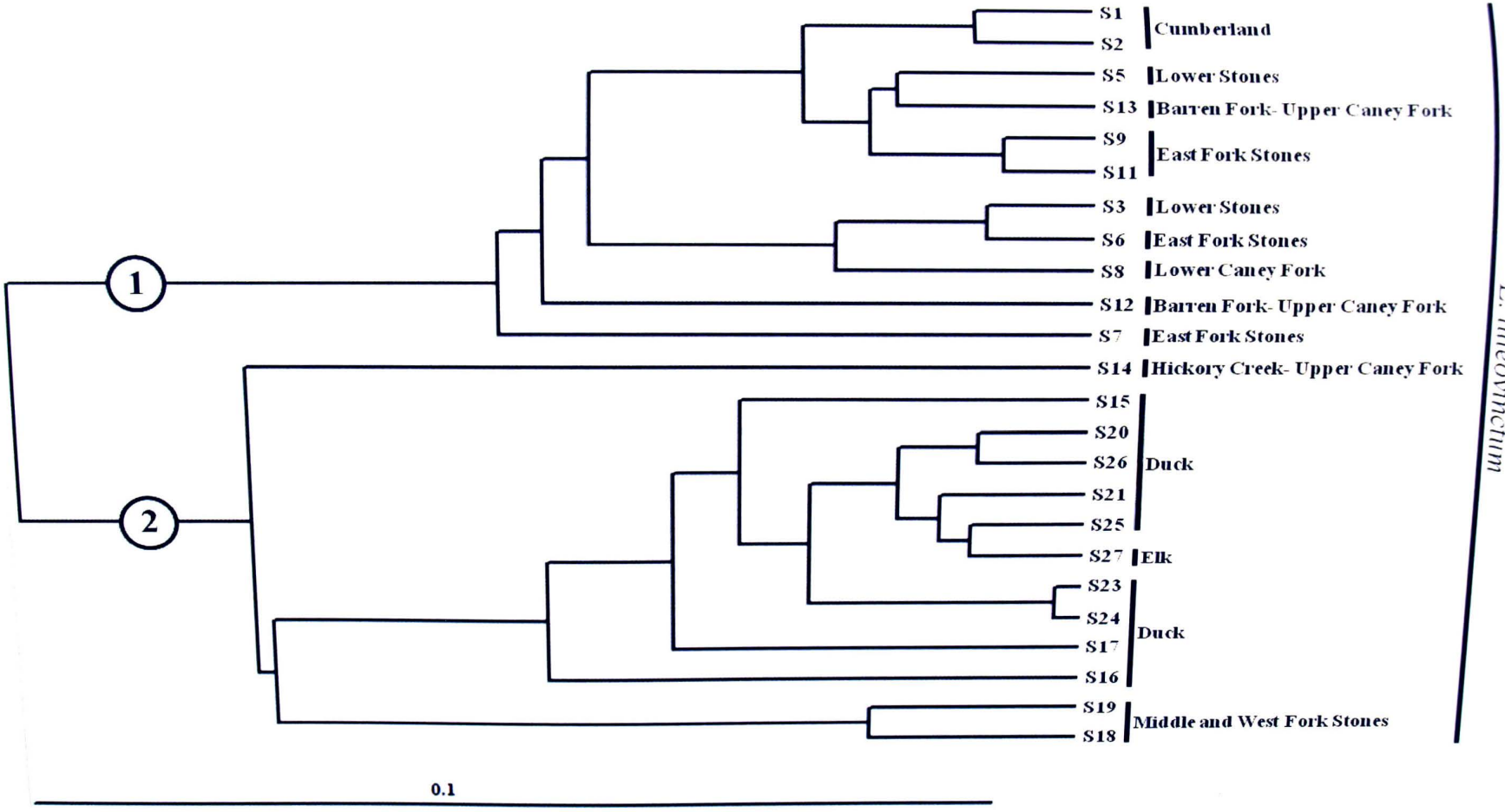

S1
S2
Cumberland
S11
S9
East Fork Stones
S12
S13
Barren Fork- Upper Caney Fork
S7
East Fork Stones
S3
Lower Stones
S8
Lower Caney Fork
S6
S5
Lower Stones
S14
Hickory Creek- Upper Caney Fork
S23
S25
S15
S17
S20
S26
S24
S21
Duck
S27
Elk
S16
Duck
S19
S18
Middle and West Fork Stones
1
2
2
0.1
E. luteovinctum

Figure 14. Unweighted Pair Group Method with Arithmetic Mean diagram generated in DAMBE 5.2.31 (Xia and Xie, 2001) based on Fst values generated by AFLP-SURV version 1.0 (Vekemens, 2002) and 569 amplified fragment length polymorphism loci amplified with EcoRI+AGG and PSAI+ATT for *Etheostoma luteovinctum*. For each group, support was assessed using 1000 permutations and all other default settings were used. Clusters labeled 1 correspond to Clade A and clusters labeled 2 correspond to Clade B of Figure 8 as recovered in the maximum parsimony analysis. The overall Fst value for among population comparisons was 0.1908 ($p<0.0001$).


S1
S2
Cumberland
S5
Lower Stones
S13
Barren Fork- Upper Caney Fork
S9
S11
East Fork Stones
S3
Lower Stones
S6
East Fork Stones
S8
Lower Caney Fork
S12
Barren Fork- Upper Caney Fork
S7
East Fork Stones
S14
Hickory Creek- Upper Caney Fork
S15
S20
S26
Duck
S21
S25
S27
Elk
S23
S24
Duck
S17
S16
S19
S18
Middle and West Fork Stones
1
2
0.1
E. luteovinctum

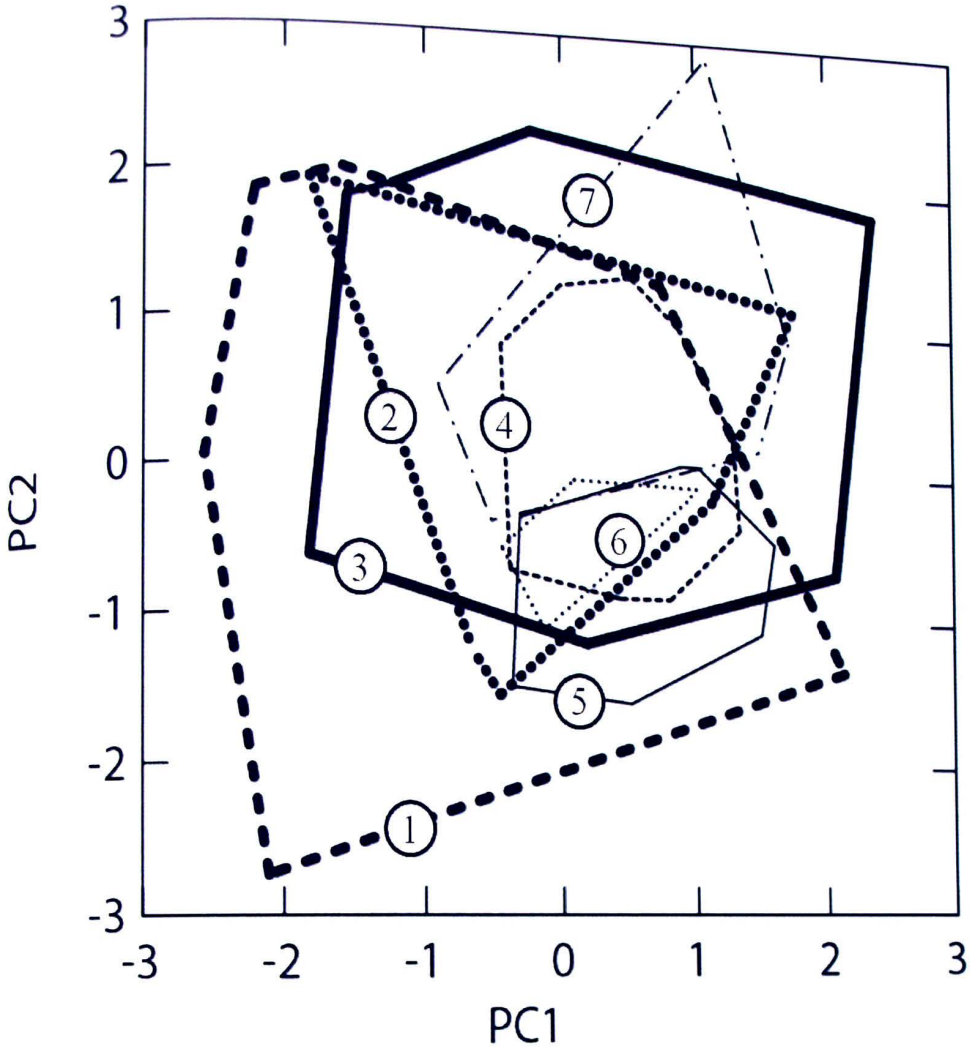


Figure 15. Plot of principal component factor scores for 10 meristic characters on PC axes 1 and 2 for *E. luteovinctum*. Polygons bound all individuals examined from a given drainage, system, river, or stream and include: (1) Eaton Creek (site 2), Lower (site 4) and East Fork (sites 7, 9, and 11) Stones River, 33 specimens; (2) Barren Fork-Upper Caney Fork River (sites 12 and 13), 17 specimens; (3) Duck River (sites 15, 21, 23, 24, 25), 39 specimens; (4) Middle (site 18) and West Fork (site 19) Stones River, 19 specimens; (5) Marshall Creek-Lower Caney Fork River (site 8), 10 specimens; (6) Hickory Creek-Upper Caney Fork River (UT 91.2518), 10 specimens; (7) Elk River (site 27), 12 specimens. Table 16 lists component loadings for meristic characters.

