A New Species of Bridled Darter Endemic to the Etowah River System in Georgia (Percidae: Etheostomatinae: *Percina*)

Thomas J. Near[1], Daniel J. MacGuigan[2], Emily L. Boring[3], Jeffrey W. Simmons[4], Brett Albanese[5], Benjamin P. Keck[6], Richard C. Harrington[2], and Gerald R. Dinkins[7]

[1]*Corresponding author:* Department of Ecology and Evolutionary Biology, Osborn Memorial Labs, Yale University, New Haven CT 06520-8106 USA; and Peabody Museum of Natural History, Yale University, New Haven, CT 06520-8118 USA —*email:* thomas.near@yale.edu

[2]Department of Ecology and Evolutionary Biology, Osborn Memorial Labs, Yale University, New Haven CT 06520-8106 USA; and Peabody Museum of Natural History, Yale University, New Haven, CT 06520-8118 USA

[3]Pierson College and Department of Ecology and Evolutionary Biology, Osborn Memorial Labs, Yale University, New Haven CT 06520-8106 USA

[4]River and Reservoir Compliance Monitoring, Tennessee Valley Authority, Chattanooga, TN 37402-2881 USA

[5]Wildlife Conservation Section, Georgia Department of Natural Resources, Social Circle, GA 30025 USA

[6]Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996-1610 USA

[7]McClung Museum of Natural History and Culture, University of Tennessee, Knoxville, TN 37996-3200 USA

Electronic supplementary materials for this article are available online.

ABSTRACT

Percina freemanorum, the Etowah Bridled Darter, is described as a new species endemic to the Etowah River system in Georgia, specifically in Long Swamp Creek, Amicalola Creek, and the upper portion of the Etowah River. The earliest collection records for *Percina freemanorum* date to 1948 and in 2007 the species was delimited as populations of *Percina kusha*. Our investigation into the systematics of *Percina kusha* is motivated by the uncertain status of populations in the Coosawattee River system and observed morphological disparity in several meristic traits between populations in the Conasauga and Etowah River systems. Our analyses of morphological divergence, nuclear genotypes, and mitochondrial DNA (mtDNA) haplotype networks confirm the distinctiveness of *Percina freemanorum*. Morphologically, *Percina freemanorum* is distinguished from *Percina kusha* through lower average numbers of lateral line scales (65.4 vs. 72.3); rows of transverse scales (18.0 vs. 21.4); scales around the caudal peduncle (22.1 vs. 24.9); and modally more pectoral fin rays (14 vs. 13). The two species are not reciprocally monophyletic in phylogenetic analysis of mtDNA sequences, but the two species do not share mtDNA haplotypes. Analysis of up to 158,000 double digest restriction-site associated DNA (ddRAD) sequencing loci resolve each of the two species as reciprocally monophyletic and genomic clustering analysis of single nucleotide polymorphisms identifies two genetic clusters that correspond to the morphologically delimited *Percina freemanorum* and *Percina kusha*.

KEYWORDS

Teleostei, species delimitation, ddRAD, phylogeography

Introduction

Darters (Percidae: Etheostomatinae) are a clade of freshwater teleost fishes endemic to eastern North America with a total of approximately 250 described and undescribed species (Scharpf 2008; Near et al. 2011). Among the 30 recognized species of darters described in the 21st century are the three bridled darters, which are distributed in three disjunct regions of the upper portions of the western and eastern Mobile Basin (Williams et al. 2007). *Percina smithvanizi* is distributed throughout the Tallapoosa River system (Smith-Vaniz 1968; Wieland and Ramsey 1987; Mettee, O'Neil,

and Pierson 1996:728; Boschung, Mayden, and Tomelleri 2004), *P. sipsi* is restricted to Sipsey Fork and Brushy Creek in the upper Black Warrior River system (Dycus and Howell 1974; Ramsey 1976; Mettee et al. 1989; Mettee et al. 1996:728; Boschung, Mayden, and Tomelleri 2004), and *P. kusha* ranges sporadically in the Conasauga, Coosawattee, and Etowah River systems (Stiles and Etnier 1971; Bryant et al. 1979; Etnier and Starnes 1993). Molecular phylogenetic analyses do not resolve the three species of bridled darters as a clade (Williams et al. 2007), but do resolve *P. kusha* and *P. smithvanizi* as sister species in a larger clade containing *P. apristis*, *P. aurolineata*, *P. crypta*, *P. lenticula*, *P. nigrofasciata*, *P. palmaris*, *P. sciera,* and *P. sipsi*. The subgenus *Hadropterus* was redefined to accommodate this lineage and the clade name or subgenus *Chalinoperca* was introduced to contain *P. kusha* and *P. smithvanizi* (Near et al. 2011).

Among the three bridled darter species, *Percina kusha* exhibits the highest degree of intraspecific variation in meristic characters. Counts of scale rows in *P. kusha*, as delimited by Williams et al. (2007), exhibit a bimodal distribution that is indicative of a contrast between species. Specifically, counts of lateral line scale rows and transverse scale rows from 19 Etowah River system specimens are much lower than observed in specimens of *P. kusha* from the Conasauga River and are more similar to *P. smithvanizi* (Williams et al. 2007). Additionally, phylogenetic analysis of the mitochondrial DNA (mtDNA) gene cytochrome *b* resolves specimens of *P. kusha* sampled from the Conasauga and Etowah River systems as reciprocally monophyletic, but the mtDNA sequence divergence between the two populations is less than 1% (Williams et al. 2007). Despite their presence in museum collections and documentation in species distribution maps (e.g., Mettee, O'Neil, and Pierson 1996:728), specimens of *P. kusha* from the Coosawattee River system were not examined by Williams et al. (2007).

In this study we examine the systematics of *Percina kusha* through an analysis of variation in meristic trait morphology, phylogenetic relationships inferred from mtDNA and double digest restriction-site associated DNA (ddRAD) loci, and genomic clustering analysis of ddRAD-generated single nucleotide polymorphisms (SNPs). We investigate the status of bridled darter populations in the Coosawattee River system and delimit two species currently classified as *P. kusha*. We investigate the phylogenetic relationships of the bridled darters (*P. kusha*, *P.* cf. *kusha*, *P. smithvanizi*, and *P. sipsi*) using DNA sequences from 11 nuclear genes. A description is provided for this new species of *Percina*.

Materials and Methods

Morphological Analyses

Comparative meristic data were collected from 176 specimens obtained from field sampling and museum collections. Institutional abbreviations follow Sabaj (2019), except that YFTC refers to the Yale Fish Tissue Collection in the Division of Vertebrate Zoology Ichthyology Collection at the Peabody Museum of Natural History, Yale University, New Haven, Connecticut, USA (YPM ICH). The sampling locations of specimens examined for morphological comparisons are presented here (Figure 1). Details on sampling locations of specimens of *Percina* cf. *kusha* from the Etowah River system are listed in the type and non-type materials examined. The specimens of *P. kusha* examined from the Conasauga and Coosawattee River systems are listed in the Appendix. 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). Terminology for body pigmentation follows Williams et al. (2007) and Etnier and Starnes (1993:591).

A principal component (PC) analysis of the meristic traits was performed 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 two species was assessed using a cross-validation linear discriminate analysis (LDA) as applied in the MASS package for R Version 3.2.0 (Venables and Ripley 2002). A Bayesian posterior probability of assignment to each of the two groups was calculated, with the group assignment determined by the highest posterior probability. The meristic traits analyzed with LDA included number of lateral line scales, scale rows above the lateral line, scale rows below the lateral line, transverse scale rows, scales

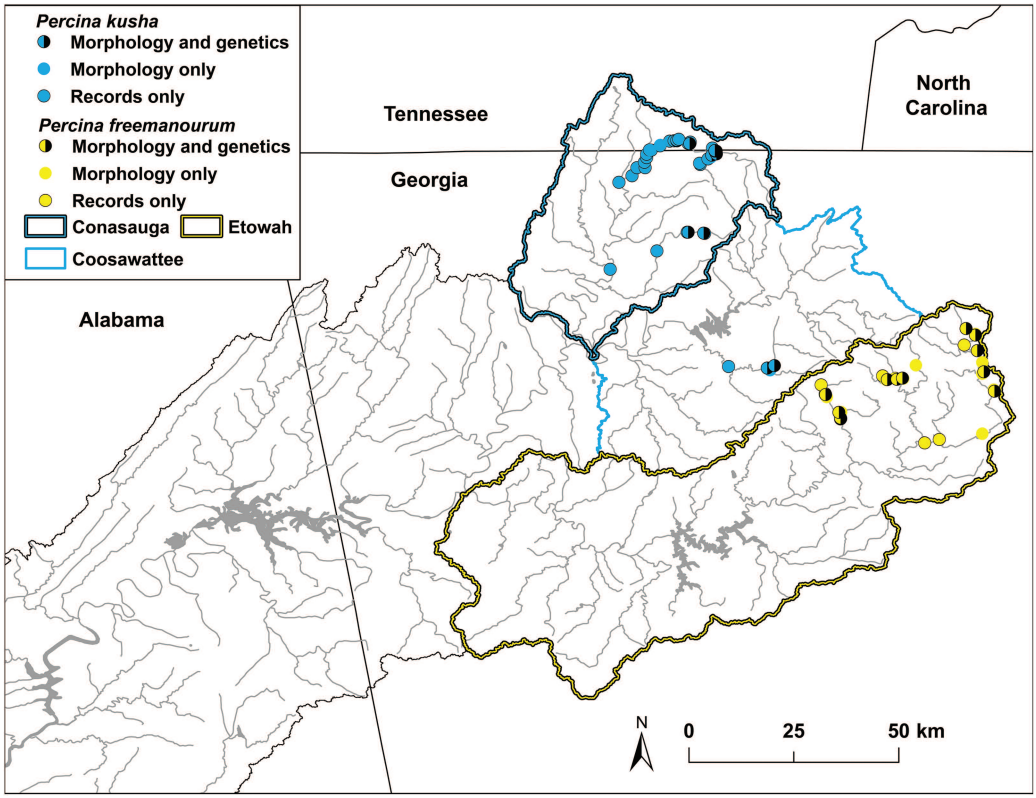

FIGURE 1. Geographic distribution of *Percina freemanorum* and *P. kusha* in the upper Coosa River system of Georgia and Tennessee.

around the caudal peduncle, first dorsal fin spines, second dorsal fin rays, and pectoral fin rays. The *a priori* groups used for LDA were *Percina kusha* consisting of specimens from the Conasauga and Coosawattee River systems and *P.* cf. *kusha* that comprised specimens sampled from the upper Etowah River, Long Swamp Creek, and Amicalola Creek.

The geographic pattern of variation in three meristic traits among populations of both *Percina kusha* and *P.* cf. *kusha*, sampled with three or more specimens each, was visualized by performing a spatial interpolation. The mean number of lateral line scales, transverse scale rows, and pectoral fin rays for populations of each species were subjected to an inverse distance weighting method in the R package gstat (Pebesma 2004). Data interpolation and weighting was proportional to the inverse of the geographic distance between the sampled populations in each of the two species. All of the meristic data are available from the Dryad Digital Depository (Near et al. 2020).

Molecular Analyses: Mitochondrial DNA

Sampling locations and numbers of specimens used for genetic analyses are presented (Table 1). Tissues were preserved in the field through either freezing whole specimens in liquid nitrogen or preserving a dissected portion of the right side pectoral fin in 95% ethanol. We extracted DNA 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: 3M 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 10 min at –80 °C. Samples

TABLE 1. Specimens of species of *Percina* used in genetic analyses. Institutional catalog number abbreviations follow Sabaj (2019).

Species	Catalog number	Sampling location	Latitude, longitude	mtDNA	Sanger nDNA	ddRAD
Percina cf. *kusha*	YPM ICH 018599	Amicalola Creek at Afton Rd., Dawson Co., Georgia	34.4988, –84.2457	5	0	5
	YPM ICH 020140	Amicalola Creek at Afton Rd., Dawson Co., Georgia	34.4988, –84.2457	8	0	6
	YPM ICH 029879	Amicalola Creek, Dawson Forest WMA, Dawson Co., Georgia	34.47402, –84.24078	3	0	3
	YPM ICH 029872	Little Amicaloca Creek, at Afton Rd., Dawson Co., Georgia	34.50145, –84.23416	1	0	1
	YPM ICH 020138	Long Swamp Creek at Sandy Bottom Rd., Pickens Co., Georgia	34.428053, –84.370421	2	0	1
	YPM ICH 021003	Long Swamp Creek, at GA 53, Pickens Co., Georgia	34.414911, –84.366803	1	0	1
	YPM ICH 027064	Long Swamp Creek E of Marbleblock Ln., Pickens Co., Georgia	34.41578, –84.36744	1	0	1
	YPM ICH 030935	Long Swamp Creek downstream of Cove Road Dam, Pickens Co., Georgia	34.46598, –84.39955	3	0	3
	YPM ICH 017509	Etowah River at Castleberry Bridge Rd., 1.3 air km W Auraria, Lumpkin Co., Georgia	34.473267, –84.037367	1	1	1
	YPM ICH 018191	Etowah River at GA Hwy 9, Lumpkin Co., Georgia	34.5151, –84.06015	2	0	1
	YPM ICH 029874	Etowah River at Hightower Church Rd., Lumpkin Co., Georgia	34.594011, –84.0778	1	0	0
	YPM ICH 029877	Etowah River at Jay Bridge Rd., Lumpkin Co., Georgia	34.560308, –84.074194	2	0	1
	YPM ICH 029881	Etowah River at Hightower Church Rd., Lumpkin Co., Georgia	34.594011, –84.0778	2	0	2
Percina kusha	UT 91.6298	Conasauga River below Minnewauga Creek, Polk Co., Tennessee	35.004033, –84.690687	1	1	0
	YPM ICH 020142	Conasauga River below Minnewauga Creek, Polk Co., Tennessee	35.004033, –84.690687	7	0	7
	YPM ICH 020139	Conasauga River at confluence with Jacks River, Polk Co., Tennessee	34.980713, –84.639983	1	0	1
	YPM ICH 020137	Conasauga River below Minnewauga Creek, Polk Co., Tennessee	35.004033, –84.690687	5	0	5

Continued

TABLE 1 CONTINUED.

