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Systematics of the Stripetail Darter, *Etheostoma kennicotti* (Putnam), and the Distinctiveness of the Upper Cumberland Endemic *Etheostoma cumberlandicum* Jordan and Swain

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The Stripetail Darter, *Etheostoma kennicotti* (Putnam), is widely distributed in tributaries of the lower Ohio River, the upper Green River system, the Clarks River system, throughout the Tennessee River system, the Laurel River system, and the upper Cumberland River system. *Etheostoma cumberlandicum* Jordan and Swain was described in 1883 from a population sampled in the Clear Fork system that drains to the upper Cumberland. A previous morphological analysis led to the placement of *E. cumberlandicum* into the synonymy of *E. kennicotti*. Results from molecular phylogenetic and relaxed molecular clock analyses, genetic variation at 25 microsatellite loci, morphological disparity in meristic traits, and variation in pigmentation from specimens sampled throughout the geographic distribution of *E. kennicotti* (s.l.) indicate *E. cumberlandicum* is a distinct species and there are multiple undescribed species masquerading as *E. kennicotti*. We elevate *Etheostoma cumberlandicum* out of synonymy and propose Moonbow Darter as the common name for the species. The results of the phylogenetic analyses are discussed in the context of the historical biogeography of rivers draining the Eastern Highlands of North America.

EASTERN North America is home to the most species-rich non-tropical freshwater fish fauna on Earth (Lundberg et al., 2000). Starting with the work of Rafinesque (1820), the discovery and delimitation of North American freshwater fish species relied entirely on external morphology such as numbers of fin elements and scale rows, proportional measurements, and patterns of pigmentation and coloration. Molecular phylogenies have resolved the relationships of the lineages that comprise the rich North American freshwater fish fauna (Near et al., 2004, 2011; Wright et al., 2012; Ghedotti and Davis, 2017; Bagley et al., 2018; Schönhuth et al., 2018; Near and Kim, 2021), but have also led to the discovery of new species and elevation of species long considered synonyms (Wood and Raley, 2000; Wood et al., 2002; Near, 2008; Near et al., 2017). North American freshwater species with large geographic ranges are prime candidates for undiscovered species diversity and the elevation of synonymized species (Piller et al., 2008; Piller and Bart, 2017). Molecular phylogenetic analyses provide a framework of lineage and species diversification, which aids in partitioning morphological trait variation potentially revealing important differences among lineages that are not apparent without a phylogenetic perspective (Berendzen et al., 2009).

The Stripetail Darter, *Etheostoma kennicotti* (Putnam), is a widely distributed species forming a circle around the Nashville Basin (Fig. 1). The species occurs throughout the Tennessee River, in the Cumberland River system above Cumberland Falls and below the falls in the Laurel River and portions of the Big South Fork, the Green River system, and tributaries of the lower Ohio River in southern Illinois and northwestern Kentucky (Page and Smith, 1976; Smith, 1979; Burr and Warren, 1986; Etnier and Starnes, 1993; Tiemann and Sherwood, 2011). *Etheostoma kennicotti* was described from specimens collected by Robert Kennicott from a tributary of the Ohio River in Union County, Illinois (Putnam, 1863; Collette and Knapp, 1966). Later, Jordan and Swain (1883) described *Etheostoma cumberlandicum* from Wolf Creek in the Clear Fork system, a tributary of the Cumberland River above Cumberland Falls. Curiously, Jordan and Swain (1883) considered *E. cumberlandicum* closely related to *E. flabellare* with no reference to Putnam's (1863) *E. kennicotti*, which was known at the time only from its type locality in southern Illinois. Later Jordan and Evermann (1898: 1098) placed *E. kennicotti* into the synonymy of *E. flabellare*, but treated *E. cumberlandicum* as a subspecies of *E. flabellare*. Forbes and Richardson (1909: 311–313) identified populations of *E. kennicotti* in Illinois as *Etheostoma obeyense* Kirsch; however, the species was resurrected from synonymy with *E. flabellare* in a list of the fishes of Illinois and a contribution on the type specimens of the species (Smith, 1965; Smith and Page, 1975). Page and Smith (1976) interpreted morphological variation in *E. kennicotti* and *E. cumberlandicum* as forming an east–west cline, reflecting hypothesized dispersal westward from an ancestral area centered in the Cumberland River drainage. As a result of the analysis of meristic trait variation, Page and Smith (1976) placed *Etheostoma cumberlandicum* into the synonymy of *E. kennicotti*.

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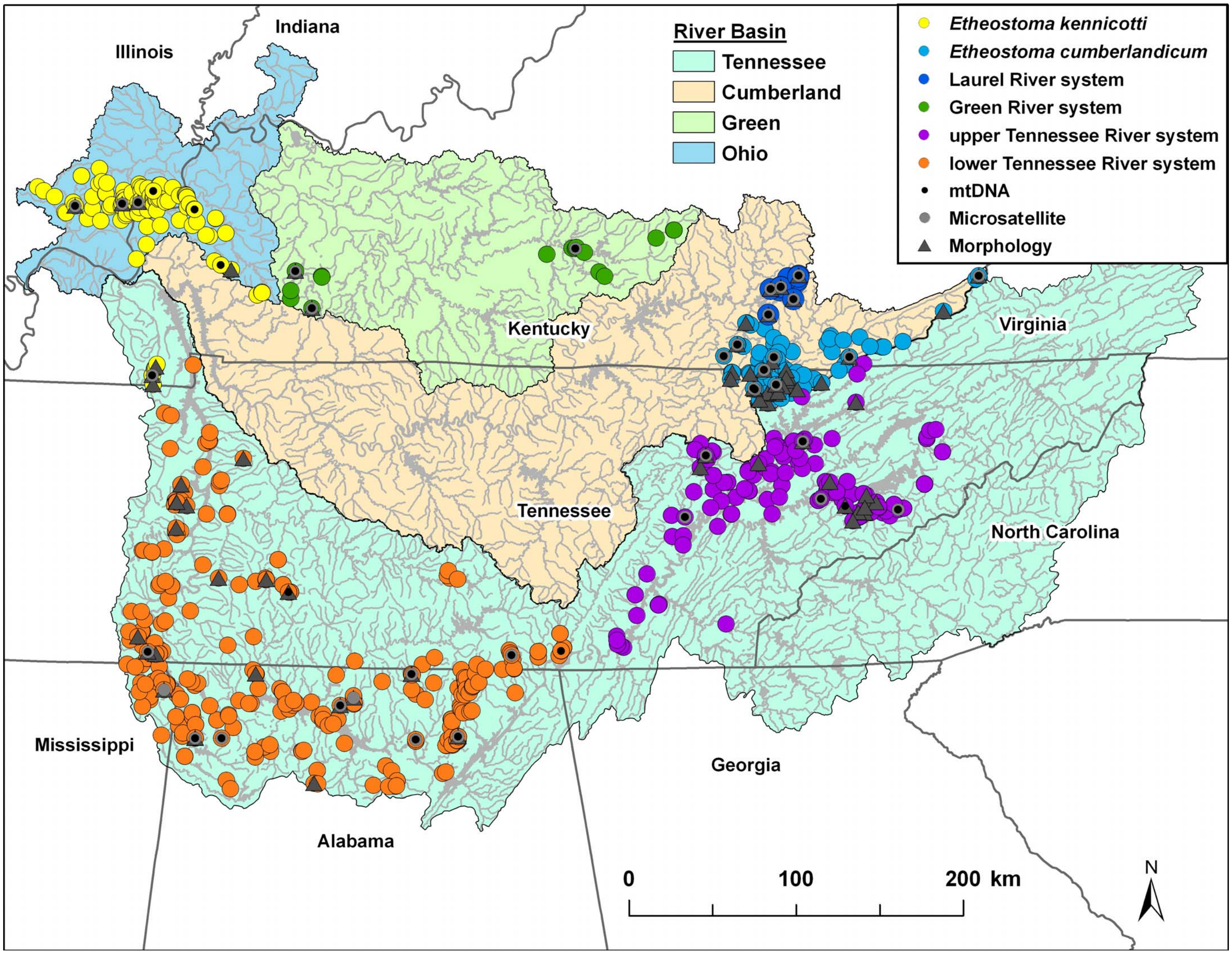

Fig. 1. Geographic distribution of *Etheostoma kennicotti sensu lato* and *Etheostoma cumberlandicum* and sampling locations of specimens used in molecular and morphological analyses. The populations in the Green River system, the Laurel River system, and the Tennessee River system (partitioned between lower and upper Tennessee River) are each considered undescribed species currently listed as *E. kennicotti*.

In this study, we reexamine the systematics of *Etheostoma kennicotti sensu lato* (s.l.) using phylogenetic analysis of the mitochondrial cytochrome *b* gene, genetic variation at 25 loci, and disparity in meristic traits. Our analyses include specimens sampled throughout the geographic distribution of *E. kennicotti* (s.l.) and indicate *Etheostoma cumberlandicum* is a distinct species. Additionally, we identify three newly discovered and undescribed species masquerading as *E. kennicotti*. The relationships of species within the *Etheostoma kennicotti* complex are discussed in the context of the pre-Pleistocene configuration of rivers in the Eastern Highlands and patterns of speciation that involve species endemic to the upper Cumberland River.

MATERIALS AND METHODS

Specimen collection.—Specimens of *Etheostoma kennicotti* (s.l.) were collected from shallow riverine habitats using a minnow seine from a time period extending from 1999 to 2019. Specimens from the main channel of the Green River in Green County, Kentucky and in the mainstem of the Tennessee River were sampled using a boat-assisted benthic trawl (i.e., mini-Missouri trawl; Herzog et al., 2005). Specimens were anesthetized in MS-222 and a portion of the right pectoral fin was dissected at the base and preserved in 95% non-denatured ethanol and cataloged into the Yale Fish Tissue Collection (YFTC). Whole-body specimens were fixed in ~10% buffered formalin for seven to 14 days, rinsed and soaked in water for one to three days, soaked in 50% ethanol for five to seven days before long term preservation in 70% ethanol. Specimens sampled from the main channel of the Green River were not fixed in formalin. An additional 15 field-collected specimens of *E. kennicotti* (s.l.) that measured less than 20 mm standard length were preserved whole in 95% non-denatured ethanol, were cataloged into the YFTC, and were available only for molecular analyses. All specimens of *E. kennicotti* and *E. cumberlandicum* collected during this study were deposited in the research collections of fishes at Austin Peay State University (APSU), the Illinois Natural History Survey (INHS), the Peabody Museum, Yale University (YPM), Tulane University (TU), and the David A. Etnier Collection of Fishes, University of Tennessee (UT).