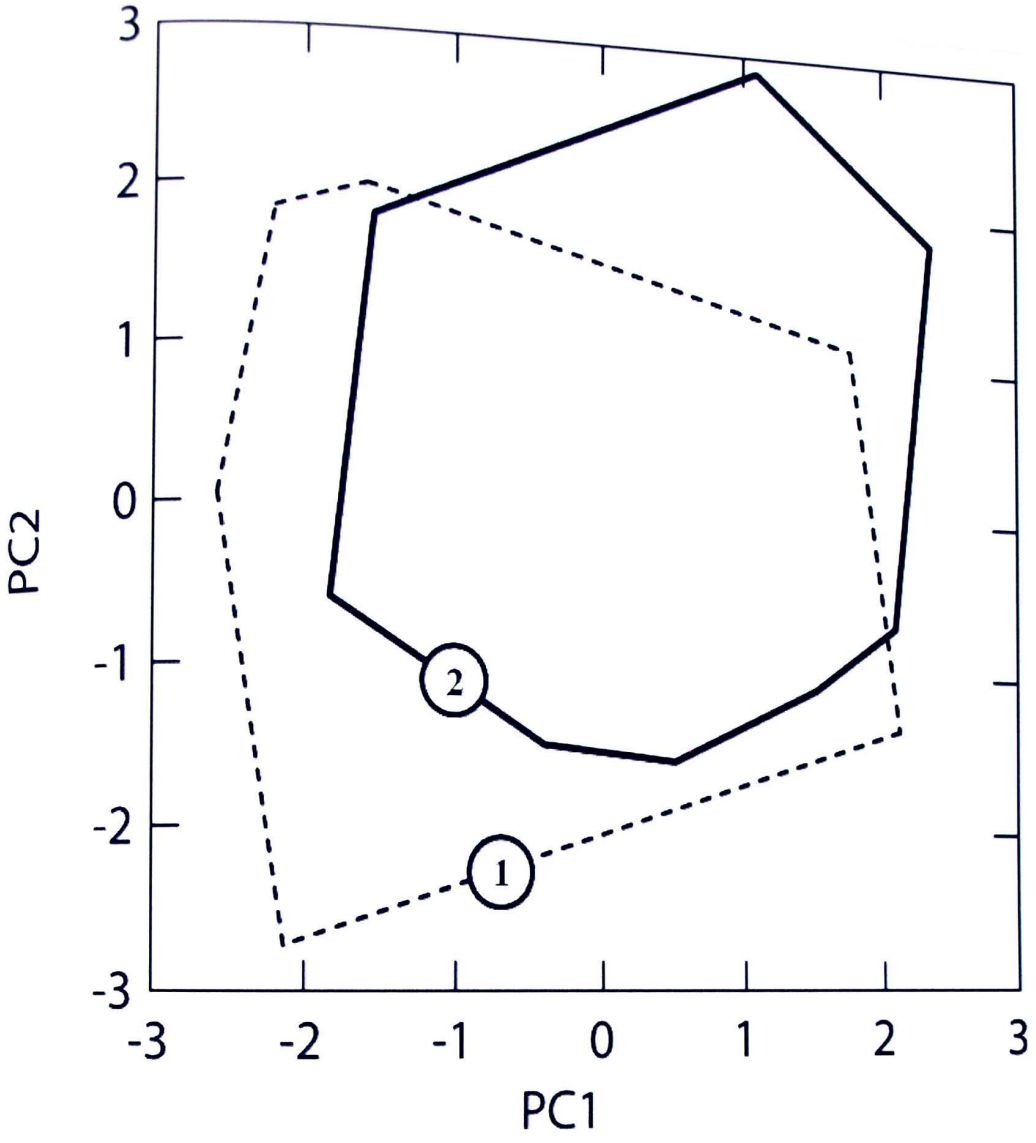


Figure 16. Plot of principal component factor scores for 10 meristic characters on PC axes 1 and 2 for *E. luteovinctum*. Polygons bound all individuals examined from a given clade (See Figures 7 and 8) and include: (1) Clade A, 60 specimens; (2) Clade B, 80 specimens. Table 16 lists component loadings for meristic characters.

CHAPTER IV

DISCUSSION

Current Distribution and Status of *E. luteovinctum*:

Results of the status survey indicated a possible 35.6% decrease in the range of *E. luteovinctum*, which supports rejection of the null hypothesis that *E. luteovinctum* is a stable species that does not require additional conservation measures. When geographically evaluated, the Cumberland River drainage populations show the most drastic decline within the range of the species (Figure 6). The potential loss of populations is likely due to the effects of anthropogenic land use practices in the region. Primary land uses in the Stones River and the Caney Fork River of the Eastern Highland Rim and the Outer Nashville Basin portions of the range of *E. luteovinctum* include pasture and cropland, while primary land uses in the Stones River of the Inner Nashville Basin include land cleared for urban development, as well as pasture and cropland (Arnwine et al., 2003; Arnwine et al., 2005).

The surveyed streams, from which *E. luteovinctum* was absent or present in low numbers, were commonly surrounded by agricultural fields with direct cattle access to streams and a lack of riparian zones between the fields and streams. The occurrence of agricultural land use has been shown to negatively affect intolerant benthic species, such as darters (Gammon and Gammon, 1990; Berkman and Rabeni, 1987; Lammert and David, 1999). Livestock access to streams has been shown to negatively affect fish communities and degrade stream habitat as animals stir up sediment, erode stream banks, and add nitrate and ammonia to the streams (Gammon et al., 2003). Decreased riparian zone width resulting in increased habitat homogeneity and increased sediment load has

been associated with decreases in abundance of benthic fishes (Richardson and Jowett, 2002.) The "widespread habitat degradation from agriculture" (Layman et al., 1993) has been previously noted in the Caney Fork River as a concern for other species of darters. In the upper Caney Fork River where the potential loss of 13 of 17 historical localities was observed, heavy agricultural impacts were observed and the distribution of such land use relative to the historical and present distribution of *E. luteovinctum* in this area are shown in Figure 17.

Drastic declines in the Stones River system are thought to be associated with a combination of agricultural practices and recent increases in urbanization. At multiple surveyed historical localities in this system, from which *E. luteovinctum* was absent, observations of the conversion of streams to drainage ditches for suburban/urban runoff were noted. Urbanization of areas typically leads to an increase in impervious surface area, which has been linked to the absence of sensitive species (Stranko et al., 2010), such as darters. Increased impervious surface area delivers increased runoff to streams (Horner et al., 1994) and has been associated with altered flow regimes, increased temperatures, and increased sediment loads (Horner et al., 1994). The influence of these factors has been linked to degradation of habitat important to the life cycles of aquatic biota, including freshwater fishes (Berkman and Rabeni, 1987; Rabeni and Smale, 1995). Stable flow regime has been associated with increased species richness (Schlosser, 1985; Tabit and Johnson, 2002). Increased temperatures have been shown to have negative effects on darter survival (Smith and Fausch, 1997), as well as darter egg production and juvenile growth (Bonner et al., 1998); while increased sediment load has been shown to have negative effects on sensitive benthic species (Berkman and Rabeni, 1987) and result

in decreased species diversity and abundance (Richardson and Jowett, 2002. Land use compromising or eliminating the small amounts of clean, loose gravel substrate necessary for Redband Darter spawning (Paxton, 1998) could explain the observed declines in the distribution of *E. luteovinctum*.

The four newly identified localities for *E. luteovinctum* in Weakley Creek (Duck River-Tennessee River Drainage), Mud Creek (site 13, Cumberland River Drainage), and the Middle and West Fork Stones rivers (sites 18 and 19, Cumberland River drainage) were comprised of typical *E. luteovinctum* habitat (Paxton, 1998) and the land use practices at these localities were typical of the Outer Nashville Basin and the Eastern Highland Rim (Arnwine et al, 2003; Arnwine et al, 2005). The Weakley Creek (Duck River-Tennessee River Drainage) and Mud Creek (site 13, Cumberland River Drainage) localities are contained within the previously known range for the species and are possibly a result of recent colonization or inadequate previous sampling. The Middle and West Fork Stones River (sites 18 and 19, Cumberland River drainage) localities do represent a range expansion for *E. luteovinctum* and are likely a result of inadequate previous sampling in the area.

Phylogeographic Relationships:

The AFLP-based phylogeny recovered well-supported, geographically structured clades of *E. luteovinctum* comparable to those observed for the species with mitochondrial DNA. This finding supports rejection of the null hypothesis that AFLPs will not provide sufficient resolution to examine phylogeographic relationships of fishes. The geographic distribution of clades (Figure 9) also supports several system and drainage transfers previously inferred from allozymes (Rogner, 1981) and the

mitochondrial ND2 gene (Lang, pers. comm.), resulting in rejection of the null hypothesis that cross system or drainage transfers have played no role in the history of *E. luteovinctum*.

This study is the first to note the presence of populations of *E. luteovinctum* in the Middle and West Fork Stones rivers (sites 18 and 19, Cumberland River drainage) and thus, their sister relationship (Figure 8) to individuals from Hickory Creek of the Caney Fork River (Cumberland River drainage, site 14). This is an interesting relationship as both populations are within the Cumberland River drainage, but separated by over 300 river-kilometers, over 40 air-kilometers, and several other populations of *E. luteovinctum* in the intervening areas. This phylogeographic relationship appears to be unique to *E. luteovinctum*, as no other fishes have been shown to display this geographic association. The factors that have shaped this relationship are largely speculative, but may be due to shared ancestral characters among these populations, populations may represent relicts of a historically more widespread lineage within *E. luteovinctum*, or other historical events such as past river connections or long-distance dispersal events. Additional research is needed to elucidate the historical processes that have shaped this phylogeographic relationship.