Species	Catalog number	Sampling location	Latitude, longitude	mtDNA	Sanger nDNA	ddRAD
	YPM ICH 020136	Conasauga River below Minnewauga Creek, Polk Co., Tennessee	35.004033, –84.690687	1	0	1
	YPM ICH 020143	Conasauga River at confluence with Jacks River, Polk Co., Tennessee	34.980713, –84.639983	9	0	9
	YPM ICH 020141	Conasauga River at confluence with Jacks River, Polk Co., Tennessee	34.980713, –84.639983	5	0	4
	YPM ICH 017590	Conasauga River at confluence with Jacks River, Polk Co., Tennessee	34.980713, –84.639983	9	0	7
	YPM ICH 027077	Conasauga River at confluence with Jacks River, Polk Co., Tennessee	34.980713, –84.639983	2	0	0
	YPM ICH 029870	Conasauga River at Cottonwood Campground, Trail 77 Head, 1.5 km upstream of Jacks River, Murray Co., Georgia	34.97935, –84.64313	2	0	2
	YPM ICH 029873	Holly Creek ~0.2 km E of Holly Creek Springs Rd., Murray Co., Georgia	34.81461, –84.68376	4	0	5
	YPM ICH 029871	Holy Creek upstream of Dile Creek confluence, Murray Co., Georgia	34.81268, –84.6605	2	0	1
	YPM ICH 018239	Talona Creek at Carn's Mill Rd., Pickens Co., Georgia	34.52753, –84.50982	2	0	2
	YPM ICH 018251	Talking Rock Creek at Ellijay Rd., Pickens Co., Georgia	34.522917, –84.524001	1	0	1
	YPM ICH 018321	Talking Rock Creek at Ellijay Rd., Pickens Co., Georgia	34.522917, –84.524001	1	0	1
Percina sipsi	INHS 48682	Sipsey Fork AL-33 Winston Co., Alabama	34.217976, –87.368675	0	1	0
	YPM ICH 017053	Sipsey Fork at Co. Rd. 60, Winston Co., Alabama	34.284700, –87.399650	0	0	1
	YPM ICH 020129	Sipsey Fork at Co. Rd. 60, Winston Co., Alabama	34.284700, –87.399650	0	1	0
Percina smithvanizi	YPM ICH 020214	Beech Creek at St. Hwy 120, Fannin Co., Georgia	33.763003, –85.223303	3	0	9
	INHS 37916	Crooked Creek at Hwy 31, Clay Co., Alabama	33.306085, –85.780966	1	1	2
	INHS 38634	Hilabee Creek at Hwy 22, Tallapoosa Co., Alabama	32.984760, –85.860287	1	0	0

Continued

TABLE 1 CONTINUED.

Species	Catalog number	Sampling location	Latitude, longitude	mtDNA	Sanger nDNA	ddRAD
	INHS 48638	Hilabee Creek at Hwy 22, Tallapoosa Co., Alabama	32.984760, –85.860287	1	0	0
	YPM ICH 020134	Cane Creek at Co. Rd., Cleburne Co., Alabama	33.725148, –85.490896	3	0	0
	YPM ICH 020133	Muscadine Creek at Co. Rd. 35, Cleburne Co., Alabama	33.769476, –85.423336	3	0	0
	YPM ICH 016700	Muscadine Creek at Co. Rd. 35, Cleburne Co., Alabama	33.769476, –85.423336	2	1	0
	YPM ICH 020132	Roberts Creek at Co. Rd. 49, Cleburne Co., Alabama	33.77369, –85.41510	3	0	0
	YPM ICH 020135	Ketchepedrakee Creek at AL-9, Clay Co., Alabama	33.462825, –85.700562	9	0	0
	YPM ICH 020131	Enitachopco Creek at AL-9, Clay Co., Alabama	33.239972, –85.859338	2	0	0
Percina palmaris	UT 91.7251	Amicalola Creek at Co. Rd. 25, Dawson Co., Georgia	34.4988, –84.2457	2	0	1
	UT 91.6553	Conasauga River below Minnewauga Creek, Polk Co., Tennessee	35.004033, –84.690687	0	0	1
	UT 91.7149	Conasauga River downstream of confluence with Jacks River, Polk Co., Tennessee	34.988868, –84.634751	2	0	0
	INHS 38631	Hilabee Creek at Hwy 22, Tallapoosa Co., Alabama	32.984760, –85.860287	1	0	0
	YPM ICH 024390	Cane Creek at Co. Rd. 444, Cleburne Co., Alabama	33.725148, –85.490896	3	0	0
Percina aurolineata	No voucher	Ellijay River along Boardtown Rd., Gilmer Co., Georgia	34.720827, –84.475663	0	1	0

were centrifuged for 30 min at 8,000 relative centrifugal field (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 5 min at 8,000 RCF, supernatant was poured off, the pellet was allowed to air dry for approximately 15 min, and the DNA pellet was resuspended with the desired amount of DNAse-free water.

The reciprocal monophyly of *Percina kusha* and *P.* cf. *kusha* was assessed with phylogenetic analyses of the mtDNA encoded cytochrome *b* (*cytb*) gene and phylogenomic analyses of several ddRAD sequencing (ddRADseq) datasets. In addition to *P. kusha* and *P.* cf. *kusha*, the closely related *P. smithvanizi* and *P. sipsi* were included in the phylogenetic analyses (Table 1). We did not include *P. sipsi* in the phylogenetic analyses of *cytb* because its mtDNA genome likely originates from historical instances of hybridization with syntopic *P. sciera* in the Sipsey Fork River system (Williams et al. 2007; Near et al. 2011). *Percina palmaris*, which is resolved as closely related to the bridled darters (Near 2002; Near et al. 2011; Smith et al. 2014; MacGuigan and Near 2019), was used as the outgroup taxon in phylogenetic analyses of both the *cytb* and the ddRAD datasets.

The *cytb* gene was amplified using previously published PCR primers and cycling conditions (Near, Porterfield, and Page 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 Version 7.2 (Kearse et al. 2012). New *cytb* sequences were aligned by eye to those previously generated in early studies of darter phylogeny (Near 2002; 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 Version 1.1 (Lanfear et al. 2012). The mitochondrial gene tree was inferred from the aligned *cytb* sequences using the computer program MrBayes Version 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 3×10^6 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 Version 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.

To visualize relationships among observed *cytb* haplotype sequences, we inferred median joining haplotype networks for *Percina* cf. *kusha* and *P. kusha* using PopArt (Bandelt, Forster, and Roehl 1999). All DNA sequence alignments used for mtDNA analyses are available from the Dryad Digital Depository (Near et al. 2020).

Molecular Analyses: ddRAD

We performed ddRADseq for 72 *Percina kusha* and *P.* cf. *kusha* individuals and 11 individuals from three outgroup species, *P. smithvanizi, P. sipsi*, and *P. palmaris* (Table 1). Specifically, we used a modified version of the ddRADseq protocol outlined in Peterson et al. (2012) and Poland et al. (2012). We digested 200 ng of DNA from each sample with the restriction enzymes *PstI* and *SbfI* for 8 hr at 37 °C. Samples were inspected visually using electrophoresis on a 2.5% agarose gel to ensure complete digestion. We ligated custom adapters (Peterson et al. 2012) using T4 DNA ligase and an incubation period of 3 h at 22 °C. The adapter sequences contained a set of 96 unique barcodes, each 8 to 10 base pairs in length. Barcodes were designed with at least two mutational differences between any pair of barcodes to avoid misassignment during demultiplexing. Sets of eight samples were pooled and cleaned using a QIAquick PCR purification kit (QIAGEN; qiagen.com). We performed PCR using 12 cycles of 30 s at 98 °C, 30 s at 62 °C, and 30 s at 72 °C and held for 10 min at 17 °C after the last cycle. After PCR, all samples were pooled to perform a 300 to 500 base pair size selection using a BluePippen 2% agarose cassette (Sage Sci-

ence, Inc.; sagescience.com). Size selection was confirmed using an ABI Bioanalyzer High Sensitivity DNA assay (Agilent Technologies, Inc.; agilent.com). The genomic library was sequenced using a single lane of Illumina HiSeq 4000 with 100 base pair single-end reads at the University of Oregon Genomics and Cell Characterization Core Facility, Eugene, Oregon, USA. The computer program FastQC (Babraham Bioinformatics 2019) was used to inspect raw read quality.

We performed de novo ddRADseq assembly using iPyrad Version 0.9.50 (Eaton, Overcast, and Schwartz 2020). After demultiplexing with iPyrad step 1, we trimmed raw reads using CutAdapt (Martin 2011). Reads were trimmed to 100 base pairs and three rounds of filtering were used to remove adapter contamination and restriction cut sites ("-b TGCAG," "-a TGCAG," and "-a CCGA GATCGGAAGAGC"). Trimmed reads were then used as input for iPyrad steps 2 through 7 (see supplementary Table S1 for assembly parameters).

Maximum likelihood phylogenetic analyses using IQTree Version 1.6.12 (Nguyen et al. 2015) were performed on several concatenated ddRAD datasets. To determine the effect of missing data on the ddRAD phylogenies, we used six datasets with different thresholds for the proportion of missing data per locus (80%, 50%, 40%, 30%, 20%, and 10% missing data) We used a GTR + gamma nucleotide substitution model and ran each tree search until 100 consecutive unsuccessful tree search iterations were completed. To assess topological support, we also performed 1,000 ultrafast bootstrap replicates (Hoang et al. 2018).

Population structure within *Percina kusha* and *P.* cf. *kusha* was investigated using several ddRAD datasets. The computer program VCFTools Version 0.1.15 (Danecek et al. 2011) was used to create datasets that retained only biallelic SNPs ("—max-alleles 2"). Since SNP singletons can confound inference of population structure (Linck and Battey 2019), we also removed SNPs with only one minor allele count ("—mac 2"). In addition, to minimize the effects of linkage biases on our inference of population structure, the datasets were thinned to include only a single SNP per ddRAD locus ("—thin 10000"). Population clustering analyses were performed using five missing data thresholds ranging between 50% and 10% ("—max-missing 0.5," "—max-missing 0.4," "—max-missing 0.3," "—max-missing 0.2," and "—max-missing 0.1"). Although this filtering removed SNPs with large amounts of missing data, some individuals in our dataset still contained large proportions of missing data, presumably caused by technical errors during library preparation. Therefore, we used the "impute" function in the R package "LEA" to replace missing genotypes with a random genotype weighted by the observed genotype probabilities (Frichot et al. 2014).

We used sparse non-negative matrix factorization (sNMF) to estimate individual ancestry coefficients for genetic cluster (K) with the R package "LEA" Version 2.6.0 (Frichot and François 2015). We performed 10 replicate analyses from $K = 1$ to $K = 10$ with a regularization parameter of 10, tolerance of 0.00001, and percentage of masked genotypes for cross-entropy validation of K values of 0.05. For each value of K, the replicate with the minimum cross-entropy score was selected for visualization. To determine the optimal K value, we applied a cross-validation approach using cross-entropy criteria. All datasets and scripts used in ddRAD analyses are available from the Dryad Digital Depository (Near et al. 2020).

Molecular Analyses: Sanger Sequenced Nuclear Genes

The introgression of *Percina sciera* mtDNA into *P. sipsi* complicates the resolution of relationships among the bridled darters using molecular data. Previous studies demonstrate that Sanger sequenced nuclear genes are informative with regard to darter phylogeny and these loci appear to be introgressed at a relatively lower rate than mtDNA (Keck and Near 2010; Near and Keck 2013). We collected DNA sequences from 10 nuclear encoded exons *ENC1*, *Glyt*, *plagl2*, *Ptr*, *RAG1*, *SH3PX3*, *Sidkey*, *sreb2*, *tbr1*, *zic1*, and from the S7 ribosomal protein intron 1. Sequence data for these 11 genes were gathered from the dataset used in Near and Keck (2013) and included 20 species of *Percina*, two species of *Etheostoma*, two species of *Nothonotus*, and *Ammocrypta beanii*. The non-darter percids *Perca flavescens* and *Sander vitreus* were included to serve as outgroups in these phylogenetic analyses (Near et al. 2011; Near and Keck 2013).

New sequences for these 11 nuclear genes were collected for the three species of bridled

darters and for an additional specimen of *Percina aurolineata* sampled from the Ellijay River in Gilmer County, Georgia (Table 1). The target genes were amplified using primers and PCR conditions in Chow and Hazama (1998), Lopez, Chen, and Ortí (2004), and Li et al. (2007, 2011). 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 Version 7.2 (Kearse et al. 2012). Alignment of the DNA sequences, determination of the optimal molecular evolutionary models and partitioning schemes, and phylogenetic analyses using MrBayes Version 3.2 were as described above for the mtDNA *cytb* gene. All files used in the MrBayes analysis that include alignments of all 11 nuclear genes, data partitioning information, and model parameters used in the analyses as well as the summary posterior tree are available on Dryad (Near et al. 2020).

Taxonomy and Results

Percina freemanorum Near and Dinkins
Etowah Bridled Darter
New species
Figures 1, 2A and B, and Tables 2–10
urn:lsid:zoobank.org:pub:
7498243F-7341-454F-9664-8E2E1F0AC179

Percina species—Mettee, O'Neil, and Pierson 1996:728–729 (Muscadine Darter, in part, distribution in Etowah River system).

Percina kusha Williams and Burkhead—Williams et al. 2007:4–12 (Bridled Darter, in part, distribution in the Etowah River system). Holotype. UF 110303 (not examined), Conasauga River, latitude 35.004048, longitude –84.690667, Polk County, Tennessee, USA.

Holotype. YPM ICH 034382 (Figure 2A), adult male, 68.0 mm standard length (SL). Amicalola Creek 0.25 km downstream of Steve Tate Road on Dawson Forest Wildlife Management Area, about 5.9 km east of the Dawson-Pickens County line, latitude 34.49827, longitude –84.26679, Dawson County, Georgia, USA (Figure 3A and B), 16 April 2020, B. Albanese, P. Dimmick, and A. Popp.

Allotype. YPM ICH 034383 (Figure 2B), adult female, 65.5 mm SL. Collected with the holotype.