Molecular analyses.—Sampling locations of specimens used for genetic analyses are listed in Table 1 and shown in Figure 1. DNA was extracted from 95% ethanol-preserved tissues using a standard DNeasy Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA, USA). To minimize downstream enzymatic inhibition, we purified DNA extractions with an ethanol precipitation: 3 M sodium acetate (pH = 5.2) was added equal to 10% of the total volume of the DNA extraction followed with 100% ethanol equal to 2.5 times the total volume of DNA. After mixing, extractions were incubated for ten minutes at –80°C. Samples were centrifuged for 30 minutes at 8,000 RCF, the supernatant was carefully poured off, and the DNA pellet was washed with 250 μL of cold 70% ethanol. Samples were centrifuged again for five minutes at 8,000 RCF, supernatant was poured off, the pellet was allowed to air dry for ~15 minutes, and the DNA pellet was resuspended with the desired amount of DNAse-free water.

The phylogenetic relationships of populations of *Etheostoma kennicotti* (s.l.) and *E. cumberlandicum* were assessed with phylogenetic analyses of the mitochondrial encoded cytochrome *b* (*cytb*) gene. The molecular phylogenetic analysis included 167 specimens of *E. kennicotti* (s.l.) and *E. cumberlandicum*. Two specimens of *E. flabellare* were sampled as outgroup taxa (Table 1). The *cytb* gene was amplified using previously published PCR primers and cycling conditions (Near et al., 2000). Amplification products were prepared for DNA sequencing using a polyethylene glycol precipitation. Contiguous sequences were assembled from individual DNA sequencing reactions using the computer program Geneious v.7.2 (Kearse et al., 2012). New *cytb* sequences were aligned by eye to those previously generated in early studies of darter phylogeny (Porterfield et al., 1999; Near et al., 2011). The optimal data partitioning scheme, among the three codon positions of the *cytb* gene, and molecular evolutionary models were determined using the Bayesian information criterion in the computer program PartitionFinder v. 2.1 (Lanfear et al., 2017). The mitochondrial gene tree was inferred from the aligned *cytb* sequences using the optimal molecular evolutionary models and partitioning scheme using the computer program MrBayes v. 3.2 (Ronquist et al., 2012), where posterior probabilities for the phylogeny and parameter values were estimated using Metropolis-couple Markov chain Monte Carlo (MC3; Larget and Simon, 1999; Huelsenbeck et al., 2001). The MrBayes analysis was run for 10^7 generations with two simultaneous runs each with four chains. Convergence of the MC3 algorithm and stationarity of the chains was assessed by monitoring the average standard deviation of the split frequencies between the two runs, which was less than 0.005 after $3x10^6$ generations. In addition, the likelihood score and all model parameter estimates were plotted against the generation number to determine when there was no increase relative to the generation number in the computer program Tracer v. 1.5 (Drummond and Rambaut, 2007). The first 50% of the sampled generations were discarded as burn-in, and the posterior phylogeny was summarized as a 50% majority-rule consensus tree. All *cytb* gene sequences generated for this study are available at GenBank MZ078304–MZ078463. The DNA sequence alignments, nexus files used for MrBayes 3.2 analysis, the MrBayes 3.2 posterior tree file, and the summarized consensus tree are available at the Dryad Digital Repository (https://doi.org/10.5061/dryad.47d7wm3jg).

A relaxed molecular clock analysis of *cytb* DNA sequences was performed to estimate the divergence times between the lineages resolved within *Etheostoma kennicotti*. Because the speciation branching model employed in the analysis assumes that only a single specimen is sampled for each species, the *cytb* sequence of a single specimen was subsampled from six lineages resolved in the MrBayes inferred *cytb* gene tree: Green River, upper Tennessee River, lower Tennessee River, Ohio River–Clarks River, the upper Cumberland–Big South Fork, and Laurel River. A single *E. flabellare* sampled from the Middle Fork Vermilion River was included as an outgroup taxon to root the time calibrated phylogeny (Table 1). The uncorrelated lognormal (UCLN) model of molecular evolutionary rate heterogeneity was implemented using the computer program BEAST v. 1.8.3 (Drummond et al., 2006, 2012). The optimal partitioning scheme and molecular evolutionary models were those used in the MrBayes analysis. A Yule pure birth diversification prior was used for the branching rates in the phylogeny. The relaxed molecular clock was calibrated with a prior on the rate of evolution of the *cytb* gene of $8.99x10^{-3}$ (95% credible interval: $7.46x10^{-3}$, $1.06x10^{-2}$) substitutions per million years estimated in a previous divergence time analysis of darters (Near et al., 2011: table 2). In the xml file, the molecular evolutionary rate was set with a normal distributed prior with a mean of 0.0089 and a standard deviation of $5.0x10^{-4}$. The BEAST analysis was run three times, with each run consisting of 10^7 generations. Convergence of model parameter values and estimated node ages to their optimal posterior distributions was assessed by plotting the marginal posterior probabilities using the computer program Tracer v. 1.6 (Drummond and Rambaut, 2007). The resulting trees and log files from each run were combined using the computer program LogCombiner v. 1.83. The posterior probability density of the combined tree and log files was summarized using TreeAnnotator v. 1.8.3. The mean and 95% HPD estimates of divergence times were visualized on the chronogram using the computer program FigTree v. 1.4. The input xml file, a sampling of the posterior trees, and the summarized consensus time tree are available at the Dryad Digital Repository (https://doi.org/10.5061/dryad.47d7wm3jg).

We genotyped 164 specimens of *Etheostoma kennicotti* for 25 microsatellite loci using primers developed for this species by Hereditec (Lansing, New York; Table 2). Genotyping was performed by a single-reaction nested PCR method (Schuelke, 2000). We used a forward primer with a universal M13 (5′–TCCCAGTCACGACGT–3′) tail at its 5′ end, a complementary M13 forward primer labeled with one of three fluorescent dyes (6FAM, VIC, or NED), and an unlabeled reverse primer. Microsatellite PCR reactions were performed in a total volume of 12.5 μl, containing 1 μL of isolated DNA, 1X colorless GoTaq Flexi Buffer (Promega Corporation), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.04 mM of both the reverse primer and the M13 tagged forward primer, 0.64 mM of the fluorescent-labeled M13 primer, and 0.06 mM of GoTaq DNA polymerase (Promega Corporation). We used an Eppendorf Mastercycler thermo cycler under the following PCR protocol: initial denaturation at 94°C (5 min), followed by 35 cycles of denaturing (94°C, 40 s), annealing, and extension (72°C, 40 s), with the annealing temperature maintained at 57°C (30 s) for the first ten cycles and subsequently lowered to 53°C (30s) for the final 25 cycles.

Table 1. Specimens of *Etheostoma kennicotti* and *E. cumberlandicum* used in genetic analyses. Institutional abbreviations follow Sabaj (2020).