In general, the AFLP phylogeny recovered relationships that were consistent with four transfers across systems or drainages previously inferred from mitochondrial ND2 analysis of variation in the species (Lang, pers. comm.). These include transfers between: 1) Duck R. and Hickory Creek (CFR), 2) Duck R. and Elk R., 3) Stones R. and Barren Fork (CFR), and 4) Stones R. and Marshall Creek (CFR). Patterns observed were also consistent with relationships inferred from allozymes (Rogner, 1981) that suggested a

close relationship between Hickory Creek (CFR) and the Duck River, and divergence of the East Fork Stones River clade.

Specifically, the AFLPs recovered a clade of individuals from Hickory Creek (site 14), Middle (site 18) and West Fork (site 19) Stones rivers sister to two clades of Duck River individuals (sites 23 and 25; Figure 8). Recovery of these three Cumberland River drainage sites within the Tennessee River clade (Clade B), rather than in the clade with other individuals from the Cumberland River is consistent with hypotheses of cross-system and cross-drainage transfers involving *E. luteovinctum*. Population-level genetic analyses of *E. luteovinctum* also showed that individuals from Hickory Creek and the Middle and West Fork Stones Rivers were more genetically similar to the Tennessee River drainage populations, than to others from the Cumberland River drainage. The observed relationship between Hickory Creek (CFR) and the Duck River is consistent with allozyme (Rogner, 1981) and mitochondrial (Lang, pers. comm.) data, in which both studies also showed affinities between Hickory Creek and the Duck River, rather than with other Caney Fork or Cumberland River drainage populations.

The distribution of *E. luteovinctum* in general and the distribution of the two major clades that comprise the species (Clade A and B) are interesting in that they span multiple river systems and the Cumberland and Tennessee River drainage divide. The recovered genetic similarity of populations distributed in different river systems and/or drainages is most likely the result of relatively recent transfers as the populations involved typically share ND2 haplotypes (Lang, pers. comm.). This would support that the relationships between systems or populations within Clade A and Clade B are intimately linked to more recent transfer events. The degree of divergence observed

between the two clades however, likely reflects older isolation of populations distributed in the Cumberland and Tennessee Rivers, which both had separate outlets to the Gulf of Mexico throughout the late Tertiary(Starnes and Etnier, 1986); the Tennessee River through the Mobile River drainage and the Cumberland River through the Ohio River drainage. The Cumberland and Tennessee Rivers did not share a common outlet to the Gulf of Mexico through the Ohio River until the down cutting of the Mississippi River in the Pleistocene, which allowed the Tennessee River to capture the North flowing Duck River and arrive at the present day configuration (Starnes and Etnier, 1986). Both the shared interdrainage distribution of several species and geologic evidence support that the lower Duck River had separate connections to both the lower Cumberland River and the lower Tennessee River at different times before reaching its current configuration in the Pleistocene (Starnes and Etnier, 1986; Mayden, 1988). If *E. luteovinctum* was historically more widespread in the lower Cumberland River or lower Duck River it is plausible that the capture of the Duck River by the lower Tennessee River in the late Tertiary may have isolated populations of *E. luteovinctum* in the lower Duck River or the lower Cumberland River. If this scenario did occur, than it would account for the level of divergence observed between the two clades in this study.

Although, cross-system and drainage transfers involving *E. luteovinctum* appear to have played a substantial role in shaping the current distribution and patterns of genetic diversity in the species, the timing and method of transfers requires further study. Several possible events may have contributed to the patterns observed, individually or in combination. Bait-bucket transfer could potentially explain the close relationship shared between systems, such as the genetic similarity between populations in the Duck and Elk

Rivers (Figures 10-14), especially given that *E. luteovinctum* is only known from a single locality in the Elk R. and records for the species in the system are relatively recent, with the oldest record from 1994 (Charles Saylor, Tennessee Valley Authority Collection). However, darters are seldom used as bait fish.

The distribution of genetically similar populations of *E. luteovinctum* distributed across system and drainage divides could also be a result of recent or historical flooding events or stream capture, allowing for the transfer of individuals between headwater streams within close proximity of each other. Headwater piracy between the headwater streams of the Cumberland and Tennessee Rivers has been previously invoked for *E. luteovinctum* (Rogner, 1981; Starnes and Etnier, 1986; Lang, pers. comm.), as well as the obligate headwater Barrens Topminnow, *Fundulus julisia* (Starnes and Etnier, 1986). Page et al. (1992) invoked stream capture between the upper Caney Fork River and the upper Duck River to explain the occurrence of *Etheostoma nigripinne* x *Etheostoma forbesi* hybrids in the upper Duck River.

Notropis rupestris, Bedrock Shiner, and *Hemitremia flammea*, Flame Chub, also have distributions spanning headwater streams of the Duck, Caney Fork and Stones rivers, providing additional support for faunal exchanges among these systems. The shared distributions of these obligate headwater species in the focal region may indicated a shared history for these species. For example common vicariant event(s) may have influenced their modern distributions. However without the addition of temporal data that provides estimates of clade ages, whether the shared distributions reflect shared geologic histories or are a result of different vicariant events that occurred at different times and resulted in similar pseudocongruent biogeographic patterns (Donoghue &

Moore 2003) for each species, remains unclear. For example, other darters within the region, *Nothonotus* darters (Keck and Near, 2010) and Barcheek darters (Hollingsworth and Near, 2009), have been shown to exhibit pseudocongruent biogeographic patterns.

The prevalence of karst environments in central Tennessee led Rogner (1981), Starnes and Etnier (1986), and Lang et al. (pers. comm.) to hypothesize that fishes in the region utilize subterranean streams to migrate between headwater streams of neighboring river systems. Using microsatellites and the mitochondrial cytochrome b gene, Palandacic et al. (2012) supported the use of underground connections for recurrent migration as an explanation of relationships between isolated populations of a Croatian cyprinid, *Delminichthys adpersus* (*Cyprinidae*).

The Spring Cavefish, *Forbesichthys agassizii*, and the troglodytic Southern Cavefish, *Typhlichthys subterraneus* both have a similar distribution to *E. luteovinctum*, spanning multiple river systems in both the Cumberland and Tennessee River drainages. These species are hypothesized to use subterranean streams as a means of dispersal, although limited gene flow has been shown to exist between populations of *T. subterraneus* both within and among drainages (Niemiller and Fitzpatrick, 2008). This lack of gene flow between populations suggests that the use of subterranean streams has occurred historically and is not responsible for contemporary connections between populations. Results recovered from analyses of five nuclear and one mitochondrial loci for populations of *T. subterraneus* in central Tennessee recovered genetically similar populations that overlap with the range of *E. luteovinctum*. Populations of *T. subterraneus* in the central Duck River were found to be most closely related to a population near the mainstem Cumberland River, while populations in the upper Caney

Fork River were found to be most closely related to populations in the East Fork Stones River (Niemiller et al., 2012). Given the karst nature of the region, evidence of underground connections used by other fishes, and the common association of *E. luteovinctum* with small spring-fed streams, the use of underground stream connections cannot be ruled out as a viable mode of inter-system or inter-drainage transfers for this species. Additional research such as that by Palandacic et al. (2012) that incorporates microsatellite data should be explored to further test this hypothesis for *E. luteovinctum* and other fishes of the region.

Long-distance dispersal events between river systems and drainages could also potentially explain the distribution *E. luteovinctum* clades, but seems less plausible given the small size and relatively low vagility of the species (Page 1983). The inability of similar headwater darter species to use large river channels for migration was supported by observations of restricted gene flow in analyses of genetic structure among populations of darters (Echelle et al. 1975; Starnes and Etnier, 1986; Blanton, 2007; Lang and Echelle, 2011; Fluker et al., 2011). Furthermore, Lang (pers. comm., unpublished data) argued that the habitat specificity of *E. luteovinctum* for limestone bedrock streams, which are not present in the lower reaches of either the Cumberland or Tennessee rivers, acts as a migrational barrier. Whether or not Redband Darters are physically able to make these migrations through large river channels and whether larger river channels act as migrational barriers or filters is unknown, but no specimen records exist from the mainstem reaches of the systems or drainages where they occur (Etnier and Starnes, 1993). Thus, the natural history and ecological limitations of the species support hypotheses of headwater stream capture or underground connections, rather than long

distance dispersal to explain a distribution crossing the Cumberland and Tennessee Rivers.

Contrary to other studies that suggest larger rivers or mainstems may serve as barriers or filters to migration of headwater darter species (Lang and Echelle, 2011; Fluker et al., 2011), migratation through larger river channels has been suggested to explain the "interdigitated" geographic distributions of *E. derivativum* and *E. smithi*. The occurrence of two divergent clades of *E. luteovinctum* in the Stones River, Clade A in the East Fork and Lower Stones River and Clade B in the Middle and West Fork Stones Rivers, reflects the distribution of two members of the Barcheek Darter species group, *E. derivativum* which occurs in the Middle and West Fork Stones Rivers and *E. smithi* which occurs in the East Fork and Lower Stones Rivers (Page et al., 2003; Hollingsworth and Near, 2009). Hollingsworth and Near (2009) argued that historical instances of dispersal through the main stem of the Cumberland River had occurred, resulting in the current distribution of the two darters, but that gene flow between the systems had been limited. Given the similar distribution of clades of *E. luteovinctum* in the Stones River, smaller scale historical migration events through the mainstem of the Stones River, and also the Caney Fork River and/or Cumberland River, may have contributed to the current patterns of genetic diversity in *E. luteovinctum*.

