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Molecular species delimitation reveals hidden endemism in *Faxonius placidus* (Hagen 1870) (Decapoda: Astacidea: Cambaridae) in southeastern USA

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ABSTRACT

Molecular surveys are critical for understanding species boundaries and evolutionary relationships of North American crayfishes, as traditional morphological characters used for taxonomy frequently misrepresent crayfish diversity. The bigclaw crayfish, *Faxonius placidus* (Hagen, 1870), is currently described as a widely distributed crayfish that is common throughout the Cumberland, Tennessee, and Lower Ohio river drainages. The geographic distribution of *F. placidus* is based on identifications using traditional morphological characters including the first-form male gonopod, chelae, and rostrum. Within *F. placidus*, color pattern variation is specific to populations separated by hydrogeographic barriers. We used DNA barcoding data (mtDNA-COI) and genome-wide molecular markers (nuclear SNPs) to examine geographic patterns of genetic variation in *F. placidus* within the Cumberland and Tennessee river drainages. The federally endangered Nashville crayfish, *F. shoupi* (Hobbs, 1948), was included in our analyses, as previous phylogenetic reconstructions suggest *F. placidus* is paraphyletic with respect to *F. shoupi*. Phylogenetic reconstructions and molecular species delimitation identified four genetically distinct lineages within *F. placidus* that are paraphyletic with respect to *F. shoupi*. Our results add to numerous studies demonstrating the utility of robust molecular analyses for understanding the biodiversity of North American crayfishes.

KEY WORDS: bigclaw crayfish, Crustacea, Cumberland River drainage, genotype sequencing, mitochondrial DNA, Nashville crayfish, Tennessee River drainage

INTRODUCTION

The application of molecular tools to crayfish taxonomy is revealing extensive biodiversity of crayfishes in the southeastern United States. Such biodiversity has not been previously identified using traditional morphological criteria. Morphological characters used for crayfish identification, including chelae and gonopodia of first-form males, often reflect evolutionary convergence rather than recent shared ancestry (Breinholt *et al.*, 2012; Bloom *et al.*, 2019). The lack of taxonomic resolution in crayfishes is a concern for conservation and management because regionally endemic, at-risk species are often overlooked in management planning and lack adequate protection (Crandall *et al.*, 2009; Stratton & DiStefano, 2021). Molecular-based phylogeographic investigations reveal that many broadly distributed crayfish species are composed of multiple, genetically distinct, and morphologically cryptic species (Glon *et al.*, 2018; Hurt *et al.*, 2019).

The bigclaw crayfish, *Faxonius placidus* (Hagen, 1870) is described as a common, stream-dwelling crayfish that is widely distributed throughout the Lower Tennessee, Cumberland, Barren, Green, and Lower Ohio river drainages (Page, 1985; Hobbs, 1989) (Fig. 1). *Faxonius placidus* is usually identified by the shapes of the chela, rostrum, and first-form male gonopod. Observations of population-specific differences in both color pattern and the morphology of the chelae suggest the presence of genetically distinct lineages within *F. placidus*, despite sharing similar gonopod morphology. Gonopod shape, though highly variable across *Faxonius*, frequently contradicts molecular-based phylogenetic relationships (Fitzpatrick, 1987; Taylor & Knouft, 2006; Taylor *et al.*, 2014;