Drainage	Catalog	Sampling location	mtDNA	Microsatellites
Ohio	YPM ICH 020762	Lusk Creek, Pope Co., Illinois 37.486839, −88.536028	10	10
Ohio	YPM ICH 020952	Big Grand Pierre Creek, Pope Co., Illinois 37.482717, −88.440317	9	9
Ohio	No voucher	Big Creek, Hardin Co., Illinois 37.544863, −88.339820	1	0
Ohio	No voucher	Phelps Creek, Caldwell Co., Kentucky 37.153468, −87.873440	1	0
Ohio	No voucher	Camp Creek, Crittenden Co., Kentucky 37.451326, −88.057999	1	0
Ohio	YPM ICH 020653	Little Cache River, Johnson Co., Illinois 37.454280, −88.865829	2	2
Clarks	YPM ICH 022246	East Fork Clarks River, Calloway Co., Kentucky 36.551949, −88.316655	6	6
Green	YPM ICH 022456	Buck Fork Pond River, Todd Co., Kentucky 36.928787, −87.257045	7	5
Green	YPM ICH 022435	West Fork Pond River, Christian Co., Kentucky 37.128005, −87.371364	3	3
Green	APSU 1002	Green River from Russell Island to Rock Hole, Green Co., Kentucky 37.27466 −85.47561 to 37.25622 −85.4848	5	5
Upper Cumberland	No voucher	Poor Fork, Letcher Co., Kentucky	1	0
Upper Cumberland	YPM ICH 027401	Poor Fork, Letcher Co., Kentucky 37.067731, −82.772031	1	1
Upper Cumberland	YPM ICH 027402	Clear Fork, Bell Co., Kentucky 36.649900, −83.656429	1	1
Upper Cumberland	UT 91.6514	Lick Fork, Campbell Co., Tennessee 36.494754, −84.320666	5	5
Upper Cumberland	YPM ICH 015854	Lick Fork, Campbell Co., Tennessee 36.491617, −84.299583	3	3
Upper Cumberland	UT 91.6517	Stinking Creek, Campbell Co., Tennessee 36.508381, −84.148977	3	3
Upper Cumberland	YPM ICH 028205	Wolf Creek, Whitley Co., Kentucky 36.65706, −84.16087	5	5
Upper Cumberland	TU 198953	Bridge Fork, McCreary Co., Kentucky 36.728301, −84.403567	1	3
Upper Cumberland	YPM ICH 015483	Capuchin Creek, Campbell Co., Tennessee 36.590767, −84.2289	1	1
Upper Cumberland	YPM ICH 015840	Laurel Creek, McCreary Co., Kentucky 36.728347 −84.403390	2	2
Big South Fork	INHS 50344	Roaring Paunch Creek, McCreary Co., Kentucky 36.669040, −84.494243	2	2
Laurel River	YPM ICH 023748	Horse Branch, Laurel Co., Kentucky 37.035802, −84.106151	1	1
Laurel River	YPM ICH 027529	Craig Creek, Laurel Co., Kentucky 37.025066, −84.173502	7	8
Laurel River	YPM ICH 027536	Little Laurel River, Laurel Co., Kentucky 37.035656, −84.106015	4	4
Laurel River	YPM ICH 027429	Lick Fork, Laurel Co., Kentucky 37.096011, −83.983483	10	10
Laurel River	YPM ICH 027545	Hazel Fork, Knox Co., Kentucky 36.970136, −84.022742	10	10
Laurel River	YPM ICH 027540	Spruce Creek, Whitely Co., Kentucky 36.88786, −84.19183	2	2
Lower Tennessee	No voucher	Buffalo River, Lawrence Co., Tennessee 35.397639, −87.387482	1	0
Lower Tennessee	UT 91.6464	Horse Creek, Hardin Co., Tennessee 35.180407, −88.209653	3	6
Lower Tennessee	UT 91.7478	Aldridge Creek, Madison Co., Alabama 34.594772, −86.545806	4	4
Lower Tennessee	UT 91.7479	Cane Creek, Colbert Co., Alabama 34.701426, −87.841723	1	1
Lower Tennessee	YPM ICH 020990	Pickens Branch, Tishomingo Co., Mississippi 34.857276, −88.190683	3	3
Lower Tennessee	YPM ICH 016297 & YPM ICH 020089	Rock Creek, Colbert Co., Alabama 34.601034, −87.981421	11	11
Lower Tennessee	YPM ICH 021221	Swan Creek, Limestone Co., Alabama 34.831744, −86.951406	2	2
Lower Tennessee	YPM ICH 027261	Horny Head Creek, Decatur Co., Tennessee 35.731942, −88.13459	2	2
Lower Tennessee	YPM ICH 021230	Round Island Creek, Limestone Co., Alabama 34.788745, −87.036873	2	2
Lower Tennessee	YPM ICH 020808	West Fork Flint River, Madison Co., Alabama 34.960742, −86.571732	4	4
Lower Tennessee	No voucher	Robinson Creek, Hardin Co., Tennessee 35.041086, −88.259258	1	0
Lower Tennessee	No voucher	Little Mud Creek, Franklin Co., Alabama 34.533928, −87.590343	1	0
Lower Tennessee	YPM ICH 027306	Leath Creek Hardin Co., Tennessee 35.059923, −88.303923	4	
Lower Tennessee	YPM ICH 020976	Yellow Branch, Jackson Co., Alabama 34.626499, −86.265960	3	3
Lower Tennessee	No voucher	Guess Creek, Jackson Co., Alabama	1	0
Lower Tennessee	UT 91.8604	Crow Creek, Franklin Co., Tennessee 35.06460, −85.91749	1	2
Lower Tennessee	YPM ICH 033147	Sequatchie River, Marion Co., Tennessee 35.087073, −85.593004	1	0
Lower Tennessee	No voucher	Sequatchie River, Marion Co., Tennessee 35.060590, −85.607480	1	0
Upper Tennessee	YPM ICH 016989	Bryams Fork, Union Co., Tennessee 36.200434, −83.979697	2	2
Upper Tennessee	UT 91.6503	Whites Creek, Roane Co., Tennessee 35.80408, −84.76809	1	1
Upper Tennessee	No voucher	Happy Creek, Sevier Co., Tennessee 35.848125, −83.707405	1	0
Upper Tennessee	No voucher	Sale Creek, Rhea Co., Tennessee 35.441622, −85.079672	1	0
Upper Tennessee	UT 91.8600	Rock Creek, Morgan Co., Tennessee 36.134089, −84.625059	0	4
Upper Tennessee	No voucher	Rock Creek, Morgan Co., Tennessee 36.134089, −84.625059	4	4
Upper Tennessee	TU 198951	Dunn Creek, Sevier Co., Tennessee 35.829535, −83.326238	1	2
Upper Tennessee	No voucher	Stock Creek, Knox Co., Tennessee 35.8890217, −83.86528	6	6
Etheostoma flabellare	No voucher	Middle Fork Vermillion River, Vermillion Co., Illinois 40.235322, −87.771730	1	0
Etheostoma flabellare	INHS 88926	Little Marrowbone Creek, Davidson Co., Tennessee 36.273807, −86.895593	1	0

Table 2. Microsatellite primer sequences used in genotyping specimens of *Etheostoma kennicotti* and *E. cumberlandicum*.

Locus	Motif	Locus size in base pairs (bp)	5′ primer	3′ primer
Eken3	AC	168–218	TCCCAGTCACGACTGTGTGAGGCTGC	ACACAATCATCACTGCAGGTC
Eken8	AC	137–161	TCCCAGTCACGACACACACGTCTCAG	GGTGTTCTACTCTCCGTCCC
Eken10	AC	147–167	TCCCAGTCACGACCCATAACCCACTC	GCCGTAGCCTCTGTAATTGG
Eken12	AC	162–170	TCCCAGTCACGACCTATGTGTTCCGC	ACAAATGTTTCGGGCGCTG
Eken14	AC	152–178	TCCCAGTCACGACGCAGTTGTGTCAG	TGAGCAGGGTCTTATCCAGC
Eken15	AC	150–204	TCCCAGTCACGACCGTGACAAACAAG	GCAAATGGTGAACGGGTGTG
Eken18	AC	152–202	TCCCAGTCACGACCCCTTTCACCAAC	ATCATAGTGCAACAGGAGAGTC
Eken19	AG	136–186	TCCCAGTCACGACTGTGATATGATGC	AGACAGACCTGAAGCAGCAC
Eken21	AC	157–165	TCCCAGTCACGACAATAGAGTCCCTG	TGTTCATAGTGTCTGTGGCC
Eken22	AC	147–211	TCCCAGTCACGACTCCCATTGTGCAG	ACTATTGTTGAGAGCCGGTG
Eken24	AC	161–233	TCCCAGTCACGACTTGGTCTGAAGGG	TTTCTACGGCACTCCTGGG
Eken27	AC	157–219	TCCCAGTCACGACCTGTTTGAGAGTG	TTACCTTGCCTTTACACAACCC
Eken28	AC	152–190	TCCCAGTCACGACTCTAACGAACAGC	ACAGTAGCCCACACAGAAGG
Eken29	AG	155–223	TCCCAGTCACGACTGCGTTTGTCTCG	GAGCCGCCATCTCCAACAG
Eken32	AG	147–175	TCCCAGTCACGACTGTATGCTGGCAC	GGGATGATTAGTTTGTTGGTGG
Eken36	AC	153–177	TCCCAGTCACGACTGCTCCATTGCTC	TTCAGTGATCCCTGAAAGCG
Eken38	AG	150–168	TCCCAGTCACGACTCTTCATGGACGC	TGTGTGCATCTCTGTCCAGG
Eken39	AC	141–171	TCCCAGTCACGACTCACATGACGAGG	CACACCCGCATTGCAAACC
Eken41	AG	167–219	TCCCAGTCACGACATCAGAGTGACCC	CTTTCTCACAAACTCAGTTGCC
Eken42	AC	161–167	TCCCAGTCACGACCTGTAAGACAAAC	CGATTCACCTTTACACAACCTC
Eken49	AC	162–172	TCCCAGTCACGACCCATAAGACCGTG	TTCGCCTGCATTGTTCTTGG
Eken52	AG	140–228	TCCCAGTCACGACCCTGCCAATAACC	TATTCGACTACAGCCAGCGG
Eken58	AC	163–213	TCCCAGTCACGACCTTCCTTTCCACG	CAACCTGCACCACCCTTCTG
Eken59	AC	158–164	TCCCAGTCACGACCTTCCGCTCTCGC	CTGGGATCAACACTCTCTGC
Eken60	AC	155–163	TCCCAGTCACGACTCTGGTGGCTGGC	CCTCCCTCTCACTGCCAAC

All microsatellite loci were genotyped on 3730xl DNA Analyzer (Applied Biosystems, Inc.) against a LIZ-500 dye size standard (Applied Biosystems, Inc.). We used GENEMAPPER v3.7 (Applied Biosystems, Inc.) to retrieve raw allele sizes and scored them by using the automatic binning function in TANDEM (Matschiner and Salzburger, 2009).

We tested for departures from Hardy-Weinberg equilibrium (HWE) using the "hw.test" function in the R package Pegas v.0.14 (http://ape-package.ird.fr/pegas.html). The proportion of polymorphic loci (P) per population was calculated with "locus_table" function in the R package poppr v. 2.8.6 (https://github.com/grunwaldlab/poppr/). The average number of alleles per locus in each population (A) and private allelic richness (pAR) were calculated using HP-RARE v. June-6-2006 (Kalinowski, 2005), using 12 loci for rarefaction. The number of private alleles per population was calculated with the "private_alleles" function in poppr v. 2.8.6. The mean population expected (H_e) and observed heterozygosity (H_o) was calculated with the "divBasic" function in the R package diveRsity v. 1.9.0 (https://rdrr.io/cran/diveRsity/). Pairwise population Weir and Cockerham (1984) FST indices were calculated using the "genet.dist" function in the R package hierfstat v. 0.5-7 (https://github.com/jgx65/hierfstat).

We assessed population structure of *Etheostoma kennicotti* using the Bayesian clustering algorithm STRUCTURE v2.3.2 (Pritchard et al., 2000; Falush et al., 2003). We used a hierarchical ΔK method to infer the number of genetic clusters by repeating STRUCTURE analyses on each of the K groups inferred in the previous step (Coulon et al., 2008). Hierarchical clustering continued until the log-likelihood for one cluster ($K = 1$) was larger than the log-likelihoods for all other values of K, or the majority of individuals were not strongly assigned to any cluster (assignment probability < 0.6). In each round of analysis, STRUCTURE was run from $K = 1$ to $K = 10$, with ten independent iterations performed for each value of K. The burn-in period was 100,000 replicates followed by 1,000,000 Markov chain Monte Carlo simulations run under a model that assumed admixture and correlated allele frequencies, without prior information on population identity. The optimal K was calculated using the ΔK method implemented in Structure Harvester v0.6.94 (Earl and vonHoldt, 2012). The scored microsatellite alleles used for the analyses are available at the Dryad Digital Repository (https://doi.org/10.5061/dryad.47d7wm3jg).

Morphological analyses.—Meristic data were collected from 703 specimens of *Etheostoma kennicotti* sampled in our fieldwork as well as specimens housed at UT. Catalog numbers of specimen lots used for morphological analyses are given in Material Examined. Detailed sampling locations are shown on Figure 1 and georeferenced data for all sampling locations are available at the Dryad Digital Repository (https://doi.org/10.5061/dryad.47d7wm3jg). The numbers of scale rows and fin elements were determined from each specimen as outlined in Hubbs and Lagler (1958) and Page (1981), with the exception of the number of transverse scale rows, which was counted as described by Page (1983: 16, fig. 2). The characterization and counting of caudal bands follows Page and Smith (1976). The meristic data for all 703 specimens are available at the Dryad Digital Repository (https://doi.org/10.5061/dryad.47d7wm3jg).