Estimates of clade divergence times for *E. luteovinctum* would help clarify the historical events that have played a role in the history of the species. The Cumberland River is an ancient system that was not covered by glaciers (Starnes and Etnier, 1986) and correspondingly ancient lineages of fishes and high levels of microendemism have been observed in Cumberland River fishes (Hollingsworth and Near, 2009). For

example, Hollingsworth and Near (2009) estimated divergence times for separate Barcheek Darter species from the focal region between 9.3 and 2.6 million years ago. It would serve as an interesting study to estimate the divergence times for *E. luteovinctum* clades recovered in the AFLP analyses and compare to results of the Hollingsworth and Near (2009).

Cryptic Species:

Mayden (2002) argues that because different selective pressures may act on the rate of evolution of different traits, such as genes or morphology, there may often be a lack of congruence in estimations of phylogeny or diversity inferred by these traits. This lack of congruence between genes and morphology has been recognized in studies utilizing molecular analyses to detect morphologically cryptic species of mites (Avanzati et al., 1994), lizards (Brehm et al., 2001), fish (Egge and Simons, 2006; Rundle et al., 2000; Ho et al., 2012), and other taxa. This brings into question whether differences in morphology are required to diagnose a species and actually depends on what species concept the researcher accepts. The phylogenetic species concept defines a species as a diagnosable lineage distinct from other such lineages (McKitrick and Zink, 1988; Cracraft, 1983), but does not require that the diagnosis be based on a morphological feature.

Whether two genetically diagnosable lineages (which are not morphologically diagnosable) can represent two separate species has been recently evaluated in a madtom systematics study (Egge and Simons, 2006). Egge and Simons (2006) argued that under the phylogenetic species concept the ability to diagnose a species is not restricted to morphology, but that a diagnosis can also be based on genetic differences. They

diagnosed two allopatric lineages of *Noturus albater* as separate species based upon differences in karyotypes, allozyme loci, and DNA sequence data that supported the reciprocal monophyly of each species.

In this study, AFLPs (based on over 2000 gene fragments from genomic DNA) did recover two parapatric and well-supported, genetically distinct lineages. These lineages are consistent with those inferred from allozymes (Rogner, 1981) and mitochondrial DNA (Lang, pers. comm.) also. This leads to a rejection of the null hypothesis that *Etheostoma luteovinctum* is a single clade of populations, as two genetically distinct and geographically definable clades were recovered. Although, modal and mean differences were observed between Clade A and B for several morphological characters, the combination of traits that varied could not be used to clearly diagnose each clade as a species. This does not necessarily mean that Clade A and B are not morphologically diagnosable, but rather that the chosen morphological characters that were examined were not diagnostic. Despite this, following arguments of Egge and Simons (2006) on the phylogenetic species concept, these two clades would represent separate species. Although the Parsimony analysis recovered Clade A and Clade B, Nei and Lei distance analyses did not recover Clade B as a single distinct lineage. Given that all analyses did not support two clades and that clades could not be clearly diagnosed morphologically, clades are not currently described formally as species.

Waples (1991) argues that an evolutionarily significant unit can be represented by a genetically distinct population that occupies a unique habitat. Clade A and Clade B (Figure 8) recovered in this study were found to be genetically divergent from one another based on analysis of AFLP data, as well as previous analyses of mitochondrial

DNA (Lang, pers. comm.) and allozymes (Rogner, 1981). For example, a lack of gene flow between the two clades is supported by a lack of shared ND2 haplotypes between populations in the two observed clades (Lang, pers. comm.). The two genetically distinct clades also meet the criteria of occupying unique habitat as the clades appear to be geographically isolated in separate parts of river systems . Thus, the two clades meet the criteria necessary to be considered as separate evolutionarily significant units.

Conservation Implications:

Although no formal taxonomic elevation is given at this time, it is recommended that the two distinct clades be recognized as separate evolutionarily significant units, and managed as such. When the 35.6% decrease in the range of *Etheostoma luteovinctum* is evaluated considering the two evolutionarily significant units, it is clear that the lineage representing Clade A (14 extant populations; Figures 6 and 9) is facing a drastic decline. These results suggest that the species status is not stable and that conservation plans are needed. Further research that includes seasonal samples that specifically examine detection probability and abundance is needed to more clearly elucidate the species status and confirm the declining trend noted herein.

An increased sediment load and lack of riparian buffer was noted at many of the field sites in this study. Conservation efforts should concentrate on the implementation and enforcement of approved agricultural practices that prevent runoff, such as fencing cattle away from streams and maintaining riparian buffer zones between the streams and agricultural fields. These changes may reduce the runoff within streams of the region and, in turn, reduce the turbidity and decrease the levels of sedimentation that negatively impact *E. luteovinctum*, as increased sediment load has been shown to have negative

effects on sensitive species (Berkman and Rabeni, 1987). Increasing the amount of riparian buffer zones, both upstream and onsite, have been shown to improve benthic species richness and density (Lee et al., 2001; Duehr et al., 2006) by reducing sediment loads and decreasing instream temperatures via increased shade. Stream temperature is important as increased temperatures have been shown to have negative effects on darter survival (Smith and Fausch, 1997), as well as darter egg production and juvenile growth (Bonner et al., 1998).

As urbanization results in increases in impervious surfaces, which in turn delivers increased runoff to streams associated with altered flow regimes, increased temperatures, and increased sediment loads (Horner et al., 1994), efforts should also focus on minimizing the runoff from these areas. Before further urban expansion occurs within the range of the species, environmental assessments should be conducted to minimize negative effects on streams and their aquatic biota.

Other conservation efforts that may help the species could include the removal of dams that fragment the range of *E. luteovinctum*, as dams have been shown to act as migrational barriers to other species of darters (Haponski et al., 2007; Beneteau et al., 2009). The removal of these dams may allow for recolonization after extirpation due to stream intermittency or other anthropogenic causes.

Behavioral Observations:

During snorkel surveys at Dry Fork Creek (Stones River-Cumberland River Drainage, S4) multiple instances in which male Redband Darters buried themselves in fine gravel were observed (Plate 4). This behavior appeared to be in response to an approaching observer and not related to spawning, as no female Redband Darters were

within visible range of the males. The observed males only buried the colorful ventral half of their bodies and would stay buried for extended periods of time (> 10 minutes). Burying behavior not related to spawning has been noted in sandy substrates for all species of *Ammocrypta* (Jordan and Copeland, 1877) and *Crystallaria* (Page, 1983), and the Glassy Darter, *Etheostoma vitreum* (Winn and Picciolo, 1960); while the Arkansas Darter, *Etheostoma cragini*, has been known to bury itself headfirst in silt (Ellis and Jaffa, 1918). Burying behavior in gravel substrate is typically noted in female darters that utilize an egg-burying reproductive behavior during spawning (Page, 1983), but seldom documented in males. The Orangethroat Darter, *Etheostoma spectabile*, is another brightly colored member of the subgenus *Oligocephalus* and males of the species have been documented burying themselves in gravel substrate. This behavior was hypothesized to be unrelated to spawning, but rather a means of avoiding predators (Simon and Wallus, 2006). The observed burying behavior of Redband Darter males observed in the absence of females and when approached by a snorkeler is consistent with an anti-predator response behavior. The evolution of such a behavior is interesting given that few predatory organisms large enough to consume Redband Darters exist in the small bedrock streams they inhabit. This is the first known observation of male Redband Darters burying only their colorful mating displays and is a valuable contribution to the known information on darter behavior.

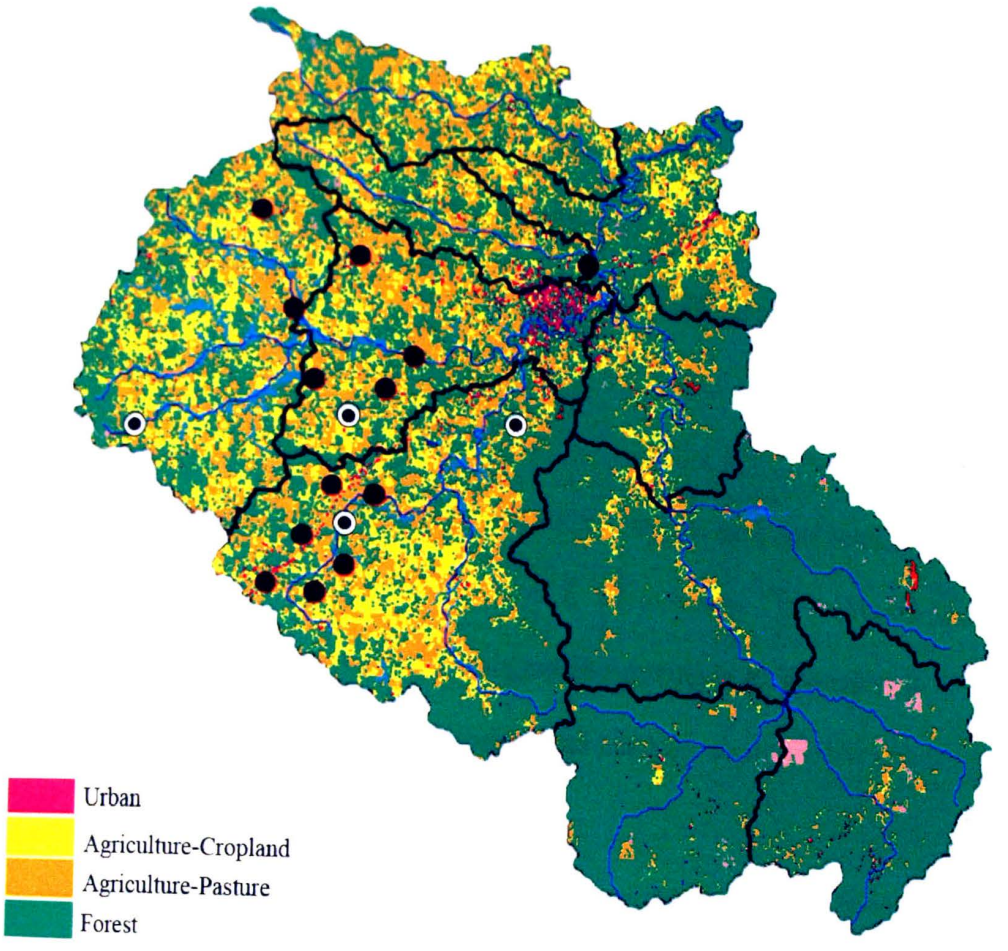


Figure 17. Land use map of the Collins River Watershed modified from Arnwine et al. (2003). Black circles represent extirpated localities and white circles with a black dot inside represent extant localities.