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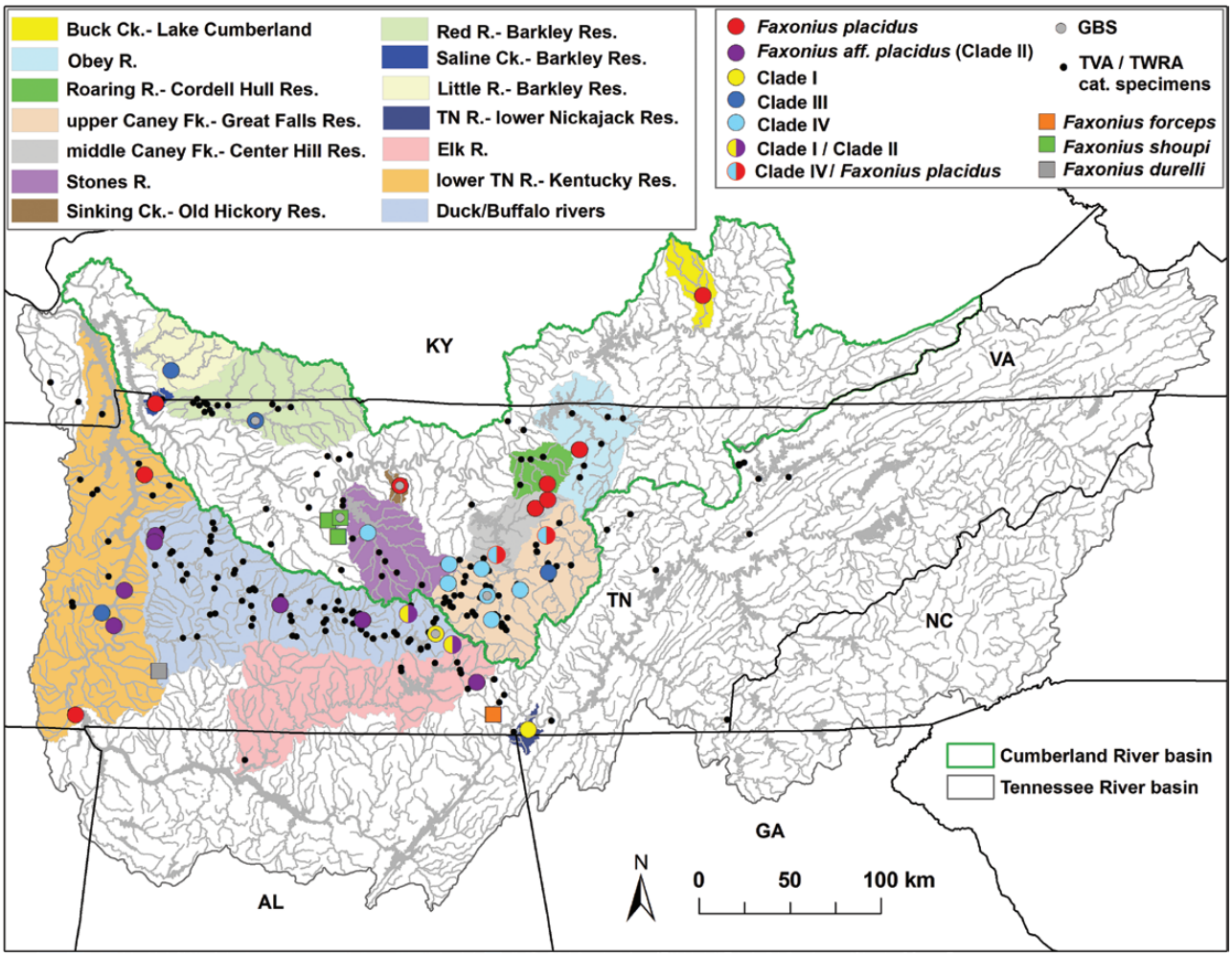

Figure 1. Map of Cumberland and Tennessee rivers with color-coded drainages represented in this study. All sites are color-coded to match the haplotype network (Fig. 4) and delimited species (Fig. 8). Circles represent *Faxonius placidus*. Squares represent *F. shoupi*, *F. forceps*, and *F. durelli* and are colored according to species; grey dots within circles represent genotyping-by-sequencing (GBS) sampling sites; black dots represent verified *F. placidus* specimens that are cataloged in Tennessee Valley Authority (TVA) or Tennessee Wildlife Resources Agency (TWRA) research collections. "Sinking Ck. Old Hickory Res." denotes the *F. placidus* type locality.

Crandall & De Grave 2017; Fetzner & Taylor, 2018; Bloom *et al.*, 2019). Color and color pattern have seldom been used for crayfish taxonomy as color is lost quickly after preservation. Recent studies have shown that in-life color patterns of crayfishes are often species-specific and consistent with molecular-based evolutionary reconstructions; for some groups, color patterns are more informative for species identification than traditional morphological characters (Schuster, 2020; Glon *et al.*, 2020).

Conversely, differences in gonopod structure have also been used to separate taxa that otherwise bear a strong resemblance to *F. placidus* (Fig. 2A). The Nashville crayfish, *Faxonius shoupi* (Hobbs, 1948) (Fig. 2B) is currently known from two disjunct areas, Mill Creek watershed and the Pickwick Tailwater located within the Cumberland and Tennessee river drainages, respectively (Hurt *et al.*, 2022). *Faxonius shoupi* was once considered to be a distant relative of *F. placidus* due to exaggerated differences in the gonopod shape of first-form males, despite similarities in chelae morphology and color pattern (Fitzpatrick, 1987; Taylor & Hardman, 2002). A molecular taxonomic investigation of a newly discovered population of *F. shoupi* suggested recent divergence from topotypic *F. placidus* (~30 kbp) (Hurt *et al.*, 2022). This finding demonstrates the need for more thorough crayfish inventory surveys of under-sampled portions of the Tennessee and Cumberland river drainages to inform conservation and management decisions, as *F. shoupi* is currently the only federally-listed endangered crayfish species in the state of Tennessee. Accurate information on species distributions is critical for informing regulatory actions and conservation action plans that directly assist managers in the production of conservation priorities and protection of natural resources. Most molecular investigations of phylogenetic relationships and species boundaries in crayfishes have been based on a limited number of mitochondrial genes and conserved nuclear loci (Larson *et al.*, 2016; Lovrenčić *et al.*, 2020). Mitochondrial sequence data is informative for species identification due to its smaller effective population size and higher mutation rate. Evolutionary reconstructions based solely on mitochondrial loci may not adequately represent the history of a species as mtDNA only reflects maternally-derived