A principal component analysis (PCA) of the meristic traits was executed using the "prcomp" function R version 3.2.0 (R Core Team, 2015). The ability of the meristic data to assign individual specimens to one of six groups based on relationships inferred in phylogenetic analysis of mtDNA gene sequences or genomic clusters estimated using microsatellite loci was assessed using a cross-validation linear discriminate analysis (LDA) as applied in the MASS package for R (Venables and Ripley, 2002). Bayesian posterior assignment probabilities for each group were calculated, with group assignment determined by the highest posterior probability. The meristic traits analyzed with PCA and LDA included: number of lateral line scales, number of transverse scale rows, number of scales around the caudal peduncle, number of first dorsal-fin spines, number of second dorsal-fin rays, number of anal-fin rays, and number of pectoral-fin rays. We calculated pairwise group mean Mahalanobis distances in full principal component space using the *pairwise.mahalanobis* function from the HDMD package (McFerrin, 2013).

RESULTS

Molecular analyses.—The PartitionFinder v. 2.1 analysis of the *cytb* alignment containing 167 specimens of *Etheostoma kennicotti* and two *E. flabellare* outgroups identifies an optimal scheme where each codon position is treated as a separate data partition. The optimal molecular evolutionary models are K80+I+G for the first codon position, HKY+I for the second codon position, and GTR+G for the third codon positions. The Bayesian inferred *cytb* gene tree, which is a 50% majority-rule consensus tree calculated from the set of 5,000 posterior trees, is shown in Figure 2A. Seven inclusive monophyletic groups within *Etheostoma kennicotti* (s.l.) are resolved in the phylogeny and are supported with high (>0.94) Bayesian posterior probabilities. A clade comprising specimens sampled from the Green River system is the sister lineage of all other populations of *E. kennicotti* (s.l.) and *E. cumberlandicum* (Fig. 2). Within the Green River clade there is a split between the geographically disjunct Pond River and upper Green River (Figs. 1, 2A).

There are three lineages from the Tennessee River system resolved in the *cytb* gene tree: a clade comprising specimens sampled from the upper Tennessee River system above Sale Creek in Rhea County, Tennessee and two lineages that comprise populations from the lower Tennessee River system (Fig. 2A). One of the lower Tennessee River clades comprises populations sampled from the Paint Rock, Flint River, and Sequatchie River systems and the Robinson Creek and Chambers Creek systems (Fig. 2A). Individual specimens collected from the same locations in Horse Creek, Hardin Co., Tennessee and Aldridge Creek, Madison Co., Alabama contain mtDNA haplotypes that resolve in both of the lower Tennessee River clades (Fig. 2A).

Populations of *Etheostoma kennicotti* (s.s.) from the Clarks River in Tennessee and Kentucky and the lower Ohio River system in Illinois and Kentucky resolve as reciprocally monophyletic sister groups in the *cytb* gene tree (Fig. 2A). The Ohio–Clarks lineage, which includes the type locality of *Etheostoma kennicotti*, is the sister lineage of a clade that encompasses all sampled populations from the Cumberland River system (Fig. 2A). The Cumberland lineage contains two clades: populations sampled from the Laurel River system and populations sampled from the Big South Fork and tributaries of the Cumberland River above Cumberland Falls that correspond to *Etheostoma cumberlandicum* (Jordan and Swain, 1883; Page and Smith, 1976; Etnier and Starnes, 1993: 500).

The relaxed molecular clock analysis in BEAST using the Yule pure birth branching model resulted in a posterior phylogeny of *Etheostoma kennicotti* (s.l.) and *E. cumberlandicum* that is congruent with phylogeny inferred using MrBayes (Fig. 2B). The time tree of *E. kennicotti* (s.l.) depicts a history of diversification that initiates in the Late Miocene and extends through the Pleistocene (Fig. 2B). The estimated age of the most recent common ancestor (MRCA) of all the lineages currently delimited as *Etheostoma kennicotti* and *E. cumberlandicum* is 6.05 million years ago (mya; 95% HPD: 4.53, 7.86 mya), which is the split between *E.* cf. *kennicotti* from the Green River and all other lineages of *E. kennicotti* and *E. cumberlandicum* (Fig. 2B). The age of the MRCA of the upper Tennessee and lower Tennessee River clades is 4.38 mya (95% HPD: 3.01, 5.83 mya). The age of the MRCA of *E. kennicotti* (s.s.) in the lower Ohio and Clarks River system and the two lineages in the Cumberland is 2.73 mya (95% HPD: 1.84, 3.76 mya). The timing of the split between *E.* cf. *kennicotti* in the Laurel River system and *E. cumberlandicum* is 1.27 mya (95% HPD: 0.69, 1.89 mya).

Twenty-five microsatellite loci were genotyped for 164 individuals of *Etheostoma kennicotti* (s.l.). The resulting dataset contains only 5% missing genotypes. For the six inclusive clades in the *cytb* gene tree (Fig. 2A), the mean number of private alleles per locus ranges from 3.2 to 5.6 (Table 3). The lower Tennessee River clade contains nearly double the number of private alleles and private allelic richness as any other population (Table 3), and 32% of the microsatellite loci (48/150) are not in HWE in at least one population (Supplemental Table 1; see Data Accessibility). None of the loci are consistently out of HWE in all populations, and one population (the lower Tennessee River) accounts for a disproportionate number (21/48) of departures from HWE. This lower Tennessee population has much lower observed (0.39) than expected (0.75) heterozygosity (Table 3).

The average pairwise F_{ST} value across the six inclusive clades of *Etheostoma kennicotti* (s.l.) and *E. cumberlandicum* in the *cytb* gene tree is 0.323 (Table 4). The lower versus upper Tennessee populations exhibit much lower differentiation ($F_{ST} = 0.145$) than all other population pairs. In contrast, *E.* cf. *kennicotti* from the Laurel River and *E. kennicotti* (s.s.) are the most differentiated ($F_{ST} = 0.438$), though several other lineage contrasts exhibit similarly high F_{ST} values (Table 4).

Hierarchical STRUCTURE analyses identify 18 clusters (K) as optimal to describe the genetic variation within *Etheostoma kennicotti* (s.l.) and *E. cumberlandicum* (Fig. 3A). Early stages of the hierarchical analyses identify genetic clusters consistent with the *cytb* phylogeny. For example, specimens of *E. kennicotti* (s.s.) from the lower Ohio and Clarks Rivers are identified as a distinct genetic cluster early in the hierarchical analyses (Fig. 3A). In the hierarchical analyses, ten of the 18 genetic clusters are localized in the Tennessee River basin, with small tributaries containing genetically distinct populations (Fig. 3A). This pattern is even stronger when analyzing K = 18 using all samples; 12 genetic clusters are restricted to the Tennessee River. Many genetic clusters in the Tennessee River represent individual sampling localities. The hierarchical analyses identify fine scale population structure within *E. kennicotti* (s.s.) in tributaries of the lower Ohio River with three genetic clusters corresponding to each sampled locality (Fig. 3A). In contrast, all specimens of *E.* cf. *kennicotti* from the Green River system from disjunct localities in the Pond River system and the upper Green River form a single

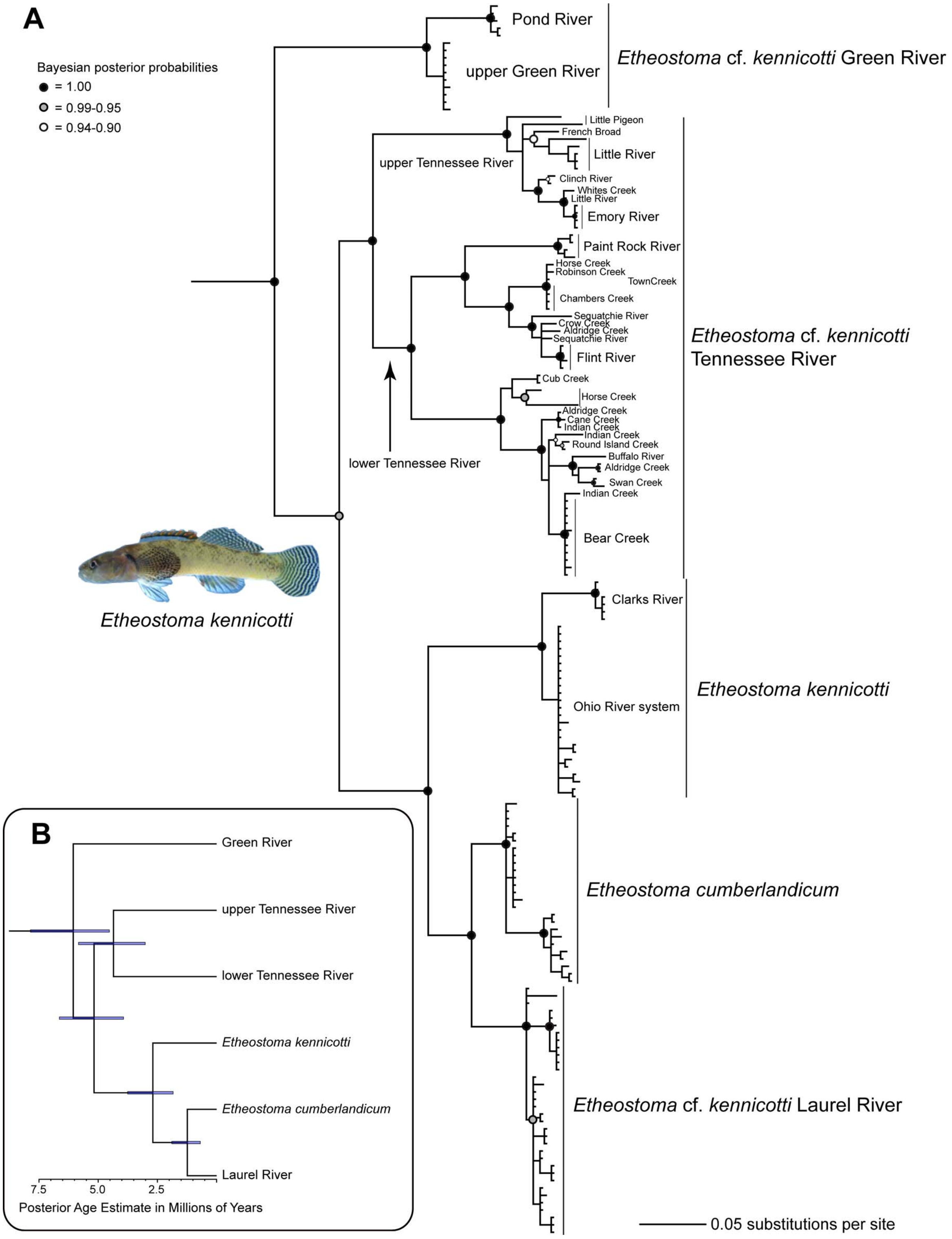

Fig. 2. Phylogenies of *Etheostoma kennicotti sensu lato* and *Etheostoma cumberlandicum* inferred from the mitochondrial *cytb* gene. (A) Bayesian inferred *cytb* gene tree. Bayesian posterior probabilities are indicated with filled circles. (B) Maximum clade credibility chronogram of *Etheostoma kennicotti sensu lato* and *Etheostoma cumberlandicum*. Bars indicate 95% posterior density of age estimates at nodes in the phylogeny.