CHAPTER V

CONCLUSIONS

- *Etheostoma luteovinctum* is not a stable species as the results of the status survey showed a 35.6% decrease in range. The localities from which *E. luteovinctum* was absent were concentrated in the Cumberland River drainage and additional conservation measures are required.

- Amplified fragment length polymorphisms did provide sufficient resolution to examine phylogeographic relationships of *E. luteovinctum*. The recovered phylogeny was well supported and recovered geographic structure in *E. luteovinctum* comparable to that observed in mitochondrial DNA.

- Analysis of the AFLP data recovered two distinct clades not confined to drainage boundaries. Clade A (Figure 8) included only populations from the Cumberland River drainage, including the direct Cumberland River tributaries (sites 1 and 2), Lower (sites 3-6) and East Fork (sites 7 and 9-11) Stones River, Lower Caney Fork River (site 8), and North Prong Barren Fork of the Caney Fork River (sites 12 and 13). Clade B (Figure 8) largely represented populations from the Tennessee River drainage including all of the Duck River (sites 15-17 and 20-26) and the Elk River (site 27), but also three populations from the Cumberland River drainage including the Middle Fork (site 18) and West Fork (site 19) Stones River, and Hickory Creek of the Caney Fork River (site 14).

- The geographic distribution of the clades recovered from the AFLP phylogeny (Figure 9) supports several system and drainage transfers previously inferred from allozymes (Rogner, 1981) and mitochondrial ND2 (Lang, pers. comm.). The generated AFLP based phylogeny was consistent with the hypotheses of four transfers across systems or drainage divides as inferred from mitochondrial ND2 (Lang, pers. comm.). These include transfer events involving E. luteovinctum between: 1) Duck R. and Hickory Creek (CFR), 2) Duck R. and Elk R., 3) Stones R. and Barren Fork (CFR), and 4) Stones R. and Marshall Creek (CFR). The AFLP phylogeny also supported a drainage transfer between the Duck River and newly discovered populations in the Middle and West Fork Stones rivers, not previously documented.

- Although, modal and mean differences were observed between Clade A and B for several morphological characters, the combination of traits that varied could not be used to clearly diagnose each clade as a species. No formal taxonomic elevation is given at this time, but it is recommended that the two distinct clades be recognized as separate evolutionarily significant units and managed as such.

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VII. APPENDIX A

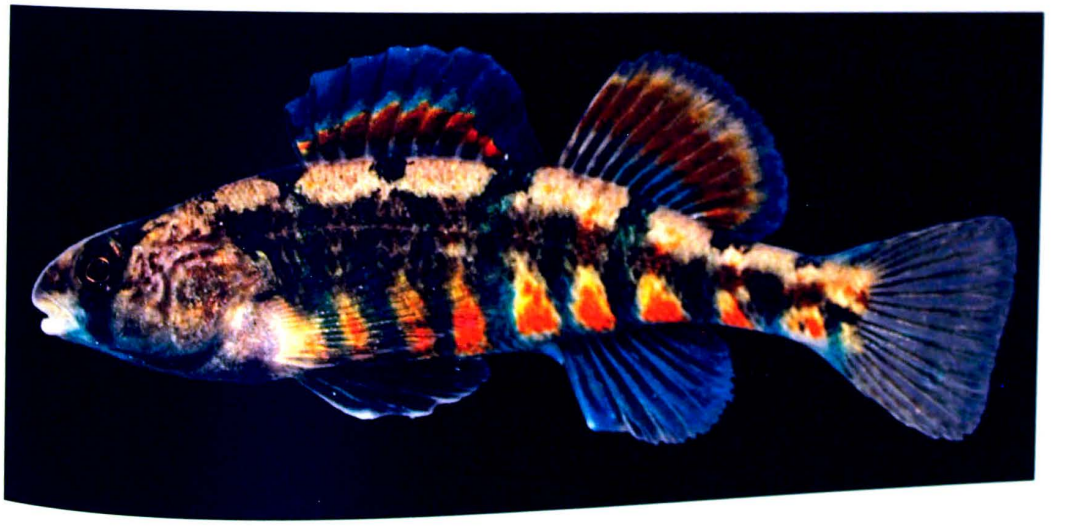
Plates 1-3

Plate 1. Examples of variation in color and pigmentation of male *Etheostoma luteovinctum* . The number below each picture corresponds to the site number given in Figure 3 and additional locality information is listed in Appendix B. General drainage and system information are listed below each picture. (Photos by M. Hoger)

A

Site 12, Caney Branch- Caney Fork River-Cumberland River Drainage

B



Site 4, Dry Fork Creek- Stones River-Cumberland River Drainage

Plate 2. Examples of variation in color and pigmentation of male *Etheostoma luteovinctum*. The number below each picture corresponds to the site number given in Figure 3 and additional locality information is listed in Appendix B. General drainage and system information are listed below each picture. (Photos by M. Hoger)

A

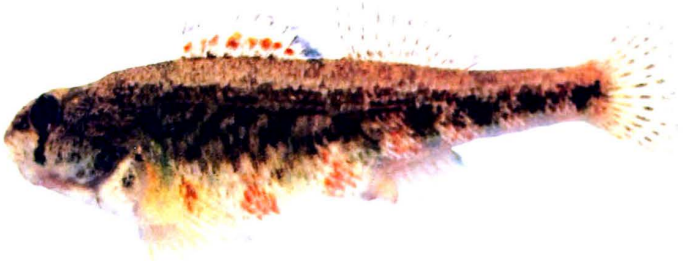
Site 27, unnamed tributary to Town Creek-Elk River-Tennessee River Drainage

B

Site 13, Mud Creek-Caney Fork River-Cumberland River Drainage

Plate 3. Examples of variation in color and pigmentation of female *Etheostoma luteovinctum*. The number below each picture corresponds to the site number given in Figure 3 and additional locality information is listed in Appendix B. General drainage and system information are listed below each picture. (Photos by M. Hoger)

A



Site 12, Caney Branch- Caney Fork River-Cumberland River Drainage

B

Site 7, Dry Fork-Stones River- Cumberland River Drainage

Plate 4. Examples of burying behavior observed for male *Etheostoma luteovinctum*. The number below the picture corresponds to the site number given in Figure 3 and additional locality information is listed in Appendix B. (Photo by M. Hoger)

Site 4, Dry Fork Creek- Stones River-Cumberland River Drainage

VIII. APPENDIX B

Materials Examined

MATERIALS EXAMINED

Materials examined including specific site locality and specimen accession information. Numbers following coordinate data are field collection numbers. Numbers following field numbers are site numbers (S#) used in plates, figures, and tables. Numbers in parentheses are number of specimens examined for morphological variation, number of specimens examined for pigmentation variation, number of specimens used to generate AFLP data, and voucher accession numbers, respectively. Institutional abbreviations follow Leviton et al. (1985).

***Etheostoma exile* specimens:**

1. MN: Hubbard Co.: Potato Lake, 7 miles North of Park Rapids at DNR boat ramp off of County Highway 40; Lat: 47.014356, Long: -95.089278; PAC09-45 (0, 0, 0, 175069 FLMNH 411)

***Etheostoma caeruleum* specimens:**

1. TN: Wilson Co.: Saunders Fork (Caney Fork R.-Cumberland R.-Ohio R.), on TN 267/96, 10 miles N of Woodbury; Lat: 35.983360 Long: - 86.066790. MDW2010-10 (0, 0, 0, APSU 00672)

Historical locality *Etheostoma luteovinctum* specimens examined:

1. TN: Davidson Co.: Sulphur Creek (Cumberland R.-Ohio R.) at intersection of Old Hickory Boulvard and Pecan Valley Road, 6 miles NW of Nashville; Lat: 36.220362 Long: -86.915925; MDW2011-27. **Site 1** (0, 5, 4, APSU 01347).

2. TN: Davidson Co.: Eaton Creek (Cumberland R.-Ohio R.) 100 m S of Eaton Creek and Rambling Brook Road, 5 miles NW of Nashville; Lat: 36.222108 Long: -86.86546; MDW2011-26, **Site 2** (10, 5, 4, APSU 01346).
3. TN: Davidson Co.: Stoners Creek and Scotts Creek Confluence (Stones R.-Cumberland R.-Ohio R.) 100 m S of Scotts Creek Parkway on Tulip Grove Road, 8 miles NE of Nashville; Lat: 36.201814 Long: -86.591471; MDW2011-04, **Site 3** (0, 0, 1, APSU 01330).
4. TN: Davidson Co.: Dry Fork Creek (Stones R.-Cumberland R.-Ohio R.) at 1084 Tulip Grove Road, 200 m S of Old Lebanon Dirt Road, 8 miles NE of Nashville; Lat: 36.182729 Long: -86.594967; MDW2011-03, **Site 4** (10, 5, 3, APSU 01329 and APSU 01333).
5. TN: Davidson Co.: McCrory Creek (Stones R.- Cumberland R.-Ohio R.) at Hurt Drive and Elm Hill Pike intersection, 5 miles E of Nashville; Lat: 36.144456 Long: -86.657046; MDW2011-13, **Site 5** (0, 5, 3, APSU 01334).
6. TN: Rutherford Co.: Rocky Fork Creek (Stones R.- Stones R.- Cumberland R.-Ohio R.) 50 m W of Red Hawk Parkway on Morton Road, 3 miles SW of Smyrna; Lat: 35.922476 Long: -86.561885; MDW2011-14, **Site 6** (0, 5, 5, APSU 01335).
7. TN: Cannon Co.: Dry Fork (Stones R.- Cumberland R.-Ohio R.) 500 m W of Bradley Creek Road on Highway 96, 3 miles E of Lascassas; Lat: 35.929735 Long: -86.238766; MDW2011-28, **Site 7** (9, 5, 4, APSU 01348).