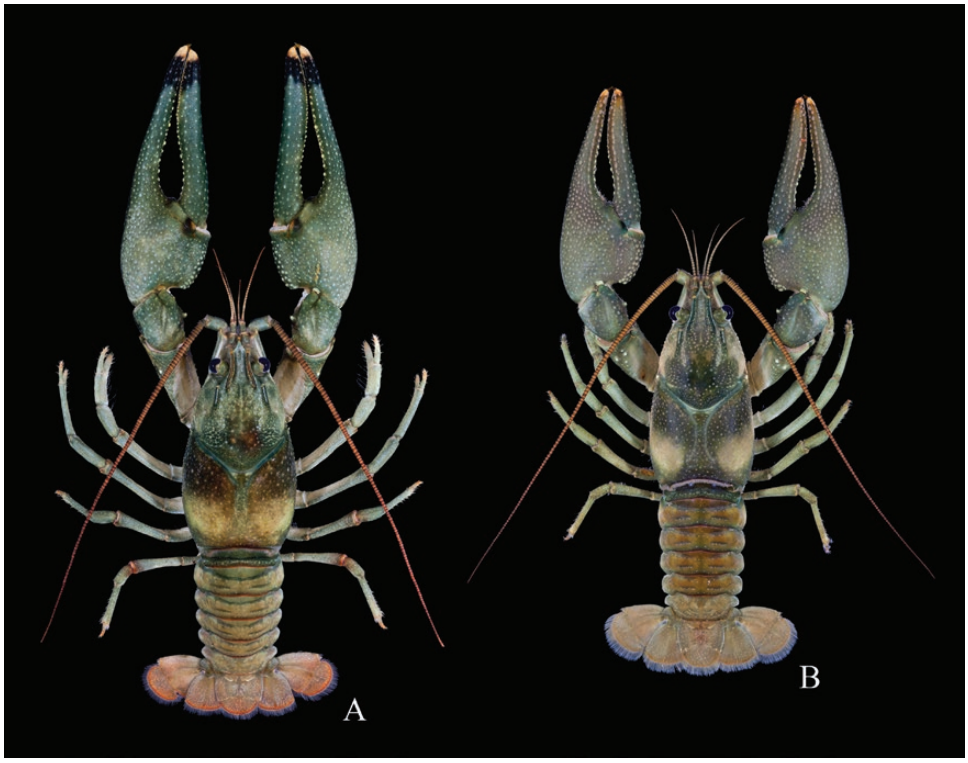

Figure 2. Dorsal views of a topotype *Faxonius placidus* collected from Sinking Creek (**A**) and of a *F. shoupi* from Sevenmile Creek, a direct tributary of Mill Creek (**B**).

inheritance patterns and can be biased by locus-specific selection pressures (Schubart *et al.*, 2009). Despite these limitations, surveys of mitochondrial loci can serve as an effective discovery approach for generating hypotheses of taxonomic diversity with multi-locus genotyping methods to further test mitochondrial-based hypotheses (Carstens *et al.*, 2013; Liu *et al.*, 2019). Reduced representation sequencing methods, such as genotyping-by-sequencing (GBS), generate genome-wide single nucleotide polymorphism (SNP) datasets that are increasingly used to address phylogeographic questions in non-model systems, including crayfish (Malone *et al.*, 2018; Unmack *et al.*, 2019). Species delimitation approaches that include both mitochondrial and nuclear data better reflect the evolutionary histories of organisms and are resilient to biases from locus-specific effects such as selection and sex-biased dispersal.

We performed a molecular taxonomic investigation of *F. placidus* within the Cumberland and Tennessee river drainages using both mitochondrial sequence data and genome-wide SNP genotypes. The evolutionary histories of morphologically-identified *F. placidus* populations and closely related *F. shoupi* were independently examined using phylogenetic analyses of mitochondrial haplotypes based on nuclear sequence data. Species delimitation analyses were used to provide a baseline molecular assessment of *F. placidus* that can inform a thorough taxonomic revision of this species group under the phylogenetic species concept (Eldredge & Cracraft, 1980; Cracraft, 1983). Results from this study have implications for the conservation of understudied crayfish groups that may harbor cryptic taxonomic diversity.