genetic cluster (Figs. 1, 3A). Likewise, specimens of *E. cumberlandicum* form a single genetic cluster (Fig. 3A). Specimens of *E.* cf. *kennicotti* from the Laurel River are separated into two genetic clusters, with one cluster containing specimens sampled from Craig Creek and the other cluster containing all other sampled populations in the Laurel River system (Fig. 3A). The ancestry assignments for the individual specimens in the hierarchical STRUCTURE analysis are presented in Supplementary Figure 1 (see Data Accessibility).

To determine whether genetic clustering is concordant with the lineages resolved in the *cytb* gene tree (Fig. 2A), we

Table 3. Microsatellite population summary statistics for *Etheostoma kennicotti* and *E. cumberlandicum*: sample size (*n*), proportion of polymorphic loci (P), mean number of alleles per locus (A), number of private alleles (nPA), private allelic richness (pAR), expected (H_e) and observed (H_o) heterozygosity.

Population	*n*	P	A	nPA	pAR	H_e	H_o
Etheostoma kennicotti							
Green River	13	0.96	3.48	27	1.27	0.51	0.42
Upper Tennessee River	19	1	3.87	12	0.93	0.6	0.32
Lower Tennessee River	44	1	5.62	97	2.22	0.75	0.39
Lower Ohio River–Clarks River	27	0.92	3.66	31	1.3	0.52	0.32
Laurel River	35	1	3.22	17	0.61	0.49	0.39
Etheostoma cumberlandicum	26	1	3.91	18	0.77	0.57	0.54

also visualized STRUCTURE results for K = 6. Four of the six genetic clusters match clades in the *cytb* phylogeny: *Etheostoma kennicotti* (s.s.) from the Clarks and Ohio Rivers, *E.* cf. *kennicotti* from the Green River, *E.* cf. *kennicotti* from the Laurel River, and *E. cumberlandicum* (Fig. 3B). There is little admixture among these genetic clusters except for one individual of *E.* cf. *kennicotti* from the Laurel River that exhibits appreciable genomic ancestry shared with *E. cumberlandicum* (Fig. 3B). Two genetic clusters are identified in the Tennessee River system. However, these clusters do not match the *cytb* gene tree (Figs. 2A, 3B).

Morphological analyses.—Scale row and fin elements counts are presented for 263 specimens of *Etheostoma cumberlandicum* and 440 specimens of *E. kennicotti* (s.l.) in Tables 5–10. The data for the number of scales above and below the lateral line, the number of scales around the caudal peduncle, and the number of anal-fin rays showed little variation and are not summarized in tables, but the meristic data for all specimens are available at the Dryad Digital Repository (https://doi.org/10.5061/dryad.47d7wm3jg). Plotting the first two principal components (PC) axes from the PC analysis shows broad overlap in the morphospace among the lineages resolved in the *cytb* gene tree and characterized as genetically distinct in the analysis of 25 microsatellite loci (Fig. 4A). The LDA of the meristic traits correctly classified 72% (180 of 250) of *E. cumberlandicum* and 80% (39 of 49) of *E.* cf. *kennicotti* from the Laurel River; however, it did not correctly identify a high proportion of the specimens of E. kennicotti (s.s.; 29%), *E.* cf. *kennicotti* from the Green River system (42%), and *E.* cf. *kennicotti* from the Tennessee River system (52%). Comparing the Mahalanobis distances of the PC values among the lineages of *E. kennicotti* (s.l.) shows the greatest disparity in the meristic traits is between the sister lineages of *E. cumberlandicum* and *E.* cf. *kennicotti* from the Laurel River (Fig. 4B), which were the two species with the highest proportions of correct specimen identification in the LDA.

Despite broad overlap in the PC plot, moderate disparity in meristic traits among most of the lineages resolved in the *cytb* gene tree, and generally poor identification in LDA for three of the five species, there is notable variation in meristic and pigmentation traits that allows for morphological characterization of four of the five lineages of *Etheostoma kennicotti* (s.l.) and *E. cumberlandicum*. As noted in Page and Smith (1976), populations of *E. kennicotti* (s.s.) from tributaries of the lower Ohio River in Illinois and Kentucky and populations from the Clarks River system in Kentucky and Tennessee have fewer lateral line scales, fewer pored lateral line scales, and fewer transverse scales than other lineages of *E. kennicotti* (s.l.) and *E. cumberlandicum* (Tables 5–7). *Etheostoma* cf. *kennicotti* in the Laurel River has a modal count of eight spines in the first dorsal fin (Table 8). The population in the upper Tennessee River system does not exhibit a modal count of dorsal-fin spines as the same number of specimens have seven and eight spines, but *E. cumberlandicum* and all other lineages of *E. kennicotti* (s.l.) have a mode of seven spines in the first dorsal fin (Table 8). *Etheostoma* cf. *kennicotti* in the Green River system have modally 13 rays in the second dorsal fin (Table 9). *Etheostoma cumberlandicum* is distinct from all lineages of *E. kennicotti* (s.l.) in having modally 13 versus 12 rays in the left pectoral fin and a mode of seven bands of dark pigment in the caudal fin of adult male specimens (Tables 10, 11). The fewer caudal bands in *E. cumberlandicum* are apparent in comparisons with other lineages of *E. kennicotti* (s.l.), including its sister species *E.* cf. *kennicotti* from the Laurel River system (Fig. 5). There are no apparent meristic traits that allow a morphological diagnosis of *E.* cf. *kennicotti* from the Tennessee River system, and the Mahalanobis distance between the upper and lower Tennessee River lineages was the lowest contrast among all lineages of *E. kennicotti* (s.l.) and *E. cumberlandicum* (Fig. 4B).

Table 4. Pairwise Weir and Cockerham (1984) F_{ST} estimated from 25 microsatellite loci for populations of *Etheostoma kennicotti* and *E. cumberlandicum*.

	GRN	UTN	LTN	OHO	LRR	CMB
Etheostoma kennicotti						
Green River (GRN)	0.000					
Upper Tennessee River (UTN)	0.344	0.000				
Lower Tennessee River (LTN)	0.219	0.145	0.000			
Lower Ohio River–Clarks River (OHO)	0.397	0.335	0.247	0.000		
Laurel River (LRR)	0.422	0.385	0.285	0.438	0.000	
Etheostoma cumberlandicum (CMB)	0.382	0.323	0.252	0.386	0.291	0.000

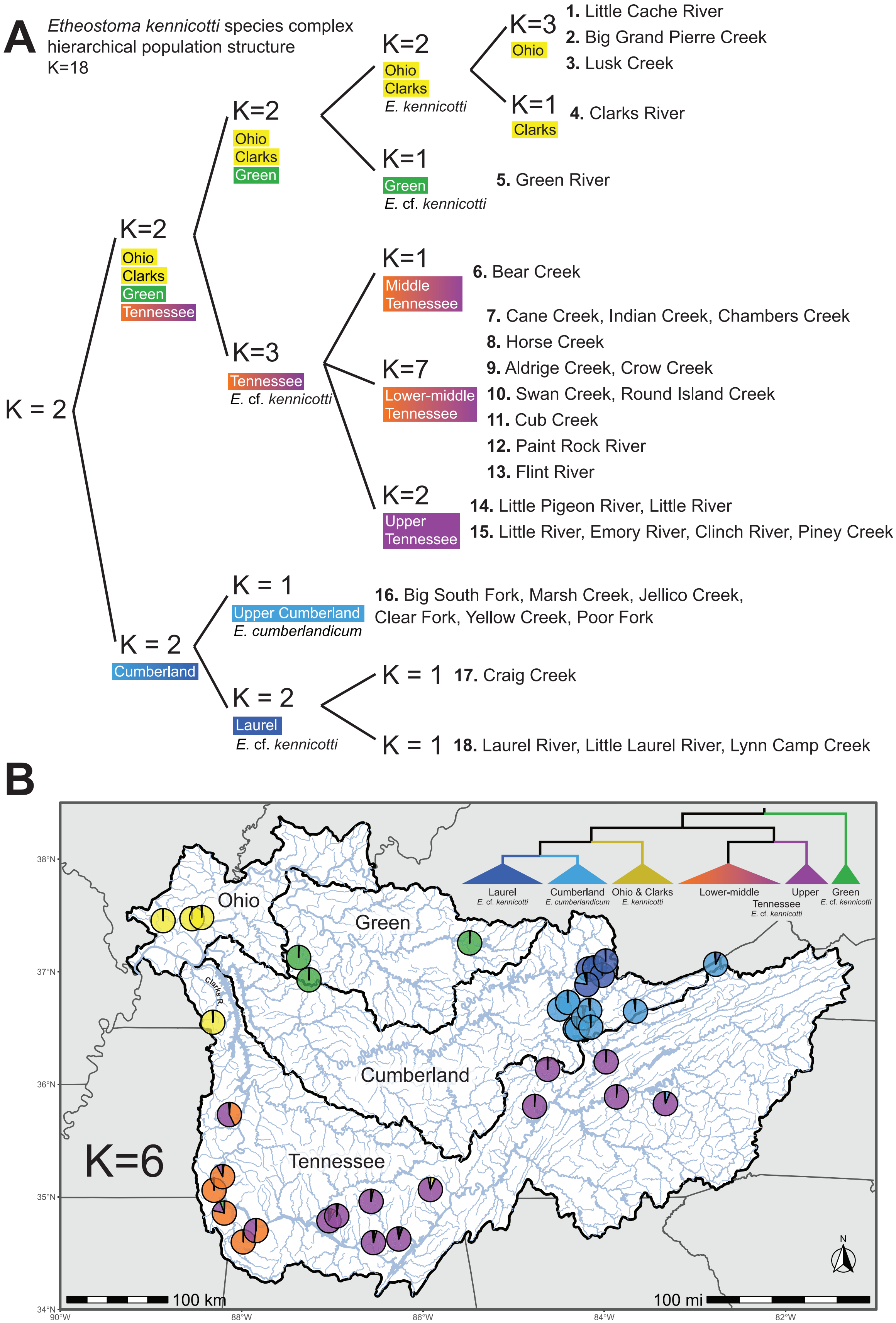

Fig. 3. Population structure inferred from 25 microsatellite loci. (A) Hierarchical STRUCTURE analysis identifies 18 genetic clusters (K). Optimal K values for each round of hierarchical clustering are shown. (B) STRUCTURE results for K = 6. Pie charts represent average ancestry coefficients for each sampling locality. The phylogeny is redrawn from Figure 2A.