8. TN: Cannon Co.: Marshall Creek (Caney Fork R.- Cumberland R.-Ohio R.) 100 m W of Marshall Creek Road on TN 96 in Auburntown; Lat: 35.950681 Long: - 86.101571; MDW2011-15, **Site 8** (10, 5, 3, APSU 01336).
9. TN: Rutherford Co.: Unamed tributary to Cripple Creek (Stones R.- Cumberland R.-Ohio R) 200 m W of intersection of Cripple Creek Road and East Lyon Road on East Lyon Road, 8 miles SW of Woodbury; Lat: 35.76338 Long: - 86.21916; MDW2011-16, **Site 9** (8, 5, 4, APSU 01337).
10. TN: Cannon Co.: Shelton Branch (Stones R.- Cumberland R.-Ohio R) 100 m SW of Dickens Hill Road on Dug Hollow Road in Bradyville; Lat: 35.735715 Long: -86.167523; MDW2011-17, **Site 10** (0, 0, 1, APSU 01338).
11. TN: Cannon Co.: Unamed tributary to Shanborne Branch (Stones R.- Cumberland R.-Ohio R) Stones River Road and Johnson Hollow Road on Johnson Hollow Road, 4 miles E of Woodbury; Lat: 35.831834 Long: -86.995818; MDW2011-18, **Site 11** (0, 3, 2, APSU 01339).
12. TN: Warren Co.: Caney Branch (Caney Fork R.- Cumberland R.-Ohio R.) at 1284 Bonner Road, 200 m W of 287 on Bonner Road, 7 miles SW of McMinnville; Lat: 35.631238 Long: - 85.925801; MDW2011-30, **Site 12** (7, 3, 4, APSU 01349).
13. TN: Coffee Co.: Meadow Branch (Caney Fork R.- Cumberland R.-Ohio R.) 100 m N of intersection of Martin Road and Smith Road on Smith Road in Morrison; Lat: 35.581819 Long: -85.961691; MDW2011-41 and MDW2011-42, **Site 14** (0, 0, 5, APSU 01355 and APSU 01356).

14. TN: Coffee Co.: Welker Branch (Duck R.- Tennessee R.-Ohio R.) 500 m N of Wayside Road on Maple Springs Road, 4 miles N of Manchester; Lat: 35.545114 Long: -86.067186; MDW2011-32, **Site 15** (5, 3, 2, APSU 01351).

15. TN: Bedford Co.: Unnamed tributary to Duck River (Duck R.- Tennessee R.-Ohio R.) 500 m N of Dement Road on Cortner Road, 30 m downstream of bridge on left, 25 miles NW of Normandy; Lat: 35.465974 Long: -86.295862; MDW2011-33, **Site 16** (0, 3, 4, APSU 01352).

16. TN: Bedford Co.: Unamed tributary to Bell Buckle Creek (Duck R.- Tennessee R.-Ohio R.) at 165 Fosterville Road, 400 m N of TN 82, W of Fosterville Road in Bell Buckle; Lat: 35.591646 Long: - 86.360866; MDW2011-11, **Site 17** (0, 5, 5, APSU 01332).

17. TN: Bedford Co.: Sinking Creek (Duck R.- Tennessee R.-Ohio R.) 1.5 miles W of TN 64 on Simms Road, 20 miles NE of Lewisburg; Lat: 35.484062 Long: -86.581508; MDW2011-25, **Site 20** (0, 3, 4, APSU 01345).

18. TN: Marshall Co.: Collins Creek (Duck R.- Tennessee R.-Ohio R.) 200 m SW of TN 272 on 31/11, 1 mile S of Lewisburg; Lat: 35.433954 Long: - 86.778986; MDW2011-24, **Site 21** (11, 5, 4, APSU 01344).

19. TN: Maury Co.: Pumpkin Creek (Duck R.- Tennessee R.-Ohio R.) 500 m W of Rally Hill Road on TN 99, 15 miles E of Columbia; Lat: 35.64264 Long: - 86.859364; MDW2011-22, **Site 22** (0, 0, 1, APSU 01343).

20. TN: Maury Co.: Grassy Branch (Duck R.- Tennessee R.-Ohio R.) 50 m W of Buckner Lane on North Old Port Royal Road, 3 miles E of Spring Hill; Lat: 35.744581 Long: -86.893679; MDW2011-19, **Site 23** (6, 4, 5, APSU 01340).

21. TN: Maury Co.: McCormick Creek (Duck R.- Tennessee R.-Ohio R.) 200 m SW of Town Center Parkway on TN 31/6, In Spring Hill; Lat: 35.74384 Long: -86.939074; MDW2011-20, **Site 24** (7, 5, 4, APSU 01341).

22. TN: Maury Co.: Bear Creek (Duck R.- Tennessee R.-Ohio R.) 50 m SE of Mount Olivet Road and Newt Hood Road intersection on Newt Hill Road, 4 miles E of Columbia; Lat: 35.63528 Long: -86.971254; MDW2011-21, **Site 25** (10, 5, 5, APSU 01342).

23. TN: Maury Co.: Unamed tributary to Hampshire Creek (Duck R.- Tennessee R.-Ohio R.) 50 m N of intersection of TN 412 and Biffle Lane in Hampshire; Lat: 35.597922 Long: -87.29191; MDW2011-37, **Site 26** (0, 2, 3, APSU 01354).

24. TN: Marshall Co.: Unnamed tributary to Town Creek (Elk R.- Tennessee R.-Ohio R.) 50 m S of Coleman Road on TN 129/31/11. Downstream of bridge in pool at Cornersville Firestation in Cornersville; Lat: 35.35365 Long: - 86.842199; MDW2011-36, **Site 27** (12, 5, 3, APSU 01353).

25. TN: Bedford Co.: Bell Buckle Creek (Duck R.-Tennessee R.-Ohio R.) at 165 Fosterville Road. 400 m N of TN 82, E of Fosterville Road in Bell Buckle; Lat: 35.592664 Long: - 86.358871; MDW2011-10, (0, 5, 0, APSU 01331).

New locality *Etheostoma luteovinctum* specimens examined:

1. TN: Coffee Co.: Mud Creek (Caney Fork R.- Cumberland R.-Ohio R.) 100 m S of Johnnie Jarrell Road on Mud Creek Road, 15 miles SW of McMinnville; Lat: 35.596008 Long: - 86.012835; MDW2011-31, **Site 13** (10, 5, 5, APSU 01350).
2. TN: Rutherford Co.: Unnamed tributary to Middle Fork Stones River (Stones R.- Cumberland R.-Ohio R.) on Christiana-Hoovers Gap Road, 6 miles S of Murfreesboro; Lat: 35.693779 Long: - 86.339137; MDW2012-03 and MDW2012-06, **Site 18** (10, 5, 2, APSU 01357 and APSU 01359).
3. TN: Rutherford Co.: Unnamed tributary to West Fork Stones River (Stones R.- Cumberland R.-Ohio R.) on Midland Road 200 m S of New Zion Road, 4 miles West of Christiana; Lat: 35.693779 Long: - 86.339137; MDW2012-03 and MDW2012-06, **Site 19** (9, 4, 4, APSU 01358).
4. TN: Bedford Co.: Weakley Creek (Duck R.-Tennessee R.-Ohio R.) at crossing on Halls Mill Road, 2 miles S of Unionville; Lat: 35.592788 Long: -86.58702; SPS2012-03, (0, 0, 0, APSU 01331).

Historical sampled localities from which *Etheostoma luteovinctum* was absent (borrowed specimens examined):

1. TN: Coffee Co.: West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) at Fountain Grove Rd., 200m West of Water Mill Rd., 5 miles SW of Morrison; Lat: 35.56023 Long: -85.945547. (10, 0, 0, UTK 91.2518)

Historical sampled localities from which *Etheostoma luteovinctum* was absent:

1. TN: Davidson Co.: Browns Creek (Cumberland R.-Ohio R.) 400 m N of Craighead Street on Bransford Avenue in Nashville; Lat: 36.127823 Long: -86.767076.
2. TN: Cannon Co.: Spring Creek (Cumberland R.-Ohio R.) on Beech Logging Road. 5 miles W of Watertown; Lat: 36.088367 Long: -86.226608.
3. TN: Cannon Co.: Locke Creek (Stones R.-Cumberland R.-Ohio R.) on Locke Creek Road, 50 m N of Country Lane, 3 miles NW of Woodbury; Lat: 35.83201 Long: -86.136143.
4. TN: Cannon Co.: East Fork Stones River (Stones R.-Cumberland R.-Ohio R.) 400 m N of Stone River Road on TN 53, 2 miles E of Woodbury; Lat: 35.83225 Long: -86.03534.
5. TN: Rutherford Co.: East Fork Stones River (Stones R.-Cumberland R.-Ohio R.) on Guy Jones (James) Road, 5 miles NE of Murfreesboro; Lat: 35.88261 Long: -86.27252.
6. TN: Rutherford Co.: Cripple Creek (Stones R.-Cumberland R.-Ohio R.) at TN 70 S, 0.5 miles W of Kittrell ; Lat: 35.824425 Long: -86.252838.
7. TN: Rutherford Co.: Bradley Creek (Stones R.-Cumberland R.-Ohio R.) at Twelve Corners Road, 2.5 miles ENE of Lascassas; Lat: 35.9513 Long: -86.2188.
8. TN: Cannon Co.: Brawleys Fork (Stones R.-Cumberland R.-Ohio R.) at crossing on 70S, 4.5 miles SW of Woodbury; Lat: 35.806381 Long: -86.155094.