Paratopotypes. YPM ICH 034384 (1 male, 65.6 mm SL) collected with the holotype; YPM ICH 029879 (2 females, 52.5–59.8 mm SL; 1 male, 70.5 mm SL) 23 September 2016.

Paratypes. **Amicalola Creek system**: GMNH 4094 (4 females, 43.8–46.9 mm SL; 10 males, 39.6–55.2 mm SL) 27 July 2000, Amicalola Creek at Fausetts Lake Road, about 3.5 air km SSW junction of Fausetts Lake Road (County Route 28) and County Route 136, latitude 34.497521, longitude –84.269151, Dawson County, Georgia; YPM ICH 020140 (4 females, 51.2–54.1 mm SL; 4 males, 47.8–58.3 mm SL) 23 July 2005, Amicalola Creek at County Road 25 (Afton Road) at Afton, latitude 34.498782, longitude –84.245895, Dawson County, Georgia; UF 165649 (2 females, 44.3–46.5 mm SL; 2 males, 47.3–55.2 mm SL) 2 May 1990, same locality as YPM ICH 020140; UAIC 10471.09 (1 female, 56.5 mm SL) 20 July 1992, same locality as YPM ICH 020140; UT 91.5160 (2 males, 47.3–53.0 mm SL) 27 March 1997, same locality as YPM ICH 020140; YPM ICH 018599 (3 females, 52.1–60.1 mm SL; 2 males, 53.5–56.3 mm SL) 26 July 2006, same locality as YPM ICH 020140. **Long Swamp Creek system**: YPM ICH 020138 (1 female, 42.9 mm SL; 1 male, 42.3 mm SL) 23 June 2005, Long Swamp Creek at County Route 34 (Sandy Bottom Road), latitude 34.42805, longitude –84.37042, Pickens County, Georgia; UT 91.6485 (1 female, 54.4 mm SL; 1 male, 53.0 mm SL) 16 September 2003, same locality as YPM ICH 020138; YPM ICH 021003 (1 male, 38.3 mm SL) 17 August 2008, Long Swamp Creek at GA 53 at Tate, latitude 34.415058, longitude –84.366781, Pickens County, Georgia; YPM ICH 027064 (1 female, 55.2 mm SL) 19 June 2014, Long Swamp Creek at Marbleblock Road near GA 53, latitude 34.415966, longitude –84.366848, Pickens County, Georgia; GMNH 4567 (1 female, 56.1 mm SL; 1 male, 52.7 mm SL) 3 October 2001, Long Swamp Creek 0.75 air km SSE Cove Road crossing, latitude 34.462739, longitude –84.397697, Pickens County, Georgia; YPM ICH 030935 (2 females, 50.8–52.9 mm SL; 1 male, 53.6 mm SL) 6 July 2017, Long Swamp Creek, downstream of Cove Road dam, access via city property toward marble quarry, latitude 34.46598, longitude –84.39955, Pickens County, Georgia. **Upper Etowah River system**: GMNH 74 (1 female, 41.9 mm SL) 8 July 1948, Etowah River at GA 53, 6.8 air km SE Dawsonville, latitude 34.38183, longitude –84.06337, Dawson County, Georgia; GMNH 4065 (2 females, 38.7–49.2 mm SL; 1 male, 49.3 mm SL) 18 July 2000, Etowah River downstream of GA Highway 9 at shoal, about 7.6 air km WSW Dahlonega, latitude 34.51098, longitude –84.06327, Lumpkin County, Georgia; YPM ICH 018191 (1 female, 57.9 mm SL; 1 male, 53.8 mm SL) 26 July 2007, Etowah River at GA Highway 9, latitude 34.5151, longitude –84.06015, Lumpkin County, Georgia; YPM ICH 029877 (1 female, 43.5 mm SL; 2 males, 34.8–50.8 mm SL) 27 July 2019, Etowah River at Jay Bridge Road, latitude 34.560308, longitude –84.074194, Lumpkin County, Georgia; UF 165648 (2 females, 36.0–57.1 mm SL; 1 male, 58.6 mm SL) 28 April 1994, same locality as YPM ICH 029877; UAIC 10621.14 (1 female, 48.1 mm SL) 29 June 1990, Etowah River, 10.6 km NW Dahlonega, 1.6 km W Whissenhunt Mountain on unpaved Forest Service Road, latitude 34.5955556, longitude –84.0780556, Lumpkin County, Georgia; YPM ICH 029881 (1 female, 48.0 mm SL; 1 male, 54.0 mm SL) 27 July 2019, Etowah River at Hightower Church Road, latitude 34.593158, longitude –84.077899, Lumpkin County, Georgia.

Material examined but not designated as types. **Amicalola Creek system**: YPM ICH 029872 (1 female, 60.2 mm SL) 23 September 2016, Little Amicalola Creek, Afton Road, latitude 34.50145, longitude –84.23416, Dawson County, Georgia; UF

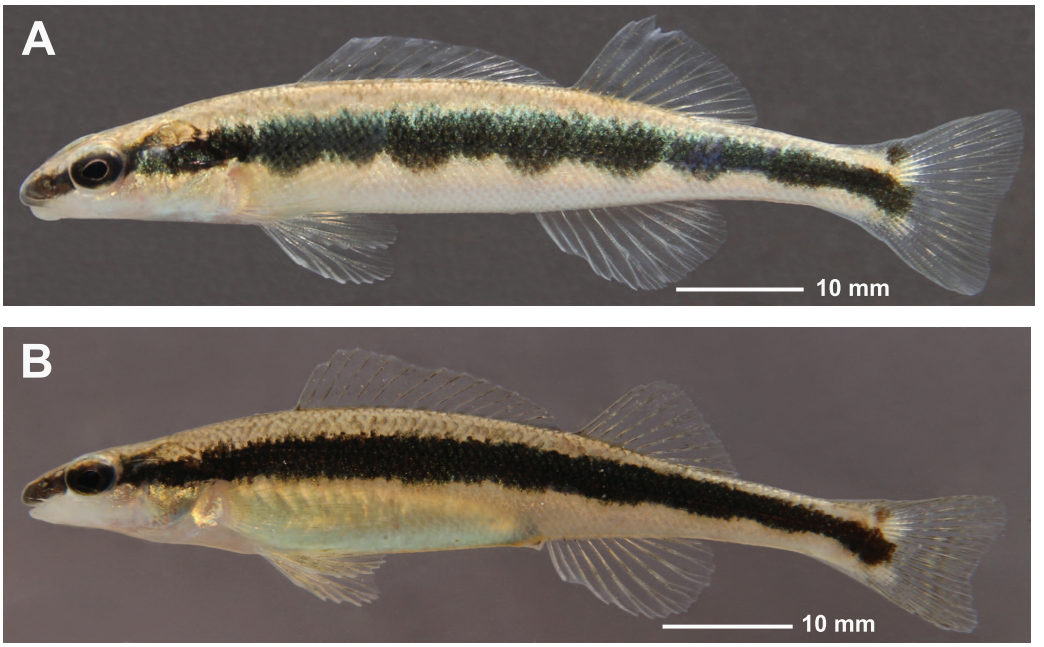

FIGURE 2. Live holotype and allotype specimens of *Percina freemanorum*. Both specimens collected from Amicalola Creek, Dawson County, Georgia, USA, 16 April 2020. **A.** *P. freemanorum* holotype, YPM ICH 034382, 68.0 mm standard length (SL) male. **B.** *P. freemanorum* allotype, YPM ICH 034383, 65.5 mm SL female. Photographs by Georgia Department of Natural Resources.

165647 (1 female, 37.0 mm SL) 3 April 1994, Amicalola Creek at County Road 25 (Afton Road) at Afton, latitude 34.498782, longitude –84.245895, Dawson County, Georgia; UF 165646 (1 female, 40.1 mm SL) 3 April 1994, Cochrans Creek at County Road 45 (New Hope Road) about 2.1 km W junction GA 342, latitude 34.519855, longitude –84.196122, Dawson County, Georgia. **Long Swamp Creek system**: GMNH 4568 (1 male, 41.7 mm SL) 20 July 1999, Long Swamp Creek at County Route 34 (Sandy Bottom Road), latitude 34.42805, longitude –84.37042, Pickens County, Georgia; GMNH 4569 (1 female, 51.2 mm SL) 21 July 1999, Darnell Creek at GA 53, 5.3 air km NNE Nelson, latitude 34.43463, longitude –84.3602, Pickens County, Georgia; GMNH 51962 (2 females, 50.0–52.3 mm SL) 30 May 2007, Long Swamp Creek, about 0.2 km upstream from County Road 294 (Cove Road), latitude 34.469804, longitude –84.400038, Pickens County, Georgia. **Upper Etowah River system**: YPM ICH 017509 (1 male, 52.6 mm SL) 26 August 2007, Etowah River at Castleberry Bridge Road 1.3 air km W Auraria, latitude 34.473267, longitude –84.037367, Lumpkin County, Georgia; GMNH 4066 (2 females, 43.0–53.7 mm SL) 18 July 2000, same locality as YPM ICH 017509; GMNH 2052 (1 male, 44.7 mm SL) 13 May 1990, Etowah River at GA 52, 7.2 air km W Dahlonega, latitude 34.53479, longitude –84.06315, Lumpkin County, Georgia; GMNH 4530 (2 males, 49.2–54.2 mm SL) 11 July 2001, Etowah River inside the Chattahoochee National Forest, 11.6 air km WNW Dahlonega, latitude 34.566357, longitude –84.098604, Lumpkin County, Georgia; YPM ICH 029874 (1 female, 47.9 mm SL) 12 July 2016, Etowah River at Hightower Church Road, latitude 34.594011, longitude –84.0778, Lumpkin County, Georgia; YPM ICH 030932 (1 female, 42.9 mm SL) 6 July 2017, Etowah River at lower crossing of Hightower Church Road, latitude 34.593071, longitude –84.077877, Lumpkin County, Georgia.

Diagnosis and description. A species of *Percina* as diagnosed by Page (1974) and the subclade *Chalinoperca*, a valid subgenus group name under the International Code of Zoological Nomenclature (ICZN 1999), as diagnosed in Near et al. (2011). Characters present in *Percina freemanorum* that are synapomorphies for *Chalinoperca* include a pre- and postorbital stripe that is continuous with a lateral stripe along the side of the body that is composed of a series of connected dark blotches of pigment (Figure 2A and B). In smaller adult males and many adult females the blotches are indistinct and the side of the body is covered with a jet-black midlateral stripe of near uniform width from the end of the opercles to the end of the caudal peduncle (Figure 2B). In both adults and juveniles the lateral stripe is continuous with a large triangular or irregularly shaped basicaudal spot that is brownish or black in color, set slightly below the midline, and extends onto the caudal fin rays. Two patches of white to cream-colored pigment flank the dorsal and ventral posterior margin of the basicaudal spot. There are two patches of dark pigment on the dorsal and ventral caudal fin base, with the dorsal patch more prominent and separated from the basicaudal

TABLE 2. Counts of lateral line scales in *Percina freemanorum* and *P. kusha*. *Abbreviations*: *N*, number of specimens; SD, standard deviation. The number of lateral line scales observed in the *P. freemanorum* holotype from Amicalola Creek is shown in bold and underlined.

		Number of lateral line scales																											
Species	**Drainage**	**57**	**58**	**59**	**60**	**61**	**62**	**63**	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**	**82**	***N***	**Mean**	**SD**
Percina freemanorum	Upper Etowah River	1						1	4	1	4	3	2	2	2	1	1		1	1								24	67.17	3.85
	Amicalola Creek		3	2	1	2	1	10	4	5	**3**	6	4		1			1										43	64.23	3.29
	Long Swamp Creek	1						1	1	1	3	2	3			1	2											15	66.67	3.77
	Total	2	3	2	1	2	1	12	9	7	10	11	9	2	3	2	3	1	1	1								82	65.54	3.77
Percina kusha	Conasauga River										2	2	6	8	9	11	8	14	4	8	3	4	2	3			1	85	72.26	3.27
	Coosawatte River															2	2		1	1								6	72.50	1.64
	Total										2	2	6	8	9	13	10	14	5	9	3	4	2	3			1	91	72.27	3.19

TABLE 3. Counts of transverse scale rows in *Percina freemanorum* and *P. kusha*. *Abbreviations*: *N*, number of specimens; SD, standard deviation. The number of transverse scale rows observed in the *P. freemanorum* holotype from Amicalola Creek is shown in bold and underlined.

		Number of transverse scale rows												
Species	**Drainage**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	***N***	**Mean**	**SD**
Percina freemanorum	Upper Etowah River	7	8	6	2	1						24	17.25	1.11
	Amicalola Creek	5	14	11	8	**5**						43	17.86	1.21
	Long Swamp Creek		1	1	5	4	3		1			15	19.73	1.44
	Total	12	23	18	15	10	3		1			82	18.02	1.48
Percina kusha	Conasauga River				6	14	26	27	6	4	2	85	21.39	1.31
	Coosawattee River					3	1	1	1			6	21.00	1.26
	Total				6	17	27	28	7	4	2	91	21.36	1.30

TABLE 4. Counts of scales above the lateral line in *Percina freemanorum* and *P. kusha*. *Abbreviations*: *N*, number of specimens; SD, standard deviation. The number of scales above the lateral line observed in the *P. freemanorum* holotype from Amicalola Creek is shown in bold and underlined.

		Number of scales above lateral line							
Species	**Drainage**	**5**	**6**	**7**	**8**	**9**	***N***	**Mean**	**SD**
Percina freemanorum	Upper Etowah River	1	14	9			24	6.33	0.56
	Amicalola Creek	7	22	**13**	1		43	6.19	0.73
	Long Swamp Creek	1	3	11			15	6.67	0.62
	Total	9	39	33	1		82	6.32	0.68
Percina kusha	Conasauga River		6	49	28	2	85	7.31	0.64
	Coosawattee River		1	3	2		6	7.17	0.75
	Total		7	52	30	2	91	7.30	0.64

spot in most individuals (Figure 2A and B). The dorsum is tan to light brown with irregular lines of brown to black pigment above the black lateral stripe that do not form blotches or saddles. The ventral area, consisting of the breast and belly, is immaculate cream to white in color. The posterior margin of scales below the midlateral stripe in nuptial condition males is peppered with melanophores (Figure 2A). The scales along the side of the body in nuptial condition males are irregularly covered with iridescent flecks that are most concentrated five to seven scale rows below the lateral line. There are iridescent scales on the postorbital area of the cheek and the margin of the opercles, which are more intense in nuptial condition males (Figure 2A). A suborbital bar is absent. Spines and rays of first and second dorsal fins are colored with alternating light black and white

TABLE 5. Counts of scales below the lateral line in *Percina freemanorum* and *P. kusha*. *Abbreviations*: *N*, number of specimens; SD, standard deviation. The number of scales below the lateral line observed in the *P. freemanorum* holotype from Amicalola Creek is shown in bold and underlined.