Table 5. Counts of lateral line scales in *Etheostoma cumberlandicum* and *E. kennicotti*. Abbreviations: *n*, number of specimens; SD, standard deviation.

		Number of lateral line scales																			
Species	Drainage	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	*n*	Mean	SD
Etheostoma cumberlandicum	Upper Cumberland		1	6	3	17	27	30	38	39	38	26	17	14	3	3	1		263	48.74	2.58
Etheostoma kennicotti	Laurel River			1	2	1	4	7	13	18	12	5	11	5	2	1			82	49.39	2.41
	Ohio–Clarks	3	3	5	10	2	3	2	1	1	2								32	44.47	2.42
	Green				3	1	2	4	4	2	1	1	1	1					20	47.65	2.54
	Lower Tennessee	4	5	11	9	15	32	24	31	16	11	5	8	6	3	1			181	47.22	2.90
	Upper Tennessee	1			3	6	13	16	17	19	15	16	7	5	4	1	1	1	125	48.95	2.70

Table 6. Counts of pored lateral line scales in *Etheostoma cumberlandicum* and *E. kennicotti*. Abbreviations: *n*, number of specimens; SD, standard deviation.

		Number of pored lateral line scales																												
Species	Drainage	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	*n*	Mean	SD
Etheostoma cumberlandicum	Upper Cumberland		1		2	2	2	2	6	6	13	17	23	25	19	18	33	22	12	18	13	10	7	3	4	1	1	1	261	24.04	4.28
Etheostoma kennicotti	Laurel River			1		1	2	3	3	3	5	4	7	5	2	5	7	2	2	1									53	21.06	3.71
	Ohio–Clarks			2	2	1		3	1	4	3	7	1	4	2	1													31	18.61	3.31
	Green							1		1	3	1		1	4	2	2	1	3										19	22.79	3.54
	Lower TN	2	1	2	4	5	6	7	6	6	7	9	13	15	8	11	10	8	4	11	4	7	6	4	3	6	2	1	168	23.07	5.82
	Upper TN			2		7	6	8	5	11	12	18	6	9	7	8	4	3	6	1	1		4	1					119	20.51	4.35

Table 7. Counts of transverse scale rows in *Etheostoma cumberlandicum* and *E. kennicotti*. Abbreviations: *n*, number of specimens; SD, standard deviation.

		Number of transverse scale rows												
Species	Drainage	12	13	14	15	16	17	18	19	20	21	22	*n*	Mean	SD
Etheostoma cumberlandicum	Upper Cumberland		1	14	32	60	82	56	13	2		2	262	16.70	1.33
Etheostoma kennicotti	Laurel River		4	3	11	26	24	13	1				82	16.29	1.30
	Ohio–Clarks			8	14	5	5						32	15.22	1.01
	Green			1	1	3	6	6	2	1			20	17.25	1.41
	Lower Tennessee	1	8	28	34	44	42	13	8	3			181	15.92	1.58
	Upper Tennessee		1	9	28	32	35	16	4				125	16.24	1.27

Table 8. Counts of first dorsal-fin spines in *Etheostoma cumberlandicum* and *E. kennicotti*. Abbreviations: *n*, number of specimens; SD, standard deviation.

		Number of dorsal-fin spines						
Species	Drainage	6	7	8	9	*n*	Mean	SD
Etheostoma cumberlandicum	Upper Cumberland	3	157	99		259	7.37	0.51
Etheostoma kennicotti	Laurel River		18	63	1	82	7.79	0.44
	Ohio–Clarks	1	27	4		32	7.09	0.39
	Green	2	16	2		20	7.00	0.49
	Lower Tennessee	7	98	73	3	181	7.40	0.59
	Upper Tennessee		60	60	4	124	7.55	0.56

Table 9. Counts of second dorsal-fin rays in *Etheostoma cumberlandicum* and *E. kennicotti*. Abbreviations: *n*, number of specimens; SD, standard deviation.

		Number of dorsal-fin rays								
Species	Drainage	10	11	12	13	14	15	*n*	Mean	SD
Etheostoma cumberlandicum	Upper Cumberland		39	158	60	4		261	12.11	0.66
Etheostoma kennicotti	Laurel River		3	42	30	2		77	12.40	0.61
	Ohio–Clarks		2	21	9			32	12.22	0.55
	Green			7	13			20	12.65	0.49
	Lower Tennessee		13	66	86	14	1	180	12.58	0.76
	Upper Tennessee	1	41	72	8	2		124	11.75	0.66

Systematic Account

Etheostoma cumberlandicum Jordan and Swain, 1883: 251

Moonbow Darter

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Figure 5A, B; Tables 5–11

Etheostoma cumberlandicum: Jordan and Swain, 1883: 251 (meristic data and species description); Page and Smith, 1976: 533 (listed as synonym of *Etheostoma kennicotti*); Page, 1983: 149 (referenced as a synonym of *Etheostoma kennicotti*); Beckham, 1983: 27 (referenced as a synonym of *Etheostoma kennicotti*); Braasch and Mayden, 1985: 53 (referenced as a synonym of *Etheostoma kennicotti*).

Etheostoma flabellare cumberlandicum: Jordan and Evermann 1898: 1098 (morphology, geographic distribution, and listed as a subspecies of *Etheostoma flabellare*); Ross and Carico, 1963: 12 (listed as a subspecies of *Etheostoma flabellare*); Collette and Knapp, 1966: 25 (listed as a subspecies of *Etheostoma flabellare*).

Catonotus kennicotti cumberlandicus: Shoup and Peyton, 1940: 111 (distribution in Jellico Creek system and listed as a subspecies of *Etheostoma kennicotti*).

Etheostoma kennicotti cumberlandicum: Page and Smith, 1976: 532 (listed as a subspecies, but it was placed into the synonymy of *Etheostoma kennicotti*); Smith, 1979: 288 (referenced as a synonym of *Etheostoma kennicotti*); Braasch and Mayden, 1985: 53 (referenced as a synonym of *Etheostoma kennicotti*); Burr and Warren, 1986: 304 (referenced as a synonym of *Etheostoma kennicotti*); Etnier and Starnes, 1993: 500 (referenced as a synonym of *Etheostoma kennicotti*).

Etheostoma kennicotti: Carter and Jones, 1969: 13, 67 (presence in Poor Fork of the upper Cumberland River system); Comiskey and Etnier, 1972: 143 (distribution in Big South Fork system); Page and Smith, 1976: tables 3–6, fig. 2 (meristic trait variation and pigmentation); Starnes and Starnes, 1978: 515 (syntopic with *Chrosomus cumberlandensis* [Starnes and Starnes] in the upper Cumberland River system); Wolfe et al., 1979 (allozyme variation); Wolfe and Branson, 1979 (LDH isozyme variation); Burr, 1980: 76 (distribution in upper Cumberland River system); Page, 1983: 149, map 80 (geographic distribution and morphological variation); Page and Schemske, 1978 (geographic distribution and body size); O'Bara and Estes, 1984: 10–12 (presence in the Clear Fork system in upper Cumberland River system); Burr and Warren, 1986: 304 (geographic distribution and habitat notes); Etnier and Starnes, 1993: 499–500, range map 227, plate 235b (photograph of nuptial condition male, geographic distribution, diet, and life history notes); Song et al., 1998: tables 1, 2, figs. 1, 3–5 (phylogenetic relationships); Strange, 1998: 101 (distribution in upper Cumberland River system); Porterfield et al., 1999: figs. 2–6 (phylogenetic relationships); Near et al., 2011: table 1, figs. 3, 4 (classification and phylogenetic relationships).

Lectotype.—Designated by Collette and Knapp (1966: 25). USNM 36502, 41 mm standard length (SL), Wolf Creek a tributary of Clear Fork, near Pleasant View, Whitley Co., Kentucky, D. S. Jordan, J. Swain, and C. H. Gilbert, May 1883.

Paralectotypes.—Designated by Collette and Knapp (1966: 25). USNM 197992, 4 specimens, 20–42 mm SL, same collection information as lectotype.

Material examined.—A total of 263 specimens, 25–62 mm SL (see Material Examined).

Table 10. Counts of left pectoral-fin rays in *Etheostoma cumberlandicum* and *E. kennicotti*. Abbreviations: *n*, number of specimens; SD, standard deviation.