9. TN: Rutherford Co.: McKnight Branch (Stones R.-Cumberland R.-Ohio R.) on Halls Hill Pike, 400 m S of Portfield Road, 4 miles NW of Woodbury; Lat: 35.87828 Long: -86.1637.

10. TN: Cannon Co.: Duke Creek (Caney Fork R.-Cumberland R.-Ohio R.) at crossing on Hollow Springs Road, 10.5 miles NE of Beechgrove; Lat: 35.66299 Long: -86.08978.

11. TN: Warren Co.: West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) on Phillip King Road, 2 miles S of Morrison; Lat: 35.580477 Long: -85.912889.

12. TN: Warren Co.: Barren Fork River at confluence with Henegar Branch (Caney Fork R.-Cumberland R.-Ohio R.), 6 miles SW of McMinnville; Lat: 35.66209 Long: -85.88061.

13. TN: Warren Co.: Garner Branch (Caney Fork R.- Cumberland R.-Ohio R.), at Comer Road, 7.6 miles WSW of McMinnville; Lat: 35.6439 Long: -85.9002.

14. TN: Warren Co.: Unnamed tributary to North Prong Barren Fork (Caney Fork R.-Cumberland R.-Ohio R.) on Petigap Road, 0.2 miles E of Oak Grove; Lat: 35.690694 Long: -85.948019.

15. TN: Warren Co.: Garner Branch (Caney Fork R.-Cumberland R.-Ohio R.) on Comer Road, 6.1 miles SW of McMinnville; Lat: 35.644254 Long: -85.899855.

16. TN: Warren Co.: Dog Branch (Caney Fork R.-Cumberland R.-Ohio R.) on TN 287, 200 m S of Underhill Road, 1 mile N of Centertown; Lat: 35.73376 Long: -85.91452.

17. TN: Warren Co.: Miller Branch (Caney Fork R.-Cumberland R.-Ohio R.) on Smithson Road, 2.4 miles NW Bates Hill; Lat: 35.75818 Long: -85.94544.

18. TN: Warren Co.: Collins River (Caney Fork R.-Cumberland R.-Ohio R.) at TN 70S, 1 mile NE of McMinnville; Lat: 35.7081 Long: -85.7317.

19. TN: Warren Co.: Unnamed tributary to West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) on Old Manchester Road, 1 mile SW of Morrison; Lat: 35.58641 Long: -85.936269.

20. TN: Warren Co.: Dry branch (Caney Fork R.-Cumberland R.-Ohio R.) at Smoot Rd, 11.2 miles SW of McMinnville; Lat: 35.662771 Long: -85.950096.

21. TN: Coffee Co.: Unnamed tributary to West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) 400 m SW of Garner Road on TN 55, 13.1 miles SW McMinnville; Lat: 35.5697 Long: -85.9542.

22. TN: Coffee Co.: West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) on Spears Road (Hickory Grove Road) 200 m S of Ramsey Road, 1.2 miles SE Summitville; Lat: 35.54204 Long: -85.9636.

23. TN: Cannon Co.: Saunders Fork (Caney Fork R.-Cumberland R.-Ohio R.) at intersection of TN 96 and TN 267, 2.5 miles NE of Auburntown; Lat: 35.9769 Long: -86.0706.

24. TN: Coffee Co.: Unnamed tributary to West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) 400 m NE of Rock Road on TN 55, 5.2 miles SW of Morrison; Lat: 35.549512 Long: -85.983227.
25. TN: Coffee Co.: West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) at Fountain Grove Rd., 200m West of Water Mill Rd., 5 miles SW of Morrison; Lat: 35.56023 Long: -85.945547.
26. TN: Coffee Co.: Norton Branch (Duck R.- Tennessee R.-Ohio R.) 400 m W of TN 64 on Norton Branch Road, 2 miles NE of Beechgrove; Lat: 35.64258 Long: -86.219047.
27. TN: Coffee Co.: Cisco Branch (Duck R.-Tennessee R.-Ohio R.) at crossing on Floyd Road, 9 miles E of Beechgrove; Lat: 35.632954 Long: -86.091803.
28. TN: Coffee Co.: Unnamed tributary to Carroll Creek (Duck R.-Tennessee R.-Ohio R.) at Craighead Road, 3.2 miles N of Tullahoma; Lat: 35.40968 Long: -86.198561.
29. TN: Coffee Co.: Garrison Fork (Duck R.- Tennessee R.-Ohio R.) at US 41 in Beechgrove; Lat: 35.62678 Long: -86.239035.
30. TN: Marshall Co.: Wilson Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on TN 270, 4 miles SE of Chapel Hill; Lat: 35.6001 Long: -86.6598.
31. TN: Maury Co.: Flat Creek (Duck R.- Tennessee R.-Ohio R.) 400 m S of Kedron Road on TN 431/106 in Rally Hill; Lat: 35.6653 Long: -86.8302.
32. TN: Maury Co.: Dry Creek (Duck R.- Tennessee R.-Ohio R.) on Gillespie Lane, 9 miles SW of Columbia; Lat: 35.539612 Long: -86.862597.

33. TN: Maury Co.: Flat Creek (Duck R.- Tennessee R.-Ohio R.) at crossing TN 412/99, 10 miles W of Columbia; Lat: 35.6425 Long: -86.8541.
34. TN: Bedford Co.: North Fork Creek (Duck R.- Tennessee R.-Ohio R.) on Unionville Deason Road, 12 miles NW of Shelbyville; Lat: 35.599348 Long: -86.535717.
35. TN: Marshall Co.: Big Rock Creek (Duck R.- Tennessee R.-Ohio R.) on Wallace Thompson Road, 6 miles NE of Lewisburg; Lat: 35.349996 Long: -86.84536.

Additional historical localities where *Etheostoma luteovinctum* was assumed present:

1. TN: Warren Co.: Locke Branch (Caney Fork R.- Cumberland R.-Ohio R.), at John Locke Road. 4.5 miles South of McMinnville; Lat: 35.621791 Long: - 85.804303.
2. TN: Bedford Co.: Duck River (Tennessee R.-Ohio R.) on Three Forks Bridge Road, 4 miles NW of Normandy; Lat: 35.4803 Long: -86.3248.
3. TN: Bedford Co.: Duck River (Tennessee R.-Ohio R.) 1 mile N of Clay Hill Road at crossing Haskins Road, 10 miles NW of Shelbyville; Lat: 35.5489 Long: -86.6407.
4. TN: Bedford Co.: Duck River (Tennessee R.-Ohio R.) 400 m S of intersection of TN 64 and TN 16 (41A), 1.5 miles E of Shelbyville; Lat: 35.4749 Long: -86.4013.

5. TN: Bedford Co.: Garrison Fork (Duck R.- Tennessee R.-Ohio R.) 400 m W of TN 64 on Walker Road, 2 miles SW of Beechgrove; Lat: 35.5832 Long: -86.261.
6. TN: Bedford Co.: North Fork Creek (Duck R.- Tennessee R.-Ohio R.) 100 m N of Kennedy Road at Crossing on TN 16 (41A), 7.5 miles NW of Shelbyville; Lat: 35.5845 Long: -86.5503.
7. TN: Bedford Co.: North Fork Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on TN 270, 10.5 miles NW of Shelbyville; Lat: 35.5845 Long: -86.5963.
8. TN: Bedford Co.: Sinking Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Wheel Road, 7.7 miles NW of Shelbyville; Lat: 35.5354 Long: -86.5902.
9. TN: Bedford Co.: Unnamed tributary to Sinking Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Sinking Creek Road, 2 miles S of Pickle Road, 1.5 miles N of Richmond; Lat: 35.39899 Long: -86.59205.
10. TN: Bedford Co.: Little Sinking Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Bethlehem Church Road, 1.5 miles S of Bedford; Lat: 35.44351 Long: -86.57777.
11. TN: Bedford Co.: Hurricane Creek (Duck R.- Tennessee R.-Ohio R.) at crossing of Old Nashville Dirt Road and Frank Martin Road, 3 miles NW of Shelbyville; Lat: 35.557158 Long: -86.49943.
12. TN: Bedford Co.: Little Hurricane Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on TN 10/231/82, 1.5 mi N Shelbyville; Lat: 35.52074 Long: -86.45491.

13. TN: Bedford Co.: Hurricane Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Airport Road, 4 miles N of Shelbyville; Lat: 35.5544 Long: -86.4331.

14. TN: Bedford Co.: Unnamed tributary to Hurricane Creek (Duck R.- Tennessee R.-Ohio R.) at TN 10/231/82 and Hurricane Grove Road, 4 miles N of Shelbyville; Lat: 35.540573 Long: -86.450704.

15. TN: Bedford Co.: Wartrace Creek (Duck R.- Tennessee R.-Ohio R.) at TN 269 and Parker Sain Road, 1 mile N of Bell Buckle; Lat: 35.60658 Long: -86.352771.

16. TN: Bedford Co.: Wartrace Creek (Duck R.- Tennessee R.-Ohio R.) at 50 m W of Couch Lane at crossing on TN 82. 1 mile E of Bell Buckle; Lat: 35.588574 Long: -86.339137.

17. TN: Maury Co.: Wartrace Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Fairfield Pike, 2 miles S of Bell Buckle; Lat: 35.588574 Long: -86.339137.