		Number of scales below lateral line									
Species	**Drainage**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	***N***	**Mean**	**SD**
Percina freemanorum	Upper Etowah River	5	6	12	1				24	9.38	0.88
	Amicalola Creek		16	13	**13**	1			43	9.98	0.89
	Long Swamp Creek		1	3	5	5	1		15	11.13	1.06
	Total	5	23	28	19	6	1		82	10.01	1.08
Percina kusha	Conasauga River			4	18	26	27	10	85	12.25	1.07
	Coosawattee River				2	4			6	11.67	0.52
	Total			4	20	30	27	10	91	12.21	1.05

TABLE 6. Counts of scale rows around the caudal peduncle in *Percina freemanorum* and *P. kusha*. *Abbreviations*: *N*, number of specimens; SD, standard deviation. The number of scales around the caudal peduncle observed in the *P. freemanorum* holotype from Amicalola Creek is shown in bold and underlined.

		Number of scales around caudal peduncle													
Species	**Drainage**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	***N***	**Mean**	**SD**
Percina freemanorum	Upper Etowah River	1	4	3	5	10	1						24	21.92	1.35
	Amicalola Creek	4	**5**	7	14	10	3						43	21.70	1.39
	Long Swamp Creek			2	2	6	3	1			1		15	23.27	1.71
	Total	5	9	12	21	26	7	1			1		82	22.05	1.54
Percina kusha	Conasauga River			1	5	11	19	23	13	11	1	1	85	24.79	1.55
	Coosawattee River							3		3			6	26.00	1.10
	Total			1	5	11	19	26	13	14	1	1	91	24.87	1.55

to cream-colored patches of pigment that give the appearance of a banding pattern in the fins. Dorsal fin membranes are mostly clear with a light peppering of black pigment; however, in nuptial condition males black pigment of moderate density in the fin membranes form a distal and basal band in the first dorsal fin and a basal band of pigment in the second dorsal fin (Figure 2A). The pectoral, pelvic, and anal fin rays are irregularly outlined with black melanophores; the fin membranes are mostly clear except in nuptial condition males in which the fin membranes are generously pigmented with dark melanophores.

The largest known specimen of *Percina freemanorum* is 70.5 mm SL (YPM ICH 029879). Modal scale counts are 63 lateral line scales (Table 2), 17 transverse scale rows (Table 3), 6 and 10 scale rows above and below the lateral line

TABLE 7. Counts of dorsal fin spines in *Percina freemanorum* and *P. kusha*. *Abbreviations*: *N*, number of specimens; SD, standard deviation. The number of dorsal fin spines observed in the *P. freemanorum* holotype from Amicalola Creek is shown in bold and underlined.

		Number of dorsal fin spines							
Species	**Drainage**	**11**	**12**	**13**	**14**	**15**	***N***	**Mean**	**SD**
Percina freemanorum	Upper Etowah River	4	7	11	2		24	12.46	0.88
	Amicalola Creek	1	10	**28**	4		43	12.81	0.63
	Long Swamp Creek	1	11	3			15	12.13	0.52
	Total	6	28	42	6		82	12.59	0.74
Percina kusha	Conasauga River	5	16	41	21	2	85	12.99	0.88
	Coosawattee River		1	5			6	12.83	0.41
	Total	5	17	46	21	2	91	12.98	0.86

TABLE 8. Counts of dorsal fin rays in *Percina freemanorum* and *P. kusha*. *Abbreviations*: *N*, number of specimens; SD, standard deviation. The number of dorsal fin rays observed in the *P. freemanorum* holotype from Amicalola Creek is shown in bold and underlined.

		Number of dorsal fin rays						
Species	**Drainage**	**9**	**10**	**11**	**12**	***N***	**Mean**	**SD**
Percina freemanorum	Upper Etowah River	2	10	12		24	10.42	0.65
	Amicalola Creek		9	**29**	5	43	10.91	0.57
	Long Swamp Creek		7	5	3	15	10.73	0.80
	Total	2	26	46	8	82	10.73	0.67
Percina kusha	Conasauga River		21	56	8	85	10.85	0.57
	Coosawattee River		2	4		6	10.67	0.52
	Total		23	60	8	91	10.84	0.56

(Tables 4 and 5), and 23 scales around the caudal peduncle (Table 6). Modal counts of fin elements are 13 first dorsal fin spines (Table 7), 11 second dorsal fin rays (Table 8), 2 anal fin spines, 9 anal fin rays (Table 9), and 14 pectoral fin rays (Table 10). There are six branchiostegal rays and the branchiostegal membrane is narrowly connected. A premaxillary frenum is present. The lateral line is complete and pored scales do not occur on the base of the caudal fin. Males with a row of 12 to 16 modified scales on the midline of the belly and nuptial tubercles are absent. The posterior margin of preopercle is without serrations. Opercles and cheeks are scaled. The nape is completely scaled in most specimens, but the anteriormost nape scales in some individuals are embedded.

There is variation in scale counts among the three disjunct populations of *Percina freemanorum*, with the average number of lateral line scales being lowest in the population from Amicalola Creek (Table 2). The population in Long Swamp Creek has slightly higher counts for transverse scale rows (Table 3), number of scales above the lateral line (Table 4), and number of scales around the caudal peduncle (Table 6). There is little to no variation in the numbers of fin elements among populations of *P. freemanorum* (Tables 7, 8, 9, and 10).

TABLE 9. Counts of anal fin rays in *Percina freemanorum* and *P. kusha*. *Abbreviations*: *N*, number of specimens; SD, standard deviation. The number of anal fin rays observed in the *P. freemanorum* holotype from Amicalola Creek is shown in bold and underlined.

		Number of anal fin rays						
Species	**Drainage**	**7**	**8**	**9**	**10**	***N***	**Mean**	**SD**
Percina freemanorum	Upper Etowah River		10	14		24	8.58	0.50
	Amicalola Creek		18	**25**		43	8.58	0.50
	Long Swamp Creek	2	4	8	1	15	8.53	0.83
	Total	2	32	47	1	82	8.57	0.57
Percina kusha	Conasauga River		22	62	1	85	8.75	0.46
	Coosawattee River		4	2		6	8.33	0.52
	Total		26	64	1	91	8.73	0.47

TABLE 10. Counts of pectoral fin rays in *Percina freemanorum* and *P. kusha*. *Abbreviations*: *N*, number of specimens; SD, standard deviation. The number of pectoral fin rays observed in the *P. freemanorum* holotype from Amicalola Creek is shown in bold and underlined.

		Number of pectoral fin rays						
Species	**Drainage**	**12**	**13**	**14**	**15**	***N***	**Mean**	**SD**
Percina freemanorum	Upper Etowah River		7	17		24	13.71	0.46
	Amicalola Creek	2	16	**24**	1	43	13.56	0.63
	Long Swamp Creek		1	14		15	13.93	0.26
	Total	2	24	55	1	82	13.67	0.55
Percina kusha	Conasauga River	3	56	26		85	13.27	0.52
	Coosawattee River		4	2		6	13.33	0.52
	Total	3	60	28		91	13.27	0.52

Comparisons. Morphologically, *Percina freemanorum* differs from *P. kusha* in having a lower average number of lateral line scales (65.54 vs. 72.27; Table 2), fewer transverse scale rows (18.02 vs. 21.36; Table 3), fewer scales above the lateral line (6.32 vs. 7.30; Table 4), fewer scales below the lateral line (10.01 vs. 12.21; Table 5), and fewer scales around the caudal peduncle (22.05 vs. 24.87; Table 6). *Percina freemanorum* has modally 14 rays in the pectoral fin compared with 13 in *P. kusha* (Table 10). The nape of *P. freemanorum* is completely scaled with exposed scales, whereas *P. kusha* has embedded scales on the anterior portion of the nape (Williams et al. 2007, tbl. 9). The two species are similar in coloration and pigmentation (Figures 2A–B and 4A).

Percina freemanorum differs from *P. sipsi* in the pigmentation of the midlateral stripe, which is more uniform in *P. freemanorum* and consists of a series of rectangular-shaped blotches as compared with a series of circular and oval-shaped pigment blotches in *P. sipsi* (Figure 4B). The dorsum of *P. freemanorum* is uniformly pigmented compared with the presence of weakly developed dorsal saddles in *P. sipsi*. The nape of *P. freemanorum* is scaled and the nape of *P. sipsi* is naked or partially scaled at the posterior portion of the nape (Williams et al. 2007). *Percina freemanorum* differs from *P. sipsi* in the modal number of fin elements with 11 versus 10 dorsal fin rays, 9 versus 8 anal fin rays, and 14 versus 13 pectoral fin rays (Tables 8, 9, and 10; Williams et al. 2007).

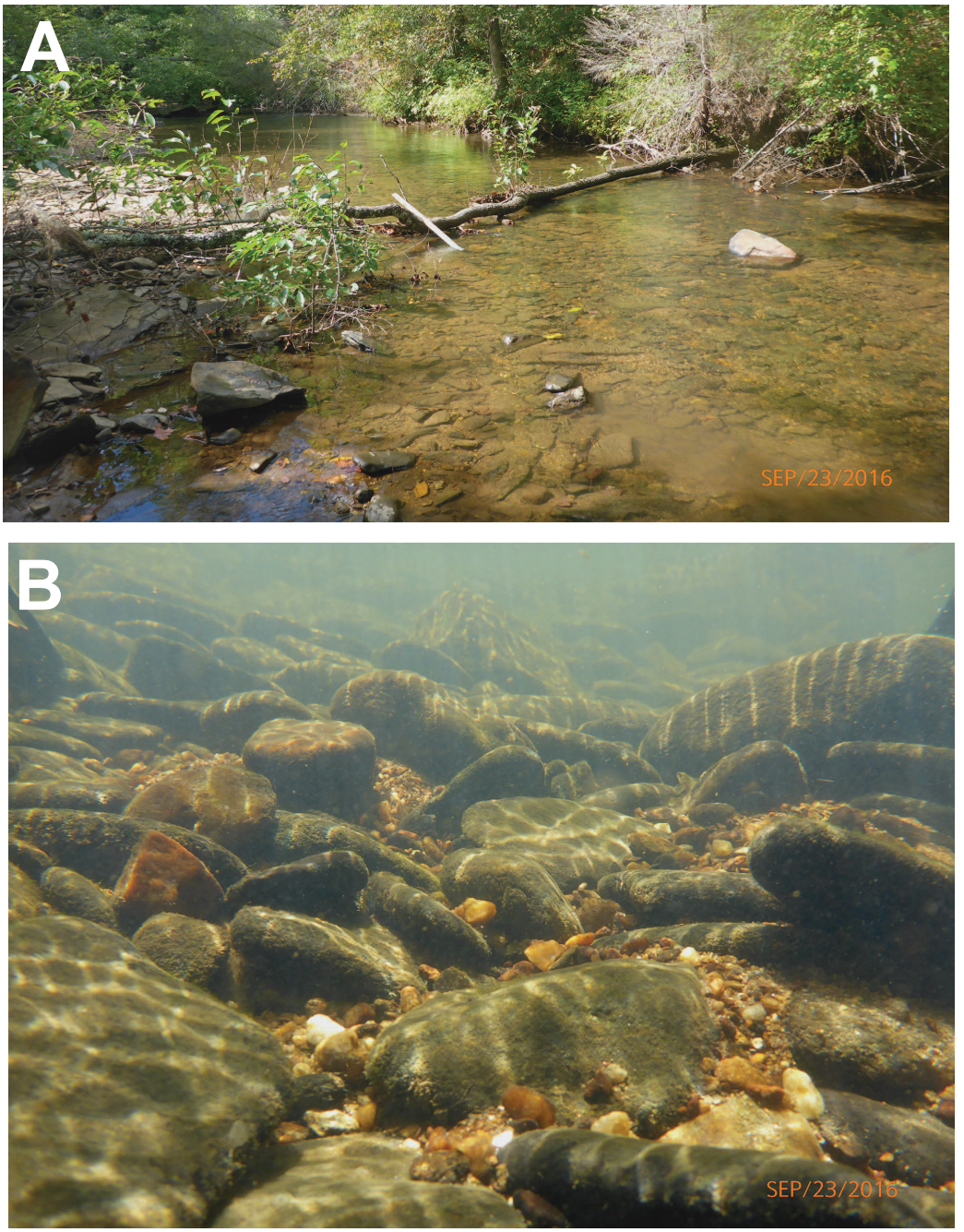

FIGURE 3. *Percina freemanorum* type locality. **A.** Amicalola Creek, latitude 34.49827, longitude –84.26679, Dawson County, Georgia, USA. **B**. Underwater, Amicalola Creek, latitude 34.49827, longitude –84.26679, Dawson County, Georgia, USA. Photographs by Georgia Department of Natural Resources.

Percina freemanorum and *P. smithvanizi* share similar patterns of pigmentation, but the marginal edges of the midlateral blotches in *P. freemanorum* have greater contact and cover an underlying midlateral line of pigmentation that is more apparent in *P. smithvanizi* (Figures 2A–B, and 4C). With regard to meristic traits, *P. freemanorum* differs from *P. smithvanizi* with modally 11 compared with 10 dorsal fin rays, 9 compared with 8 anal fin rays, and 14 compared with 13 pectoral fin rays (Tables 8, 9, and 10; Williams et al. 2007).

Percina kusha

Percina sipsi

Percina smithvanizi

FIGURE 4. Species of *Chalinoperca*. **A.** *Percina kusha* YPM ICH 017590, YFTC 10980, 58.9 mm SL male, Conasauga River, Polk County, Tennessee, USA, 4 September 2007. **B.** *Percina sipsi* YPM ICH 017052, YFTC 10861, 51.5 mm SL male, Sipsey Fork, Winston County, Alabama, USA, 28 July 2007. **C.** *Percina smithvanizi* YPM ICH 016700, YFTC 9124, 57.0 mm SL female, Muscadine Creek, Cleburne County, Alabama, USA, 26 July 2007.