		Number of left pectoral-fin rays						
Species	Drainage	11	12	13	14	*n*	Mean	SD
Etheostoma cumberlandicum	Upper Cumberland	2	69	169	17	257	12.78	0.57
Etheostoma kennicotti	Laurel River	2	61	18		81	12.20	0.46
	Ohio–Clarks	3	17	12		32	12.28	0.63
	Green	2	11	7		20	12.25	0.64
	Lower Tennessee	15	119	43	1	178	12.17	0.57
	Upper Tennessee	11	91	16	2	120	12.08	0.54

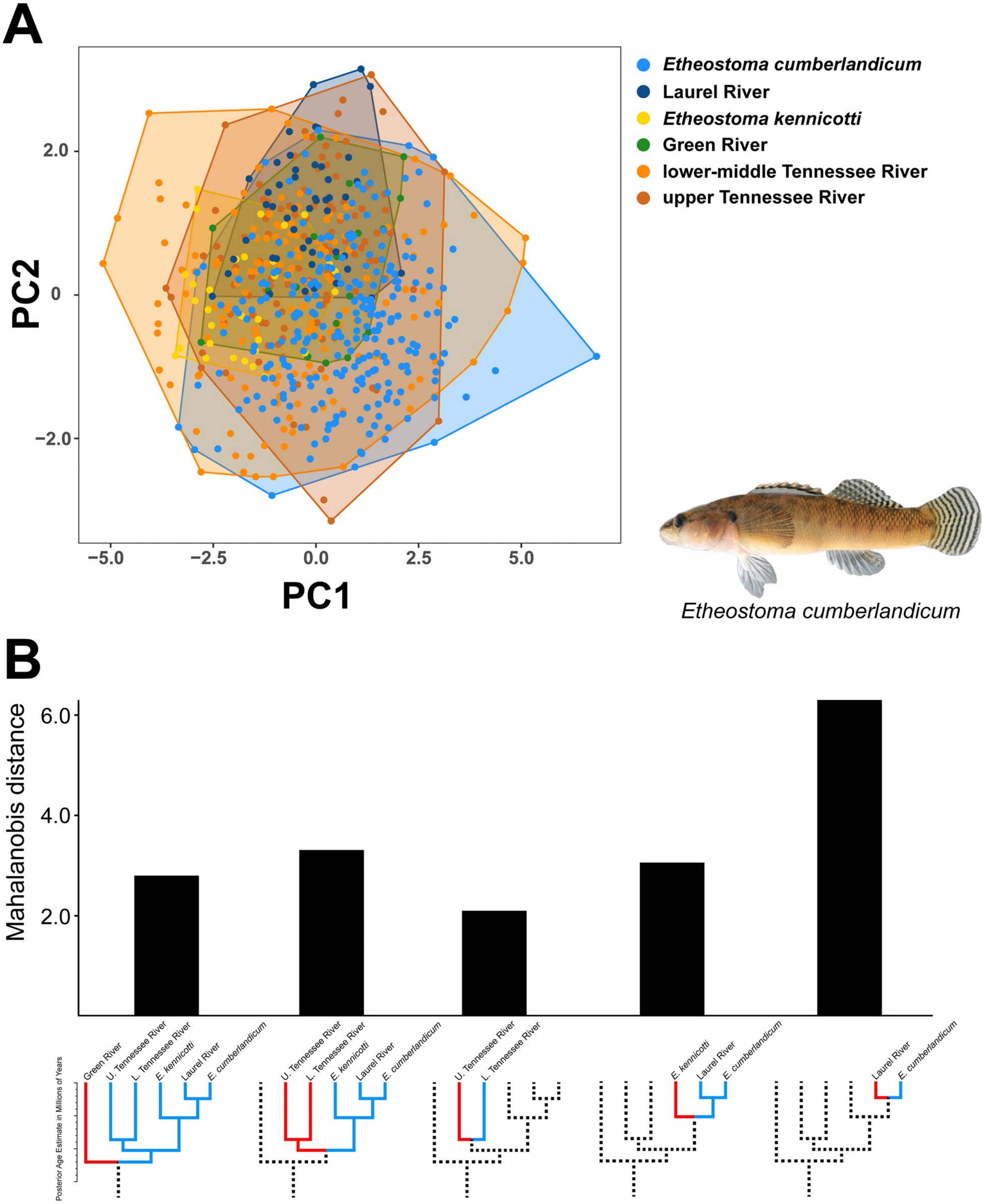

Fig. 4. Morphological disparity in *Etheostoma kennicotti sensu lato* and *Etheostoma cumberlandicum*. (A) Plot of first and second principal component scores of meristic traits in *Etheostoma kennicotti sensu lato* and *Etheostoma cumberlandicum*. (B) Mahalanobis distances of PC scores for contrasts of species in the *Etheostoma kennicotti* complex. In each comparison, the red and blue lineages on the *cytb* phylogeny are contrasted. A lineage that is a dashed branch is not included in the contrast. The comparison between the upper (U.) Tennessee and lower (L.) Tennessee River is considered an intraspecific contrast.

Diagnosis.—*Etheostoma cumberlandicum* is distinguished from all other species referred to as *E. kennicotti* by a modal count of 13 versus 12 rays in the pectoral fin and modally seven caudal bands versus modally nine or ten caudal bands (Tables 10, 11), 88.3% of specimens of *E. cumberlandicum* have eight or fewer caudal bands and 82.7% specimens of *E. kennicotti* (s.l.) have nine or more caudal bands.

Distribution.—*Etheostoma cumberlandicum* is distributed in the Roaring Paunch Creek system, a tributary of the Big South

Table 11. Counts of caudal bands in *Etheostoma cumberlandicum* and *E. kennicotti*. Abbreviations: *n*, number of specimens; SD, standard deviation. Only specimens 44.0 mm and greather in standard length included. Data included from Page and Smith (1976: table 7).

		Number of caudal bands								
Species	Drainage	6	7	8	9	10	11	*n*	Mean	SD
Etheostoma cumberlandicum	Upper Cumberland	4	55	39	11	2		111	7.57	0.79
Etheostoma kennicotti	Laurel River		1	4	5	8		18	9.11	0.96
	Ohio–Clarks				16	23	2	41	9.66	0.57
	Green		1	1	2	1		5	8.60	1.40
	Lower Tennessee		1	4	1		2	8	8.75	1.49
	Upper Tennessee			6	15	9	2	32	9.22	0.83

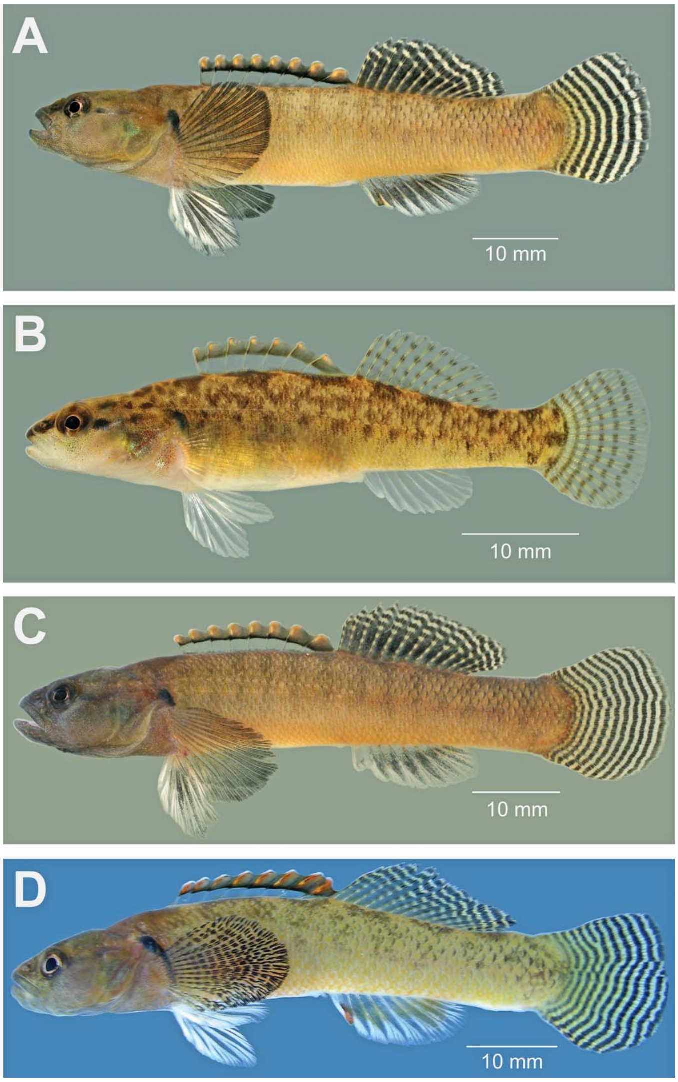

Fig. 5. Photographs of live specimens of *Etheostoma cumberlandicum*, *E.* cf. *kennicotti* Laurel River, and *E. kennicotti*. (A) *Etheostoma cumberlandicum*, YPM ICH 028205, 62 mm SL, male, Wolf Creek, Whitley Co., Kentucky, USA, 28 April 2015. (B) *Etheostoma cumberlandicum*, YPM ICH 028204, 46 mm SL, female, Little Wolf Creek, Whitley Co., Kentucky, USA, 25 March 2015. (C) *Etheostoma* cf. *kennicotti*, Laurel River species, YPM ICH 028202, 63 mm SL, male, Lick Fork, Laurel Co., Kentucky, USA, 14 May 2015. (D) *Etheostoma kennicotti*, UF 167303, 61 mm SL, Bay Creek, Pope Co., Illinois, USA, 18 April 2007.

Fork and in the Cumberland River system above Cumberland Falls. Collections of *E. cumberlandicum* closest to Cumberland Falls include Eagle Creek, McCreary Co., Kentucky (UT 91.3177) and Bunches Creek, Whitely Co., Kentucky (UT 91.3122). In addition to the main stem of the Cumberland River, major tributary systems above the Cumberland Falls occupied by *E. cumberlandicum* include Marsh Creek, Jellico Creek, Clear Fork, Greasy Creek, Clear Creek, Yellow Creek, Brownies Creek, and Poor Fork of the Cumberland River (Fig. 1). Collections of *E. cumberlandicum* include locations in Bell, Harlan, Letcher, McCreary, and Whitely Counties, Kentucky and Campbell, Claiborne, and Scott Counties, Tennessee (Fig. 1).

Etymology.—While not stated directly, it is clear the specific epithet *Etheostoma cumberlandicum* is in reference to the location of the species in the Cumberland River system (Jordan and Swain, 1883). The common name Moonbow Darter is in reference to the rare "moonbow" associated with Cumberland Falls. On bright moonlit nights, mist rising from the water plunging over the waterfall refracts the moonlight producing an effect similar to a rainbow, but with less vivid colors.

DISCUSSION

Phylogenetic relationships and divergence times inferred from mitochondrial DNA sequences, population structure at 25 microsatellite loci, and divergence of meristic and pigmentation traits support the conclusion that *Etheostoma kennicotti* (s.l.) is composed of multiple species and *E. cumberlandicum* warrants recognition as a distinct species. The mitochondrial *cytb* gene tree resolves six major lineages which began diversifying approximately six million years ago, with the most recent speciation involving *E.* cf. *kennicotti* from the Laurel River system and *E. cumberlandicum* ~1.3 million years ago (Fig. 2B). With the exception of samples from the Tennessee River system, population structure inferred from microsatellite loci is consistent with the mtDNA phylogenetic structure (Fig. 3). We suggest there are at least five species in the *Etheostoma kennicotti* complex: *E. kennicotti* (s.s.), *E. cumberlandicum*, *E.* cf. *kennicotti* distributed in the Tennessee River system, *E.* cf. *kennicotti* distributed in the Green River system, and *E.* cf. *kennicotti* distributed in the Laurel River system (Fig. 1).