18. TN: Bedford Co.: Bear Creek (Duck R.- Tennessee R.-Ohio R.) at Old Bear Creek Road, 2.5 miles NE Columbia; Lat: 35.634924 Long: -87.002629.

19. TN: Maury Co.: Bear Creek (Duck R.- Tennessee R.-Ohio R.) at Berea Church S of TN 412, W of Cothran Road, 4.4 miles NE of Columbia; Lat: 35.6347 Long: -86.9634.

20. TN: Maury Co.: Snow Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Craig Bridge Road, 2 miles E of Williamsport; Lat: 35.6946 Long: -87.188.

21. TN: Maury Co.: Sugar Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Enterprise Road upstream of Arrow Lake, 3.2 miles SE of Mount Pleasant; Lat: 35.48653 Long: -87.18271.

22. TN: Maury Co.: Carters Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Butler Road, 6 miles N of Columbia; Lat: 35.7172 Long: -86.9956.

23. TN: Maury Co.: Titan Creek (Duck R.- Tennessee R.-Ohio R.) at Saturn Parkway upstream from Railroad overpass, 1.5 miles SW of Spring Hill; Lat: 35.740034 Long: -86.95659.

24. TN: Maury Co.: Unnamed tributary to Knob Creek (Duck R.- Tennessee R.-Ohio R.) on Haywood Hollow Road, 6 miles W of Spring Hill; Lat: 35.742123 Long: -87.059629.

25. TN: Maury Co.: Johnson Branch (Duck R.- Tennessee R.-Ohio R.) at intersection of Denning Road and Station Loop, 3 miles SW of Spring Hill; Lat: 35.716239 Long: -86.954916.

26. TN: Maury Co.: Fountain Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Old Highway 50 (Old Lewisburg Highway), 4 miles SE of Columbia; Lat: 35.54459 Long: -86.96528.

27. TN: Coffee Co.: Shanklin Branch (Duck R.- Tennessee R.-Ohio R.) at crossing on New Bushy Branch Road. 4 miles East of Manchester; Lat: 35.493975 Long: -86.014851.

28. TN: Coffee Co.: Duck River (Tennessee R.-Ohio R.) at crossing on TN 40 in Manchester; Lat: 35.4864 Long: -86.0911.

29. TN: Coffee Co.: Parks Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on TN 53, 4 miles N of Manchester; Lat: 35.55078 Long: -86.08252.
30. TN: Davidson Co.: Eatons Creek (Cumberland R.-Ohio R.) on Eatons Creek Road, 5 miles NW of Bordeaux; Lat: 36.2568 Long: -86.885.
31. TN: Hickman Co.: Dunlap Creek (Duck R.- Tennessee R.-Ohio R.) at intersection of TN 50 and Leatherwood Road, 11 miles SE of Centerville; Lat: 35.72378 Long: -87.27872.
32. TN: Marshall Co.: Dunlap Creek (Duck R.- Tennessee R.-Ohio R.) at intersection of TN 50 and Leatherwood Road, 11 miles SE of Centerville; Lat: 35.72378 Long: -87.27872.
33. TN: Marshall Co.: Big Rock Creek (Duck R.- Tennessee R.-Ohio R.) at UT Dairy Experiment Station, 2.6 miles SW of Lewisburg; Lat: 35.419272 Long: -86.807622.
34. TN: Marshall Co.: Big Rock Creek (Duck R.- Tennessee R.-Ohio R.) 200 m N of dead end on Mcbride Road, 3.7 miles NE of Lewisburg; Lat: 35.496447 Long: -86.761084.
35. TN: Marshall Co.: Big Rock Creek (Duck R.- Tennessee R.-Ohio R.) 1 mile N of Anes Station Road on TN 272; Lat: 35.5379 Long: -86.769.
36. TN: Marshall Co.: Big Rock Creek (Duck R.- Tennessee R.-Ohio R.) at W end of Water Street (Water Treatment Plant) in Lewisburg; Lat: 35.451336 Long: -86.78686.

37. TN: Marshall Co.: East Rock Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Anes Station Road, 7.7 miles NE of Lewisburg; Lat: 35.5541 Long: -86.7586.
38. TN: Marshall Co.: Caney Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Lunns Store Road, 14 miles W of Columbia; Lat: 35.6145 Long: -86.7658.
39. TN: Marshall Co.: Lick Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on Beasley Road, 4 miles N of Chapel Hill; Lat: 35.68133 Long: -86.66298.
40. TN: Marshall Co.: Spring Creek (Duck R.- Tennessee R.-Ohio R.) at crossing on TN 270, 3 miles S of Chapel Hill; Lat: 35.6033 Long: -86.6962.

Additional localities sampled from which *Etheostoma luteovinctum* was absent:

1. TN: Bedford Co.: Fall Creek (Duck R.- Tennessee R.-Ohio R.) on Gregory Mill Road, 6 miles NW of Shelbyville; Lat: 35.564315 Long: -86.516484.
2. TN: Bedford Co.: Hurricane Creek (Duck R.- Tennessee R.-Ohio R.) on TN 82, 2.5 miles N of Shelbyville; Lat: 35.543227 Long: -86.450787.
3. TN: Marshall Co.: Big Rock Creek (Duck R.- Tennessee R.-Ohio R.) on 31/11 and Cochran Cemetary Road, 4 miles SW of Lewisburg; Lat: 35.504701 Long: -86.767603.
4. TN: Marshall Co.: Spring Creek (Duck R.- Tennessee R.-Ohio R.) on Eagleview Pike/99, 1 mile N of Chapel Hill; Lat: 35.645457 Long: -86.683661.
5. TN: Marshall Co.: Town Creek (Elk R.- Tennessee R.-Ohio R.) on 129/31/11, 200 m S of Valley View Drive in Cornersville; Lat: 35.401565 Long: -86.808733.

6. TN: Wilson Co.: Little Caney Branch (Cumberland R.-Ohio R.) on Bell Road, 3.8 miles N of Watertown; Lat: 36.147444 Long: -86.131672.
7. TN: Wilson Co.: Big Caney Branch (Cumberland R.-Ohio R.) on Bell Road, 3.5 miles N of Watertown; Lat: 36.141271 Long: -86.12386.
8. TN: Wilson Co.: Round Lick Creek (Cumberland R.-Ohio R.) on Knee Road, 1 mile N of Watertown; Lat: 36.114433 Long: -86.131368.
9. TN: Cannon Co.: Brawleys Fork (Stones R.-Cumberland R.-Ohio R.) on Barker Road, 0.5 miles S of 70S, 3 miles E of Woodbury; Lat: 35.801908 Long: -86.151039.
10. TN: Rutherford Co.: Unnamed tributary to East Fork Stones River (Stones R.-Cumberland R.-Ohio R.) on Guy Jones (James) Road, 5 miles NE of Murfreesboro; Lat: 35.878862 Long: -86.27545.
11. TN: Warren Co.: Unnamed tributary to West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) on Tic Tac Mill Road, 400m W of King Road. 2 miles S of Morrison; Lat: 35.574159 Long: -85.926278.
12. TN: Warren Co.: Keel Branch (Caney Fork R.-Cumberland R.-Ohio R.) on Vervilla Road, 3 miles SE of Morrison; Lat: 35.574159 Long: -85.926278.
13. TN: Warren Co.: Unnamed tributary to North Prong Barren Fork (Caney Fork R.-Cumberland R.-Ohio R.) at crossing on 10 Penny Road, 6.1 miles SW of McMinnville; Lat: 35.646329 Long: -85.878998.
14. TN: Warren Co.: Unnamed tributary to North Prong Barren Fork (Caney Fork R.-Cumberland R.-Ohio R.) at crossing on Henegar Road, 6 miles SW of McMinnville; Lat: 35.637976 Long: -85.880693.

15. TN: Coffee Co.: West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) on Garner Road, 10 m N of Grove Road intersection, 2.5 miles SW of Morrison; Lat: 35.565484 Long: -85.943221.

16. TN: Coffee Co.: Unnamed tributary to West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) on Garner Road, 200 m N of Grove Road intersection, 2.5 miles SW of Morrison; Lat: 35.569045 Long: -85.943328.

17. TN: Coffee Co.: West Fork Hickory Creek (Caney Fork R.-Cumberland R.-Ohio R.) on Rock Road, 200 m South of TN 55, 6 miles SW of Morrison, 2.5 miles SW of Morrison; Lat: 35.54547 Long: -85.987688.

IX.VITA

Matthew David Wagner was born on 11 September 1987 in Reading, Pennsylvania. He graduated from Muhlenberg High School in 2006. He then attended Juniata College in Pennsylvania on the Calvert Ellis academic scholarship. In May 2010, he graduated with a Bachelor of Science degree in Biology. In August 2010, he moved to Clarksville, Tennessee to continue his education at Austin Peay State University. He was awarded a teaching assistantship with the Biology Department and worked under Dr. Rebecca Blanton Johansen. During the summer of 2011, he made major contributions to the curation of the ichthyology collection in the David H. Snyder Museum of Zoology. In addition to his assistantship, Matthew received additional academic and research support from the Graduate Student Research and Creativity Grant (APSU). He was awarded a Master of Science degree in Biology in August 2012 with a 4.0 GPA. Matthew has presented his research at Southeastern Fishes Council (2011), Tennessee American Fisheries Society (2012), APSU Graduate Student Research Extravaganza (2012), and Society for Freshwater Science (2012). He placed third in the student poster competition at SFC and placed first at the APSU Graduate Student Research Extravaganza. In June 2012, he moved to Brookings, South Dakota to start a doctorate program in the Department of Natural Resource Management at South Dakota State University. At SDSU he will be in charge of establishing and curating the ichthyology collection as well as writing an updated version of "The Fishes of the Dakotas".