Etymology. *Percina freemanorum* is named in honor of Mary C. Freeman and Byron (Bud) J. Freeman, who have made substantial contributions to the study of freshwater fishes in the southeastern United States. In particular, their work has shed light on and significantly aided in the conservation of the biodiverse rich Etowah River system.

Distribution and habitat. *Percina freemanorum* is limited to three populations in the upper portion of the Etowah River system in Dawson, Lumpkin, and Pickens Counties, Georgia (Figure 1). From west to east in the upper Etowah River system, *P. freemanorum* is found in the Long Swamp Creek system, the Amicalola Creek system, and the upper main stem of the Etowah River (Figure 1). In the Long Swamp Creek system, *P. freemanorum* occurs in a limited stretch of approximately 8 river km from the lower portion of Champion Creek downstream to the Georgia Highway 53 crossing of Long Swamp Creek near Tate, Georgia (Figure 1). In the Amicalola Creek system, *P. freemanorum* is distributed among a set of spatially proximate locations in Amicalola Creek and Little Amicalola Creek (Figure 1). In addition, there is a 1994 collection of a single small female specimen of *P. freemanorum* (UF 165646, 40.1 mm SL) from the Amicalola Creek tributary Cochrans Creek. In the main stem of the upper Etowah River, *P. freemanorum* is

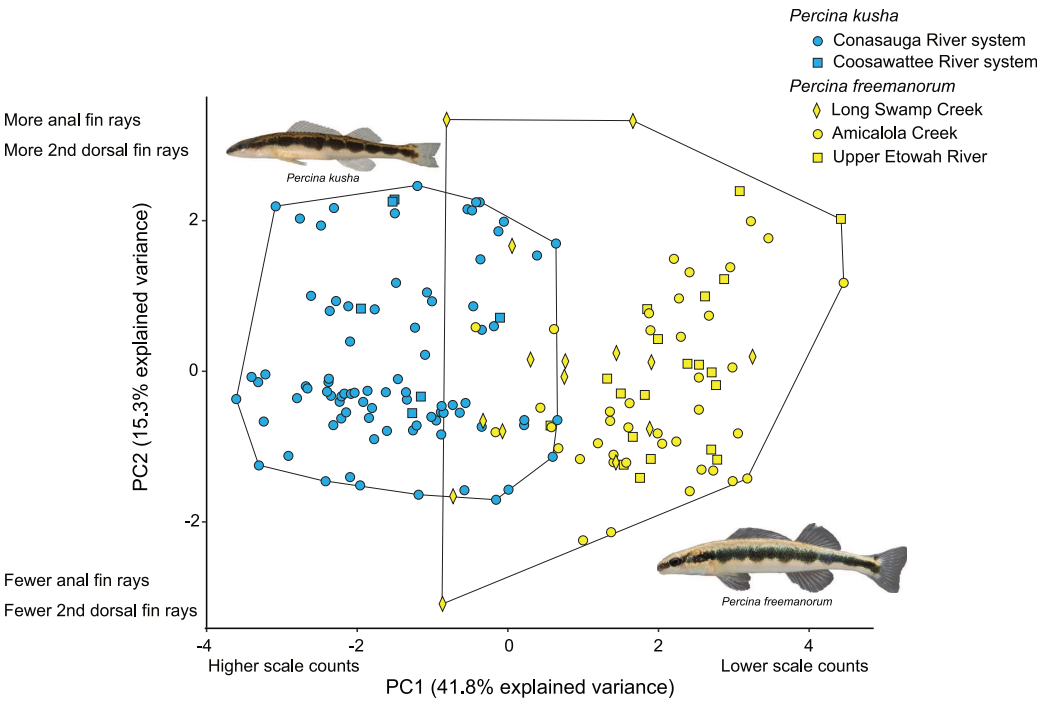

FIGURE 5. Plot of first and second principal component (PC) scores of meristic traits for *Percina kusha* and *P. freemanorum*.

distributed from the upper crossing of Hightower Church Road in Lumpkin County, Georgia, to the Castleberry Bridge Road crossing just west of Auraria, Georgia, in Lumpkin County. The earliest collection of *P. freemanorum* was made in 1948 (GMNH 3815) several kilometers downstream of the Castleberry Bridge Road location at the Georgia Highway 53 at Dougherty, Dawson County, Georgia. There are two single collections of *P. freemanorum* made in 1995 (GMNH 3799 and GMNH 5844) from the main stem of the upper Etowah River near the confluences of Shoal Creek and Amicalola Creek, respectively. It is not known whether there is a viable population of *P. freemanorum* in this portion of the upper Etowah River or if the presence at these localities reflect downstream dispersal between populations (Albanese et al. 2018).

Based on collection records and underwater observations using masks and snorkels, *Percina freemanorum* occupies small to medium-sized rivers spending much of the time off of the substrate. Preferred habitat in Long Swamp Creek, Amicalola Creek, and the upper portion of the Etowah River includes midwater areas of flowing runs and pools over substrates of bedrock, mixtures of sand and gravel, or small cobble (Figure 3A and B). We assessed microhabitat use of *P. freemanorum* during July through August 2016 at five sites in Amicalola Creek and the upper Etowah River by dropping a weighted flag at locations where 46 individual fish were observed while snorkeling. Depths ranged from 22 to 124 cm (mean 58 cm, SE = 4.1). Current velocities ranged from 0 to 43 cm/s (mean 15 cm/s, SE = 0.01). *Percina freemanorum* most frequently use portions of the stream with a sand substrate (43% of observations), followed by cobble (26%), and bedrock (17%). Other substrate types (e.g., silt, gravel, boulder) comprise less than 5% of the observations. Average stream width at these sites ranges from 5.8 to 18.9 m (mean = 11.8 m, SE = 1.2).

Our field observations are consistent with a quantitative analysis of habitat use in the closely related *Percina kusha* in the Conasauga River (Johnston, Kleiner, and Herrington 2002). In this study, Johnston et al. (2002) observe that spawning in *P. kusha* occurs from mid-May through at least the third week of June with water temperature ranging from 16 °C to 22 °C. Etnier and Starnes (1993:592) report the diet of *P. kusha* consists of small prey items dominated by baetid mayfly and blackfly larvae.

Conservation status. A species status assessment (SSA) for *Percina kusha* that included all known populations of *P. freemanorum* was completed by the United States Fish and Wildlife Service (USFWS) in response to a petition to list the species under the US Endangered Species Act (USFWS 2017a, 2017b). The SSA process involved compilation of all available collection data, targeted surveys and habitat assessments for all populations during 2016 and 2017, and examination of land cover and other watershed-level threats (USFWS 2017a; Albanese et al. 2018). The USFWS determined that listing of *P. kusha s.l.* was not warranted (USFWS 2017a); however, the SSA indicates that

TABLE 11. Principal component (PC) loadings for the first two PC axes for meristic traits in *Percina freemanorum* and *P. kusha*.

Trait	PC1	PC2
Lateral line scales	–0.39132609	0.03114870
Scales above lateral line	–0.40471640	–0.11242557
Scales below lateral line	–0.44868676	0.11420469
Transverse scales	–0.47635555	0.08574184
Scales around caudal peduncle	–0.40944392	0.17877871
Number of spines in first dorsal fin	–0.19585678	–0.03726958
Number of rays in second dorsal fin	–0.09660924	–0.65320508
Number of anal fin rays	–0.13191118	–0.68455137
Number of pectoral fin rays	0.14864726	–0.19328107

all populations of *P. kusha* and *P. freemanorum* have low resiliency to future environmental changes because of their limited distribution and low abundance and stressors to habitat quality (USFWS 2017b). The SSA also predicted the population of *P. freemanorum* in Long Swamp Creek and *P. kusha* in Talking Rock Creek (Coosawattee River system) will likely be extirpated under future scenarios of climate change and urbanization (USFWS 2017b).

It is important to evaluate the status of *Percina freemanorum* in the context of its small geographic range and the low probability of demographic support among populations (USFWS 2017b). The Long Swamp Creek population is at high risk of extirpation because of its limited spatial extent (about 8 river km), a low-head dam that fragments its distribution and prevents colonization of upstream habitats (latitude 34.46727, longitude –84.40011), and lack of habitat protection in the watershed. Collection efforts indicate a low census population size, with only four individuals captured during seven recent surveys targeting the species (Albanese et al. 2018). In contrast, the Amicalola Creek population occupies a slightly greater extent of higher quality habitat (about 10 river km), has a relatively high proportion of conservation land in its watershed (about 35%), and is not fragmented by any dams within reaches occupied by *P. freemanorum*. Snorkeling is the most effective method for assessing this population owing to water clarity, with recent surveys documenting more than 20 individuals within single stream reaches. Similarly, the upper Etowah River population occupies about 15 river km of high-quality habitat with conservation lands protecting headwater reaches (about 30% of watershed). The upper Etowah also supports relatively large populations, with recent snorkeling observations documenting up to 30 individuals within a small stretch of stream. Although the upper Etowah and Amicalola Creek populations appear currently stable, these populations warrant additional land protection efforts and monitoring (Albanese 2008).

Analysis of meristic trait variation. The first two PCs account for 57.1% of the variance among the meristic traits (Figure 5). All of the scale count traits load heavily on PC1 and the number of anal fin rays and second dorsal fin rays load most heavily on PC2 (Table 11; Figure 5). Plotting PC2 in comparison with PC1 reveals clustering of specimens from each of the two species that reflects their phenotypic disparity and morphological distinctiveness (Figure 5). There is overlap between *Percina freemanorum* and *P. kusha* in the bivariate PC plot that is consistent with both the limited overlap in meristic traits that exhibit the greatest divergence between the two species and the overlap in bivariate PC plots between closely related species of darters (e.g., Mayden 2010, fig. 4; Layman and Mayden 2012, fig. 21; Kozal et al. 2017, fig. 7; Near et al. 2017, fig. 8).

The LDA correctly classified 88.5% (69 of 78) of the specimens of *Percina freemanorum* and 96.7% (88 of 91) of the specimens of *P. kusha*. The results of the LDA and the PC analysis demonstrate that meristic traits provide strong resolution in delimiting the two species.

Geographic interpolation of the average number of lateral line scales, transverse scales, and pectoral fin rays shows a distinct break between *Percina freemanorum* and *P. kusha* (Figure 6). The variation between the two species is not clinal, as demonstrated within other darter species (Near et al. 2016, fig. 5). In addition, there is no observed geographically structured intraspecific variation in these traits.

Analyses of mtDNA variation. Bayesian phylogenetic analysis of the mtDNA *cytb* gene resolves a clade containing all sampled specimens of *Percina freemanorum* and *P. kusha*, but relationships within this clade are unresolved (Figure 7). The lack of reciprocal monophyly in both *P. freemanorum* and *P. kusha* in the mtDNA gene tree is reflected in the haplotype network where there are fewer DNA site differences between *P. freemanorum* from Amicalola Creek and *P. kusha* from the Coosawattee River system than the Coosawattee *P. kusha* population and the most common haplotype from *P. kusha* in the Conasauga River system (Figure 7). While genetic divergence among the six mtDNA haplotypes is relatively shallow, no haplotypes are shared between the two species.

ddRADseq phylogeny and population structure. Removing low-quality reads and filtering for adapter contamination results in retention of on average 3.3 million reads per specimen (SD = 2.6 million reads). All specimens retained at least 100,000 reads.

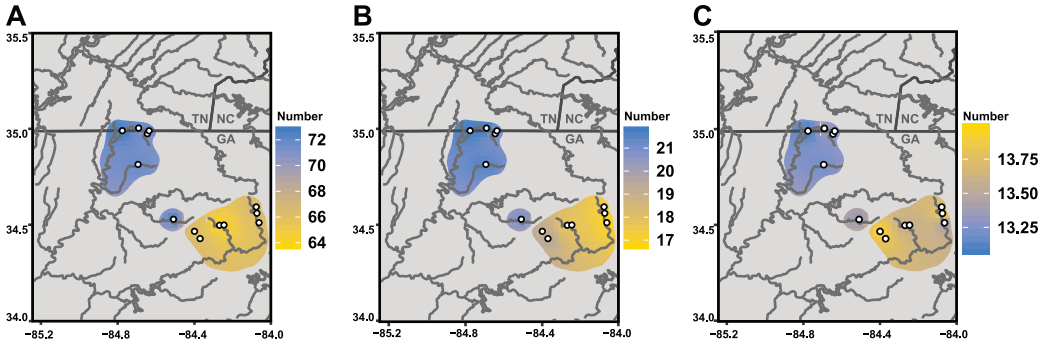

FIGURE 6. Geographic interpolation of meristic traits in *Percina kusha* (blue) and *P. freemanorum* (yellow). **A.** Number of lateral line scales. **B.** Number of transverse scales. **C.** Number of pectoral fin rays.

After clustering and exclusion of loci with less than 6 × coverage, there is 20 × coverage on average per locus across all specimens. The total number of loci in the alignments varies depending on the filtering threshold for missing data (Table 12). The largest and most sparse alignment with about 50% missing sites is approximately 14 million base pairs in length, whereas the smallest and least sparse alignment with about 10% missing sites is approximately 42,000 base pairs in length (Table 12).

All ddRAD phylogenies resolve *Percina kusha* and *P. freemanorum* as a clade. Most of the phylogenies also resolve *P. kusha* and *P. freemanorum* as reciprocally monophyletic except for the smallest dataset with less than 10% missing samples per locus (Table 12). This alignment contains only 469 ddRAD loci and, similarly to the mtDNA, appears to have low power to infer the recent history of *P. freemanorum* and *P. kusha* diversification. In most phylogenies, specimens of *P. freemanorum* from Amicalola Creek, Long Swamp Creek, and the upper Etowah River resolve as reciprocally monophyletic; however, there is topological incongruence among the phylogenies inferred from the different alignments. Populations from Long Swamp Creek and the upper Etowah are resolved as sister lineages in most phylogenies, though not always with strong support. However, the 20% maximum missing data alignment resolves the Amicalola Creek and Long Swamp Creek populations as a clade, with a single individual from Long Swamp Creek rendering that population as non-monophyletic. Within *P. kusha*, the specimens sampled from the Conasauga and Coosawattee River systems are each reciprocally monophyletic with high bootstrap node support (Figure 8A).