The *cytb* gene tree resolves three deep branching lineages in the clade that comprises *E.* cf. *kennicotti* from the Tennessee River system (Fig. 2A). The clades resolved in the Tennessee River lineage correspond to a split between populations from tributaries of the upper Tennessee River and those from the lower portions of the Tennessee River (Fig. 2A). It is long accepted that phylogeographic structure can evolve without barriers to gene flow in nonrecombining genetic units such as mtDNA (Irwin, 2002). The splits in the *cytb* gene tree likely do not reflect geographic isolation among the populations in the lower Tennessee River system as haplotypes sampled from two populations, Horse Creek and Aldridge Creek, resolve in both of the lower Tennessee River system clades. A very similar pattern where there is little associating between geography and distribution of haplotypes in the Tennessee River system was observed in *Etheostoma simoterum* (Harrington and Near, 2012).

Populations of *Etheostoma* cf. *kennicotti* from the Tennessee River system contain the highest diversity of microsatellite alleles (Table 3) and the highest number of genetic clusters (Fig. 3A). However, genetic structure does not strongly match geography, and there are signals of admixture throughout the lower Tennessee River (Fig. 3A). Pairwise F_{ST} between the lower and upper Tennessee River is the lowest of any comparison among the six lineages resolved in the *cytb* phylogeny (Table 4). These patterns suggest the Tennessee River contains disjunct populations of *E.* cf. *kennicotti* connected by gene flow. The Tennessee River is well suited to produce such mosaic genetic structure, with many small but geologically stable tributary systems separated by larger, shifting river courses that may facilitate a mosaic of localized isolation with periods of gene flow among populations in the Tennessee River system. Despite high levels of genetic variation, we recognize all populations of *E.* cf. *kennicotti* in the Tennessee River, sans the Clarks River system, as a single undescribed species.

While the phylogenetic relationships and substantial genetic diversity strongly suggest that *Etheostoma kennicotti* (s.l.) is composed of five distinct species, meristic and pigmentation traits traditionally utilized to discover and delimit species of ray-finned fishes allow for diagnosis of only four of these lineages. The nominal *E. kennicotti* differs from all other species in the clade by having a lower average number of lateral line scales (Table 5). The species *E.* cf. *kennicotti* from the Green River system is distinguished by a larger number of transverse scale rows and modally 13 versus 12 rays in the second dorsal fin (Table 9). The species *E.* cf. *kennicotti* endemic to the Laurel River differs from all other lineages of *E. kennicotti* and *E. cumberlandicum* by having modally eight versus seven dorsal-fin spines (Table 8). *Etheostoma cumberlandicum* is diagnosed with 13 versus 12 pectoral-fin rays and modally seven versus nine or ten caudal bands (Tables 10, 11).

Page and Smith (1976) interpreted the lower scale counts in *Etheostoma kennicotti* (s.s.), the intermediate scale counts of *E.* cf. *kennicotti* from the Tennessee River system, and the higher scale counts in *E.* cf. *kennicotti* in the Laurel River and *E. cumberlandicum* as evidence for a pattern of east–west clinal variation in *E. kennicotti* (s.l.). This hypothesized cline was used to justify treating *E. cumberlandicum* as a synonym of *E. kennicotti* (s.l.). While the morphological variation in the scale counts detected in our study is similar to that presented by Page and Smith (1976), phylogenetic relationships inferred in the *cytb* gene tree do not support that *E.* cf. *kennicotti* from the Tennessee River system is "intermediate." Instead, the Tennessee River lineage is sister to a clade containing *E. kennicotti* (s.s.), *E.* cf. *kennicotti* from the Laurel River system, and *E. cumberlandicum*, which are the lineages that exhibit the lowest and highest scale counts (Fig. 2A, Tables 5, 7).

The phylogenetic relationships and timing of diversification of species in the *Etheostoma kennicotti* complex are congruent with the paleogeographic history of rivers of the Eastern Highlands of North America. The pre-Pleistocene configurations of the present-day Green, Cumberland, and Tennessee River systems are sufficient to explain the origin of the lineages endemic to these systems (Mayden, 1988). The pre-glacial Green River was a tributary of the Old Ohio River that drained to the Old Mississippi River and was independent of the Old Cumberland and Old Tennessee Rivers (Burr and Page, 1986; Burr and Warren, 1986; Starnes and Etnier, 1986; Mayden, 1988). *Etheostoma* cf. *kennicotti* in the Green River system diverged from all other lineages of *E. kennicotti* in the Late Miocene (Fig. 2A, B), consistent with the fractured nature of the pre-glacial Eastern Highland River systems and the long isolation of the Green River system (Mayden, 1985; Mayden and Matson, 1992). In addition to *E.* cf. *kennicotti*, there are five other species of freshwater fishes endemic to the Green River system: *E. barrenense* Burr and Page, *E. rafinesquei* Burr and Page, *E. barbouri* Kuehne and Small, *Nothonotus bellus* (Zorach), and *Thoburnia atripinnis* (Bailey). The divergence times of these Green River endemics range from ~15 mya for *T. atripinnis* and a clade comprising all three species of *Hypentelium* that is widespread throughout freshwater habitats in eastern North America (Bagley et al., 2018), 12.7 mya for the split between the clade containing the Green–Barren endemics *E. rafinesquei* and *E. barrenense* and the three species of the *Etheostoma simoterum* complex (*E. simoterum*, *E. atripinne*, and *E. planasaxatile*) distributed in the Tennessee, Cumberland, and Duck River systems (Near et al., 2011), 9.8 mya for the MRCA of *E. barbouri* and the *Etheostoma basilare* complex that is endemic to the Caney Fork River system (Hollingsworth and Near, 2009; Near et al., 2011), to 1.6 mya for the MRCA of the Green River endemic *N. bellus* and *N. camurus* that is widespread through the Ohio, Cumberland, and upper Tennessee River systems (Near et al., 2011). The relationships and divergence time of *E.* cf. *kennicotti* contribute to a set of observations that indicate the endemism of fishes in the Green River system is not the result of a single shared paleogeographic event or biogeographic process.

The elevation of *Etheostoma cumberlandicum* out of synonymy with *E. kennicotti* highlights patterns of endemism of freshwater fishes in the Cumberland River system above Cumberland Falls, which is a barrier to upstream dispersal of aquatic organisms. The darter *E. susanae* (Jordan and Swain) is endemic to the upper Cumberland upstream and in the immediate vicinity of the Cumberland Falls (Jordan and Swain, 1883; Starnes and Starnes, 1979; O'Bara, 1991; Strange, 1998). *Etheostoma cumberlandicum* and *E. sagitta* (Jordan and Swain) are near endemics to the upper Cumberland above the falls, but both species are distributed in the Roaring Paunch Creek system, a tributary of the Big South Fork that empties into the Cumberland River below the Cumberland Falls (Jordan and Swain, 1883; Kuehne and Bailey, 1961; Burr and Warren, 1986). Headwater stream capture between the upper Kentucky River system and the upper Cumberland is invoked as a mechanism of allopatric speciation leading to the origin of *E. susanae* and *E. sagitta* (Kuehne and Bailey, 1961; Starnes and Starnes, 1979; Strange, 1998); however, the origin of *Etheostoma cumberlandicum* involves allopatric speciation within the Cumberland River system. The Laurel River endemic *Etheostoma* cf. *kennicotti* and *E. cumberlandicum* are sister species with an estimated divergence time of 1.3 mya (Fig. 2A, B). This implies that the Cumberland Falls was an important geographic isolating barrier in the diversification of these two species (Fig. 1). The sister lineages of both *E. susanae* and *E. sagitta* are endemic to the upper Kentucky River system (Kuehne and Bailey, 1961; Strange, 1998; Heckman et al., 2009). As determined for Green River endemics, it appears endemism of freshwater fishes in the upper Cumberland River system is not the result of a single event involving vicariance between two specific river basins, but several events that involved allopatric processes between different areas adjacent to the upper Cumberland River system.

Conclusions.—Phylogenetic analysis of mtDNA sequence data and assessment of genetic clusters using microsatellite loci identify five distinct lineages in the *Etheostoma kennicotti* complex that we treat as allopatrically distributed species. Combining inferences of phylogenetic resolution, genetic clustering, and morphological distinctiveness, we elevate *Etheostoma cumberlandicum* out of synonymy with *E. kennicotti*. The delimitation and description of the three new species distributed in the Laurel River system, the Tennessee River system, and the Green River system (Fig. 1) require more data on male nuptial pigmentation and testing the phylogenetic resolution of the *cytb* gene tree using a method such as ddRADseq to collect nuclear DNA sequences from tens of thousands of loci (e.g., Near et al., 2021). The results of the phylogenetic and morphological study of *E. kennicotti* are part of a larger story where the applications of molecular and phenotypic data to species of darters with widespread distributions in the Eastern Highlands of North America results in the discovery of additional biodiversity masquerading as a single species. Ichthyologists studying the North American freshwater fish fauna are entering the last stages of describing all species of darters. As reflected by research of the past decade, this last phase of biodiversity discovery in darters will likely combine traditional morphological data with genetic and genomic analyses as has been deployed over the past decade (Keck and Near, 2013; Robison et al., 2014; Near and Thomas, 2015; Powers et al., 2015; Kozal et al., 2017; Near et al., 2017, 2021; Sterling and Warren, 2020).

DATA ACCESSIBILITY

Supplemental material is available at https://www.ichthyologyandherpetology.org/i2021053 and at the Dryad Digital Repository (https://doi.org/10.5061/dryad.47d7wm3jg). Unless an alternative copyright or statement noting that a figure is reprinted from a previous source is noted in a figure caption, the published images and illustrations in this article are licensed by the American Society of Ichthyologists and Herpetologists for use if the use includes a citation to the original source (American Society of Ichthyologists and Herpetologists, the DOI of the *Ichthyology & Herpetology* article, and any individual image credits listed in the figure caption) in accordance with the Creative Commons Attribution CC BY License.

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