We performed population structure analyses using five different sets of SNPs ranging from 50% missing data (27,643 biallelic SNPs) to 10% missing data (240 biallelic SNPs). The cross-entropy plots exhibit an "elbow" at $K = 2$ (50% and 40% missing data) or at $K = 3$ (30%, 20%, and 10% missing data). Thus, two or three genetic clusters are optimal to describe the data.

At $K = 2$, the inferred genetic clusters correspond largely to the two species, *Percina freemanorum* and *P. kusha* (Figure 8B). The two genetic clusters do not have a sharp break, with some specimens from both species exhibiting heterogeneous genomic ancestry. However, there is no clear spatial pattern to the individuals with a heterogeneous genomic ancestry (Figure 8B). Within *P. freemanorum*, the most heterogeneous individuals are found in the upper Etowah River, which is also the population furthest from any populations of *P. kusha.* Within *P. kusha*, heterogeneous individuals are found in both of the populations from the Conasauga and Coosawattee Rivers. Qualitatively, the degree of genomic heterogeneity increases in datasets with a higher proportion of missing data.

The results of the genetic clustering analyses recapitulate the patterns of geographic structure in the ddRAD phylogeny (Figures 8A and B). Because all datasets show the largest decrease in cross-entropy from $K = 1$ to $K = 2$, we choose to focus our discussion primarily on $K = 2$. However, there is additional population structure at higher K values. At $K = 3$, most of the analyses infer two genetic clusters within *Percina freemanorum* that correspond to specimens sampled from Amicalola Creek as compared with those from Long Swamp Creek and the upper Etowah River. Surprisingly, this result does not match geography as Amicalola Creek is situated between the upper Etowah River and Long Swamp Creek. At higher values of K, other populations from the Coosawattee River and the Conasauga River tributary Holly Creek form distinct genetic clusters.

Phylogeny of *Chalinoperca* inferred from Sanger sequenced nuclear genes. Bayesian phylogenetic analysis of DNA sequences sampled from 11 nuclear genes strongly resolves *Chalinoperca*, including *Percina sipsi*, as a clade with a posterior probability of 1.0 (Figure 9). Near et al. (2011) recognizes *Chalinoperca* as a subclade of *Hadropterus,* and this relationship is reflected in the nuclear gene inferred phylogeny. In particular the clade comprising *P. sciera* and *P. aurolineata* is resolved as the sister lineage of *Chalinoperca* (Figure 8). Within *Chalinoperca*, *P. sipsi,* and *P. smithvanizi* are resolved as sister species, and this clade is the sister lineage to the clade containing *P. freemanorum* and *P. kusha* (Figure 8).

Discussion

When the bridled darters, *Percina kusha*, *P. sipsi*, and *P. smithvanizi*, were described, Williams et al. (2007) noted the substantial disparity in meristic

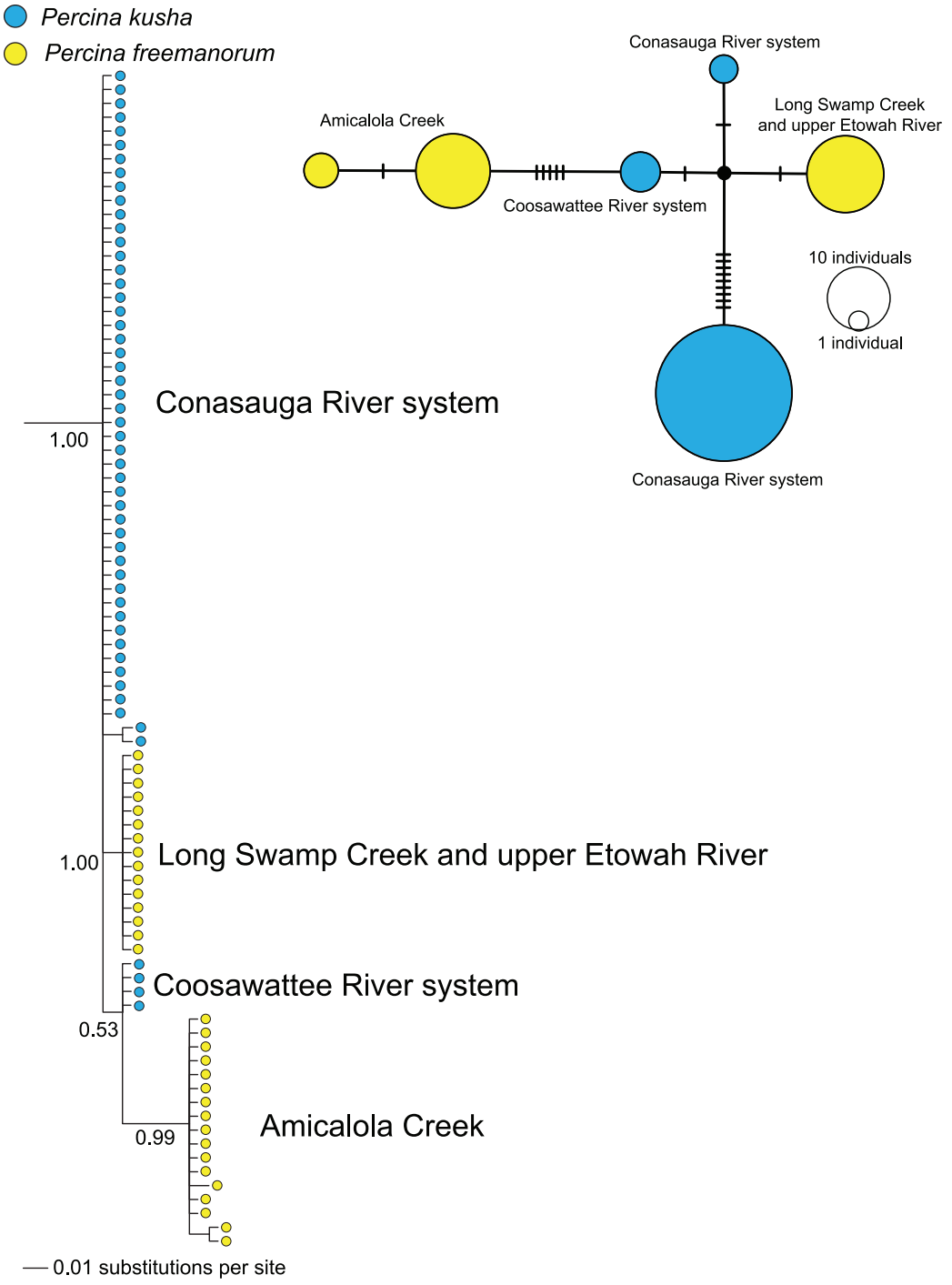

FIGURE 7. Bayesian inferred mitochondrial DNA gene tree and mtDNA haplotype network for *Percina kusha* and *P. freemanorum*. Numbers at nodes in the phylogeny are Bayesian posterior support values. The outgroup species, *P. palmaris*, is not shown in the phylogeny.

TABLE 12. Statistics on concatenated ddRADseq datasets for *Percina freemanorum* and *P. kusha*.

Maximum percentage of missing samples per locus	Number of loci	Parsimony variable sites	Alignment informative sites	Length (bp)	Percentage of missing sites in alignment
80	158,791	406,890	222,445	14,065,976	50
50	82,597	226,762	136,993	7,333,439	37
40	51,228	143,061	88,550	4,554,584	31
30	27,664	79,266	50,194	2,460,084	25
20	8,638	25,932	16,796	767,900	19
10	469	1,457	973	41,681	10

traits between *P. kusha* populations in the Conasauga River system and populations we delimit here as *P. freemanorum* in the Etowah River system. This variation was interpreted as intraspecific and justified by the observation that "mitochondrial DNA revealed very little differentiation between these populations, although they were recovered as reciprocally monophyletic groups" (Williams et al. 2007:9). Our analyses of mtDNA variation do not resolve the two species as reciprocally monophyletic; however, no mtDNA haplotypes are shared between the two species (Figure 5). More generally, no specific magnitude of mtDNA genetic divergence is cited in Williams et al. (2007) as an appropriate operational criterion for delimiting species. We hypothesize that the shared alleles driving the pattern of genomic heterogeneity is a result of unsorted ancestral polymorphism and not admixture from secondary contact and introgression (Figure 8B). The two species have only recently diverged with relaxed molecular clock age estimates for the common ancestry of *P. freemanorum* and *P. kusha* at approximately 350,000 years (Near et al. 2011). Following a unified general lineage concept of species (de Queiroz 1998, 2007), we treat the conceptualization of *P. freemanorum* and *P. kusha* as separately evolving metapopulation lineages as the only necessary property for the recognition of each as a distinct species. The lines of evidence offered as operational criteria that delimit the two closely related species includes disparity in meristic traits (Tables 2, 3, 4, 5, 6, and 10; Figures 5 and 6), the non-sharing of mtDNA haplotypes (Figure 7), and reciprocal monophyly of both *P. freemanorum* and *P. kusha* in phylogenomic analyses of ddRADseq loci (Figure 8A).

Along with *Etheostoma scotti* and *Nothontous etowahae* (Wood and Mayden 1993; Bauer et al. 1995), *Percina freemanorum* is one of at least three species of darters endemic to the upper Etowah River system. *E. brevirostrum* is distributed in the Coosa River system, specifically the Choccolocco Creek system, the Conasauga River system, tributaries of the Coosawattee River, and the upper Etowah River system. Citing a personal comment, Boschung and Mayden (2004:516) state there are multiple undescribed species masquerading as *E. brevirostrum*, which includes two distinct and undescribed species in Amicalola Creek and the mainstream of the upper Etowah River (Burkhead et al. 1997; Freeman et al. 2005; Jelks et al. 2008:414; Anderson et al. 2012). In addition to the undescribed diversity in *E. brevirostrum*, analyses of mtDNA gene sequences resolve three distinct lineages of *E. scotti* that correspond to color and pigmentation traits in nuptial males that are allopatrically distributed in the upper Etowah system (Storey 2003). The pattern of speciation involving the Etowah River system observed in several other darter lineages is consistent with the recognition of *P. freemanorum* as a distinct species.

In addition to delimiting *Percina freemanorum* as a distinct species, our study provides important conclusions regarding the taxonomy of *P. kusha* in the Coosawattee River system. The range map of *P. kusha* presented in Williams et al. (2007, fig. 3) does not include records for the species in the Coosawattee River system, despite their presence in a range map published 11 years before their study (Mettee, O'Neil, and Pierson 1996:728). When we initiated our investigation, there were two museum collections of *P. kusha*

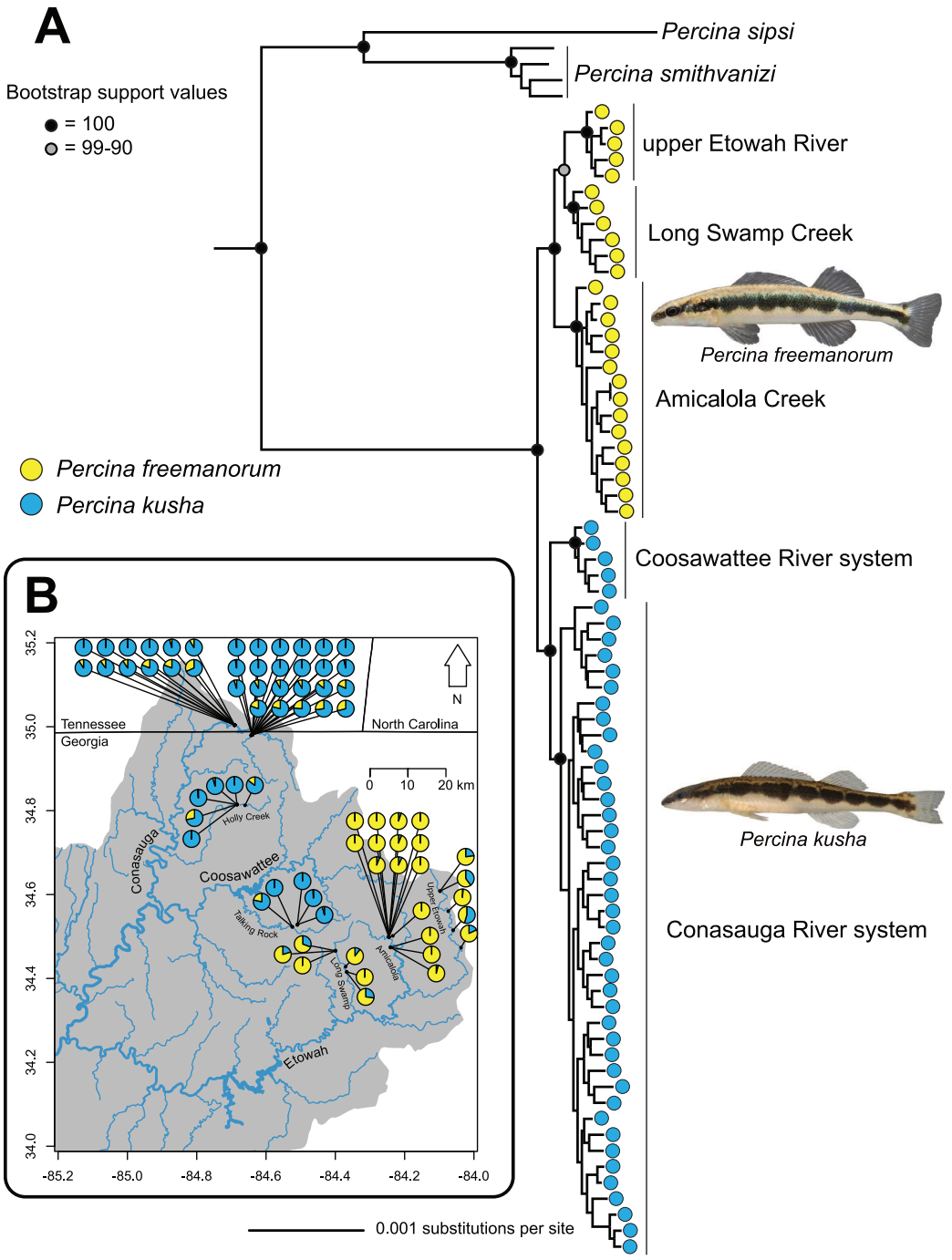

FIGURE 8. ddRAD analyses. **A.** Maximum likelihood inferred phylogeny of *Percina kusha* and *P. freemanorum* using a concatenated ddRAD dataset comprising 82,597 loci and 50% missing data. Bootstrap support for nodes are indicated with black (100%) or gray (90%–99%) filled circles. The outgroup species, *P. palmaris*, is not shown in the phylogeny. **B.** Ancestry coefficients using sparse non-negative matrix factorization for $K = 2$ with a minimum 90% complete single nucleotide polymorphism matrix. Pie chart colors represent estimated genomic ancestry. Each pie chart is an individual specimen; localities are indicated by small black dots. Gray region on the map covers the Coosa River system.

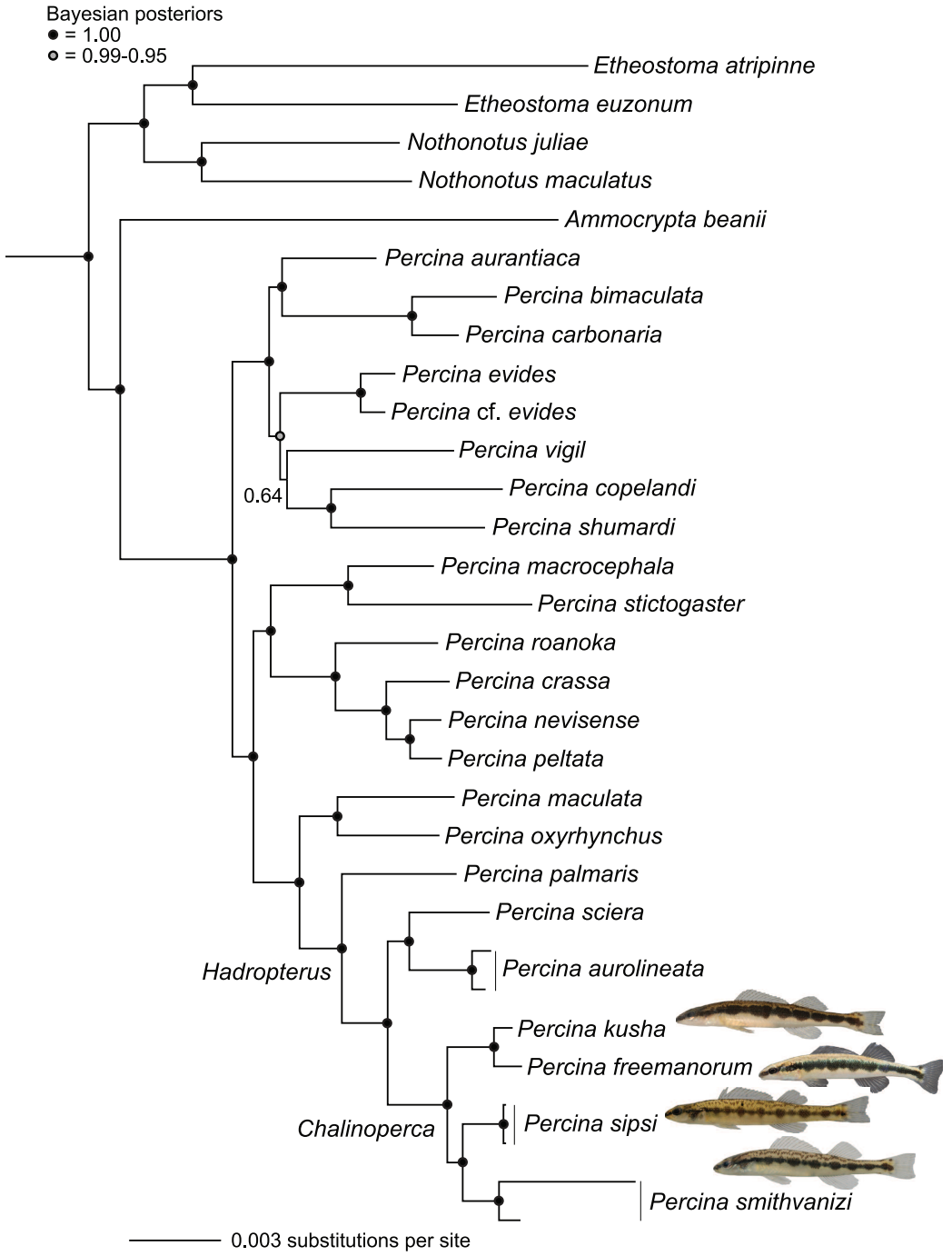

FIGURE 9. Phylogeny of *Percina* and species of *Chalinoperca* inferred from Bayesian analysis of 11 Sanger sequenced nuclear genes. The outgroup species, *Perca flavescens* and *Sander vitreus*, are not shown in the phylogeny. Bayesian posterior node support values are indicated with black (1.00) or gray (0.95–0.99) filled circles. Node support values less than 0.90 are indicated numerically.

from the Talking Rock Creek system, one from Talking Rock Creek (TU 204429, ex TU 38351) and the other from Talona Creek (GMNH 3383). Our fieldwork in 2007 resulted in four specimens, two from Talking Rock Creek (YPM ICH 018251 and YPM ICH 018321) and the other two from Talona Creek (YPM ICH 018239), but a survey by the Georgia Department of Natural Resources in 2016 and 2017 did not capture or observe any individuals in either creek. Morphologic and molecular data delimits the Coosawattee population as *P. kusha*; however, the results from recent field surveys are reflected in the rather grim prediction that this population faces a serious threat of extirpation (USFWS 2017b). Analyses of both mtDNA and nuclear ddRAD loci show the two populations of *P. kusha* in the Coosawattee and Conasauga are genetically distinct, presumably precluding any efforts of reintroduction of the species into the Coosawattee using a Conasauga source population (George et al. 2009). Analysis of genetic variation within *P. kusha*, specifically assessing if there is historic gene flow between these populations, will provide a greater perspective on the role of genomics in aiding the conservation of the imperiled population of *P. kusha* in the Coosawattee River system.

Inferring the phylogenetic relationships of the bridled darter species, *Chalinoperca*, using mtDNA is complicated by introgression with other species of *Percina*. Mitochondrial gene trees strongly resolve a clade containing *P. smithvanizi*, *P. kusha*, and *P. freemanorum*; however, *P. sipsi* is nested in a clade containing five specimens of *P. sciera* (Williams et al. 2007). *Percina sipsi* and *P. sciera* are syntopic in the Sipsey Fork of the Black Warrior River system and the non-monophyly of *Chalinoperca* appears the result of mtDNA introgression from *P. sciera* to *P. sipsi* (Williams et al. 2007; Near et al. 2011, tbl. 4). Our analyses of 11 Sanger sequenced nuclear genes resolves *Chalinoperca* as a monophyletic group. The clade and subgenus definition of *Chalinoperca* as the "least inclusive clade containing *P. kusha* Williams & Burkhead 2007 and *P. smithvanizi* Williams & Walsh 2007" (Near et al. 2011:592) does not require modification on the basis of the new phylogenetic analysis, but the composition of *Chalinoperca* is modified to include *P. sipsi* in addition to *P. smithvanizi*, *P. kusha*, and *P. freemanorum*.

In addition to resolving *Chalinoperca* as monophyletic, the 11 nuclear gene phylogenetic analysis also identifies *Percina smithvanizi* and *P. sipsi* as sister species (Figure 9). *Percina smithvanizi* is distributed in the Tallapoosa River system. Therefore, it was expected that it would be related to the clade containing *P. freemanorum* and *P. kusha* that is collectively distributed in the adjacent Coosa River system. Contrary to this expectation, *P. smithvanizi* is the sister species of the non-adjacently distributed *P. sipsi*, which is endemic to the Sipsey Fork of the Black Warrior River system (Figure 9).

The description of *Percina freemanorum* provides a prospectus for the continued discovery and delimitation of new species of darters. There were 129 recognized species of darters when Page (1983) published the important monograph detailing the diversity, distribution, and biology of darters. With the description of *P. freemanorum*, there are now 228 darter species, a growth of more than 43%. An average of 2.7 new species of darters have been introduced every year since 1983, with 15 new species over the last ten years. We estimate there are approximately 30 undescribed species of darters, and many of these are well known among ichthyologists working on North American freshwater fishes (e.g., Clabaugh et al. 1996; Ceas and Burr 2002; Boschung, Mayden, and Tomelleri 2004:509–510, 515–516; Hollingsworth and Near 2009; Near et al. 2011, tbl. 1). The continued discovery and description of the species that comprise the rich eastern North American freshwater fish fauna is enabled by access to an unparalleled database of museum collections that document species distributions and provide specimens for phenotypic and molecular analyses. We anticipate the last push to describe all species of North American freshwater fishes is imminent, and the optimal approach to this important work will involve a combination of morphological and genetic analyses as deployed frequently over the past 10 years (Mayden 2010; Powers, Kuhajda, and Ahlbrand 2012; Keck and Near 2013; Robison et al. 2014; Near and Thomas 2015; Kozal et al. 2017; Near et al. 2017).

Acknowledgments

We thank Gregory Watkins-Colwell for support with managing collections and securing

appropriate collecting permits from various state agencies. Z. Abouhamdan, C.M. Bossu, A. Cressler, R. Carlson, P. Dimmick, K. Heckman, P.R. Hollingsworth, L. Kruse, A.M. Near, P. Marcinek, A. Popp, M. Rowe, and D. Weiler provided assistance in field work. A. Ghezelayagh provided assistance in the laboratory and preliminary data analysis. We thank J. Parris (UT), L. Page and R. Robins (UF), H. Bart and J. Mann (TU), B. Freeman (GMNH), and C. Dillman (CUVM) for loans of specimens under their care. The US Fish and Wildlife Service funded this project through a Section 6 grant administered by the Georgia Department of Natural Resources. Financial support was also provided from the Bingham Oceanographic Fund of the Peabody Museum of Natural History, Yale University. J. Williams and an anonymous reviewer provided comments on the manuscript. "Talona Creek Steve" provided historical and cultural aspects of several tributaries in the Coosawattee River system.

Received 17 June 2020; revised and accepted 10 September 2020.

Appendix

Specimens of *Percina kusha* Included in Morphological Analyses

Numbers in parentheses indicate numbers of specimens examined.

Percina kusha. **Conasauga River system, Tennessee:** Conasauga River, Polk County: UAIC 12834.01 (1), YPM ICH 017590 (10), YPM ICH 020139 (1), YPM ICH 020141 (5), YPM ICH 020143 (10), YPM ICH 027077 (2), YPM ICH 029870 (2), YPM ICH 020136 (1), YPM ICH 020142 (10); Conasauga River, Bradley County: CUMV 69883 (3), TU 58965 (3), TU 65939 (7), UF 42757 (2), UT 91.6298 (7), YPM ICH 020137 (7), UF 22793 (2); Minnewauga Creek, Polk County: UF 165734 (4). **Conasauga River system, Georgia:** Conasauga River, Murray County: UF 110286 (1), UF 165704 (6); Holly Creek, Murray County: YPM ICH 029873 (5), YPM ICH 029871 (2). **Coosawattee River system, Georgia:** Talking Rock Creek, Pickens County: TU 204429 (1), YPM ICH 018251 (1), YPM ICH 018321 (1); Talona Creek, Pickens County: GMNH 3383 (1), YPM ICH 018239 (2).

Literature Cited

Albanese, B. 2008. Species profile for *Percina kusha* Williams and Burkhead. Georgia Biodiversity Portal, Georgia Wildlife Resources Division, Wildlife Conservation Section, Social Circle. https://georgiabiodiversity.a2hosted.com/natels/profile?es_id=32024

Albanese, B., Z. Abouhamdan, P. Marcinek, P. Dimmick, and D. Weiler. 2018. Conservation status of the bridled darter, holiday darter, trispot darter, and frecklebelly madtom. Social Circle, GA: Georgia Department of Natural Resources, Wildlife Conservation Section. 59 pp.

Anderson, G.B., M.C. Freeman, M.M. Hagler, and B.J. Freeman. 2012. Occupancy modeling and estimation of the holiday darter species complex within the Etowah River system. Transactions of the American Fisheries Society 141(1):34–45.

Babraham Informatics. 2019. FastQC. Version 0.11.9. Cambridge, UK: Babraham Institute. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Bandelt, H.J., P. Forster, and A. Roehl. 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16(1):37–48.

Bauer, B.H., D.A. Etnier, N.M. Burkhead, J.S. Ramsey, G.W. Hubbs, J.S. Burke, and L.J. Davenport. 1995. *Etheostoma* (*Ulocentra*) *scotti* (Osteichthyes, Percidae): a new darter from the Etowah River system in Georgia. Bulletin of the Alabama Museum of Natural History 17:1–16.

Boschung, H.T., and R.L. Mayden. 2004. Fishes of Alabama. Washington, DC: Smithsonian Books. 736 pp.

Bryant, R.T., B.H. Bauer, M.G. Ryon, and W.C. Starnes. 1979. Distributional notes on fishes from northern Georgia with comments on the status of rare species. Southeastern Fishes Council Proceedings 1(8):1–4.

Burkhead, N.M., S.J. Walsh, B.J. Freeman, and J.D. Williams. 1997. Status and restoration of the Etowah River, an imperiled southern Appalachian ecosystem. In: G.W. Benz and D.E. Collins, eds. Aquatic Fauna in Peril: The Southeastern Perspective. Decatur, GA: Lenz Design & Communications. pp. 375–444. (Southeast Aquatic Research Institute Special Publication 1.)

Ceas, P.A., and B.M. Burr. 2002. *Etheostoma lawrencei*, a new species of darter in the *E. spectabile* species complex (Percidae: subgenus *Oligocephalus*), from Kentucky and Tennessee. Ichthyological Exploration of Freshwaters 13(3): 203–216.

Chow, S., and K. Hazama. 1998. Universal PCR primers for S7 ribosomal protein gene introns in fish. Molecular Ecology 7(9):1255–1256.

Clabaugh, J.P., K.E. Knott, R.M. Wood, and R.L. Mayden. 1996. Systematics and biogeography of snubnose darters, genus *Etheostoma* (Teleostei: Percidae) from the Black Warrior River system, Alabama. Biochemical Systematics and Ecology 24(2):119–134.

Danecek, P., A. Auton, G. Abecasis, C.A. Albers, E. Banks, M.A. DePristo, R.E. Handsaker, G. Lunter, G.T. Marth, S.T. Sherry, et al. 2011. The variant call format and VCFtools. Bioinformatics 27(15):2156–2158.

de Queiroz, K. 1998. The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. In:

D.J. Howard and S.H. Berlocher, eds. Endless Forms: Species and Speciation. New York: Oxford University Press. pp. 57–75.

—2007. Species concepts and species delimitation. Systematic Biology 56(6):879–886.

Drummond, A.J., and A. Rambaut. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7(1):214.

Dycus, D.L., and W.M. Howell. 1974. Fishes of the Bankhead National Forest of Alabama. Montgomery: Alabama Department of Conservation and Natural Resources. 51 pp.

Eaton, D.A.R., I. Overcast, and R. Schwartz. 2020. ipyrad: Interactive assembly and analysis of RADseq datasets. Bioinformatics 36(8):2592–2594.

Etnier, D.A., and W.C. Starnes. 1993. The Fishes of Tennessee. Knoxville: University of Tennessee Press. 681 pp.

Freeman, M.C., E.R. Irwin, N.M. Burkhead, B.J. Freeman, and H.L. Bart Jr. 2005. Status and conservation of the fish fauna of the Alabama River system. In: J.N. Rinne, R.M. Hughes, and B. Calamusso, eds. Historical Changes in Large River Fish Assemblages of the Americas. Bethesda, MD: American Fisheries Society. pp. 557–585. (American Fisheries Society Symposium 45.)

Frichot, E., and O. François. 2015. LEA: An R package for landscape and ecological association studies. Methods in Ecology and Evolution 6(8):925–929.

Frichot, E., F. Mathieu, T. Trouillon, G. Bouchard, and O. François. 2014. Fast and Efficient Estimation of Individual Ancestry Coefficients. Genetics 196(4):973–983.

George, A.L., B.R. Kuhajda, J.D. Williams, M.A. Cantrell, P.L. Rakes, and J.R. Shute. 2009. Guidelines for propagation and translocation for freshwater fish conservation. Fisheries 34(11):529–545.

Hoang, D.T., O. Chernomor, A. von Haeseler, B.Q. Minh and L.S. Vinh. 2018. UFBoot2: improving the ultrafast bootstrap approximation. Molecular Biology and Evolution 35(2):518–522.

Hollingsworth, P.R., and T.J. Near. 2009. Temporal patterns of diversification and microendemism in eastern highland endemic barcheek darters (Percidae: Etheostomatinae). Evolution 63(1):228–243.

Hubbs, C.L., and K.F. Lagler. 1958. Fishes of the Great Lakes Region. Ann Arbor: University of Michigan. 213 pp.

Huelsenbeck, J.P., F. Ronquist, R. Nielsen, and J.P. Bollback. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294(5550):2310–2314.

Jelks, H.J., S.J. Walsh, N.M. Burkhead, S. Contreras-Balderas, E. Diáz-Pardo, D.A. Hendrickson, J. Lyons, N. E. Mandrak, F. McCormick, J. S. Nelson, et al. 2008. Conservation status of imperiled North American freshwater and diadromous fishes. Fisheries 33(8):372–407.

[ICZN] International Commission on Zoological Nomenclature. 1999. International Code of Zoological Nomenclature. 4th ed. London: International Trust for Zoological Nomenclature. 306 pp.

Johnston, C.E., K.J. Kleiner, and S.J. Herrington. 2002. Seasonal, diel and spawning habitat of the rare muscadine darter (*Percina* sp.) in the Conasauga River, Georgia. Southeastern Fishes Council Proceedings 1(44):1–5.

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, et al. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12):1647–1649.

Keck, B.P., and T.J. Near. 2010. Geographic and temporal aspects of mitochondrial replacement in *Nothonotus* darters (Teleostei: Percidae: Etheostomatinae). Evolution 64(5): 1410–1428.

—2013. A new species of *Nothonotus* darter (Teleostei: Percidae) from the Caney Fork in Tennessee, USA. Bulletin of the Peabody Museum of Natural History 54(1):3–21.

Kozal, L.C., J.W. Simmons, J.M. Mollish, D.J. MacGuigan, E. Benavides, B.P. Keck, and T.J. Near. 2017. Phylogenetic and morphological diversity of the *Etheostoma zonistium* species complex with the description of a new species endemic to the Cumberland Plateau of Alabama. Bulletin of the Peabody Museum of Natural History 58(2):263–286.

Lanfear, R., B. Calcott, S.Y.W. Ho, and S. Guindon. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Molecular Biology and Evolution 29(6):1695–1701.

Larget, B., and D.L. Simon. 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. Molecular Biology and Evolution 16(6):750–759.

Layman, S.R., and R.L. Mayden. 2012. Morphological diversity and phylogenetics of the darter subgenus *Doration* (Percidae: *Etheostoma*), with descriptions of five new species. Bulletin of the Alabama Museum of Natural History 30:1–83.

Li, C., G. Ortí, G. Zhang, and G. Lu. 2007. A practical approach to phylogenomics: the phylogeny of ray-finned fish (Actinopterygii) as a case study. BMC Evolutionary Biology 7(1):44.

Li, C., B.R. Ricardo, W.L. Smith, and G. Ortí. 2011. Monophyly and interrelationships of snook and barramundi (Centropomidae *sensu* Greenwood) and five new markers for fish phylogenetics. Molecular Phylogenetics and Evolution 60(3):463–471.

Linck, E., and C.J. Battey. 2019. Minor allele frequency thresholds strongly affect population structure inference with genomic data sets. Molecular Ecology Resources 19(3):639–647.

Lopez, J.A., W.J. Chen, and G. Ortí. 2004. Esociform phylogeny. Copeia 2004(3):449–464.

MacGuigan, D.J., and T.J. Near. 2019. Phylogenomic signatures of ancient introgression in a rogue lineage of darters (Teleostei: Percidae). Systematic Biology 68(2):329–346.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17(1):10–12.

Mayden, R.L. 2010. Systematics of the *Etheostoma punctulatum* species group (Teleostei: Percidae), with descriptions of two new species. Copeia 2010(4):716–734.

Mettee, M.F. Jr., P.E. O'Neil, and J.M. Pierson. 1996. Fishes of Alabama and the Mobile Basin. Birmingham, AL: Oxmoor House. 820 pp.

Mettee, M.F. Jr., P.E. O'Neil, J.M. Pierson, and R.D. Suttkus. 1989. Fishes of the Black Warrior River System in Alabama. Alabama Geological Survey Bulletin 133:1–201.

Near, T.J. 2002. Phylogenetic relationships of *Percina* (Percidae: Etheostomatinae). Copeia 2002(1):1–14.

Near, T.J., C.M. Bossu, G.S. Bradburd, R.L. Carlson, R.C. Harrington, P.R. Hollingsworth Jr., B.P. Keck, and

D.A. Etnier. 2011. Phylogeny and temporal diversification of darters (Percidae: Etheostomatinae). Systematic Biology 60(5):565–595.

Near, T.J., E.D. France, B.P. Keck, and R.C. Harrington. 2016. Systematics and taxonomy of the snubnose darter, *Etheostoma simoterum* (Cope). Bulletin of the Peabody Museum of Natural History 57(2):127–145.

Near, T.J., and B.P. Keck. 2013. Free from mitochondrial DNA: nuclear genes and the inference of species trees among closely related darter lineages (Teleostei: Percidae: Etheostomatinae). Molecular Phylogenetics and Evolution 66(3):868–876.

Near, T., D. MacGuigan, E. Boring, J. Simmons, B. Albanese, B. Keck, R. Harrington, and G. Dinkins. 2020. A new species of bridled darter endemic to the Etowah River system in Georgia (Percidae: Etheostomatinae: *Percina*) [dataset deposited at Dryad Digital Depository]. https://doi.org/10.5061/dryad.kd51c5b3z

Near, T.J., J.C. Porterfield, and L.M. Page. 2000. Evolution of cytochrome *b* and the molecular systematics of *Ammocrypta* (Percidae: Etheostomatinae). Copeia 2000(3): 701–711.

Near, T.J., J.W. Simmons, J.M. Mollish, M.A. Correa, E. Benavides, R.C. Harrington, and B.P. Keck. 2017. A new species of logperch endemic to Tennessee (Percidae: Etheostomatinae: *Percina*). Bulletin of the Peabody Museum of Natural History 58(2):287–309.

Near, T.J., and M.R. Thomas. 2015. A new barcheek darter species from Buck Creek (Cumberland River system), Kentucky (Percidae: Etheostomatinae: *Catonotus*: *Oopareia*). Bulletin of the Peabody Museum of Natural History 56(2):127–146.

Nguyen, L.T., H.A. Schmidt, A. von Haeseler, and B.Q. Minh. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Molecular Biology and Evolution 32(1):268–274.

Page, L.M. 1974. The subgenera of *Percina* (Percidae: Etheostomatin). Copeia 1974(1):66–86.

—1981. The genera and subgenera of darters (Percidae, Etheostomatini). Lawrence: Museum of Natural History, University of Kansas. 69 pp. (Occasional Papers 90.)

—1983. Handbook of Darters. Neptune City, NJ: T.F.H. Publications. 271 pp.

Pebesma, E.J. 2004. Multivariable geostatistics in S: the gstat package. Computers & Geosciences 30(7):683–691.

Peterson, B.K., J.N. Weber, E.H. Kay, H.S. Fisher, and H.E. Hoekstra. 2012. Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. PLoS One 7(5):e37135.

Poland, J.A., P.J. Brown, M.E. Sorrells, J.L. Jannink, and T. Yin. 2012. Development of high-density genetic maps for barley and wheat using a novel two-enzyme genotyping-by-sequencing approach. PLoS One 7(2):e32253.

Powers, S.L., B.R. Kuhajda, and S.E. Ahlbrand. 2012. Systematics of the *Etheostoma cinereum* (Teleostei: Percidae) species complex (subgenus *Allohistium*). Zootaxa 3277(1):43–55.

R Core Team. 2015. R: A Language and Environment for Statistical Computing. Version 3.2.0. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org

Ramsey, J.S. 1976. Freshwater fishes. In: H. Boschung, ed. Endangered and Threatened Plants and Animals of Alabama. University, AL: University of Alabama. pp. 53–65. (Bulletin of the Alabama Museum of Natural History 2.)

Robison, H.W., R.C. Cashner, M.E. Raley, and T.J. Near. 2014. A new species of darter from the Ouachita Highlands in Arkansas related to *Percina nasuta* (Percidae: Etheostomatinae). Bulletin of the Peabody Museum of Natural History 55(2):237–252.

Ronquist, F., M. Teslenko, P. Van Der Mark, D.L. Ayres, A. Darling, S. Hohna, B. Larget, L. Liu, M.A. Suchard, and J.P. Huelsenbeck. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61(3):539–542.

Sabaj, M.H. 2019 March 21. Standard symbolic codes for institutional resource collections in herpetology and ichthyology: an online reference. Version 7.1. Washington, DC: American Society of Ichthyologists and Herpetologists. https://asih.org

Scharpf, C. 2008. Annotated checklist of North American freshwater fishes, including subspecies and undescribed forms. Part IV: Cottidae through Percidae. American Currents 34(4):1–44.

Smith, T.A., P.J. Ciccotto, T.C. Mendelson, and L.M. Page. 2014. Dense taxon sampling using AFLPs leads to greater accuracy in phylogeny estimation and classification of darters (Percidae: Etheostomatinae). Copeia 2014(2):257–268.

Smith-Vaniz, W.F. 1968. Freshwater Fishes of Alabama. Auburn, AL: Agricultural Experiment Station, Auburn University. 211 pp.

Stiles, R.A., and D.A. Etnier. 1971. Fishes of the Conasauga River drainage, Polk and Bradley counties, Tennessee. Journal of the Tennessee Academy of Science 46:12–16.

Storey, C.M. 2003. Genetic Population Structure and Life History Aspects of the Federally Threatened Cherokee Darter, *Etheostoma scotti* [master's thesis]. Athens, GA: University of Georgia. 89 pp. https://galileo-usg-uga-primo.hosted.exlibrisgroup.com/permalink/f/v7b6bh/01GALI_USG_ALMA71119794150002931

[USFWS] United States Fish and Wildlife Service. 2017a. Endangered and Threatened Wildlife and Plants; 12 Month Findings on Petitions to List the Holiday Darter, Trispot Darter, and Bridled Darter; Threatened Species Status for Trispot Darter; Proposed Rule. Federal Register 82, No. 191 (October 4, 2017):46183–46197 (50 CFR 17). https://www.govinfo.gov/content/pkg/FR-2017-10-04/pdf/2017-21350.pdf

—2017b. Species Status Assessment Report for the Bridled Darter (*Percina kusha*), Version 1.0. Atlanta, GA: US Fish and Wildlife Service. 83 pp. (Document ID FWS-R4-ES-2017-0063-0002.)

Venables, W.N., and B.D. Ripley. 2002. Modern applied statistics with S. 4th ed. New York: Springer. 498 pp.

Wieland, W., and J.S. Ramsey. 1987. Ecology of the muscadine darter, *Percina* sp. cf. *P. macrocephala*, in the Tallapoosa River, Alabama, with comments on related species. Southeastern Fishes Council Proceedings 1(17):5–11.

Williams, J.D., D.A. Neely, S.J. Walsh, and N.M. Burkhead. 2007. Three new percid fishes (Percidae: *Percina*) from the Mobile Basin drainage of Alabama, Georgia, and Tennessee. Zootaxa 1549(1):1–28.

Wood, R.M., and R.L. Mayden. 1993. Systematics of the *Etheostoma jordani* species group (Teleostei: Percidae), with descriptions of three new species. Bulletin of the Alabama Museum of Natural History 16:31–46.