MATERIALS AND METHODS

Tissue collections

We collected *F. placidus* tissue samples (1–3 individuals per location) from 38 sites distributed throughout the Cumberland and Tennessee river drainages (Table 1; Fig. 1). Results from our phylogenetic analysis of mitochondrial haplotypes were used to identify four populations that represented major clades for inclusion in SNP genotyping (a total of 19 or 20 individuals per population): Sinking Creek (SKCR), Little Duck River (LDRV), Passenger Creek (PGCR), and Barren Fork (BFRV) (Table 1). We also included 25 *F. shoupi* tissue samples from the type locality at Mill Creek (MLCR). *Faxonius shoupi* individuals were collected by biologists from the Tennessee Wildlife Resources Agency (TWRA) and were released immediately after removal of a single leg at a self-autotomizing joint (Durand, 1960). Before preservation of each *F. placidus* individual, one leg was removed and fixed in RNA later (Invitrogen, Waltham, MA, USA) prior to DNA extraction. All *F. placidus* individual included in the SNP analyses were photographed to document life color patterns. Whole *F. placidus* specimens were preserved in 70% ethyl alcohol. Vouchers were deposited in the TWRA Crayfish Research Collection, Morristown, TN.

Molecular methods

Genomic DNA was extracted using the E.Z.N.A Tissue DNA kit (Omega Biotek, Norcross, GA, USA) following the manufacturer's instructions, except that nuclease-free water was used for the final elution step instead of the elution buffer. A 680 bp region of the mitochondrial gene cytochrome oxidase I (COI) was

Table 1. Collection sites for *Faxonius spp.* used for molecular analyses. *n* and *N* represent the number of individuals used for mitochondrial sequencing and SNP genotyping, respectively. Sites abbreviations in parentheses correspond to the abbreviations in Figure 1. Major drainage (MD) abbreviations are Cumberland River (CR) and Tennessee River (TN).

Waterbody (Site Abbrev.)	*n/N*	Haplotypes	Latitude	Longitude	MD
F. placidus					
Sinking Creek (SKCR)	3/20	PA16, PA14	36.2191	-86.3093	CR
Nettlecarrier Creek	3	PA6, PA13, PA16	36.3953	-85.2097	CR
Turkey Creek	3	PA9	36.2265	-85.4077	CR
Whiteoak Creek	3	PA7, PA15, PA16	36.2589	-87.8712	CR
Falling Water River	2	PA5, PA16	36.1061	-85.4824	CR
Falling Water River	1	PA4	36.1476	-85.4061	CR
Sink Creek	2	PA10, C4	35.8753	-85.7215	CR
Warren Branch	3	PB10, A5, PB9	35.4322	-85.9894	CR
Little River	3	B2, B1	36.7784	-87.7221	CR
Stewart Creek	3	C3, C2	35.9856	-86.5038	CR
Sink Creek	1	C5	35.8743	-85.7221	CR
Bullpen Creek	2	C6	35.7368	-86.016	CR
East Fork Stones River	3	C8, C7	35.8302	86.00769	CR
Mountain Creek	3	C4	35.8076	-85.8119	CR
Cove Creek	3	C4	35.5557	-85.7516	CR
Barren Fork (BFRV)	3/20	C8, C9	35.6746	-85.7767	CR
Passenger Creek (PGCR)	3/19	B5, B2	36.5356	-87.1978	CR
Cane Creek	3	B3, B2	35.7897	-85.404	CR
Garrison Fork	3	A3, PB6, PB8	35.5841	-86.2572	CR
Saline Creek	3	PA3, PA1, PA2	36.612	-87.8127	CR
Buck Creek	3	PA8, PA16	37.1518	-84.4382	CR
Calfkiller River	3	PA11, PA16, C4	35.9712	-85.4168	CR
Rocky River	3	C6, C1	35.7017	-85.578	CR
Rushing Creek	1	B4	35.5699	-88.1148	CR
Unnamed tributary to Gillian Creek	3	PB8, PB11	35.2462	-85.8432	TN
Little Duck River (LDRV)	3/19	A2, A4, A1	35.4863	-86.0911	TN
Tennessee River (Kentucky Reservoir inflow)	1	PA12	35.0643	-88.2602	TN
Running Water Creek	1	A2	35.0113	-85.531	TN
Duck River	3	PB2	35.9278	-87.8036	TN
Lick Creek	3	PB1	35.6852	-87.9806	TN
Duck River	3	PB3, PB4	35.6242	-87.0342	TN
Fall Creek	3	PB8, PB7	35.5523	-86.5339	TN
Whites Creek	1	PB2	35.5092	-88.0415	TN
Hurricane Creek	1	PB3	35.9598	-87.7983	TN
F. shoupi					
Mill Creek (MLCR)	2/25	S2, S4	36.0598	-86.6726	CR
Sevenmile Creek	1	S1	36.0451	-86.746	CR
Unnamed tributary to Mill Creek	1	S3	35.9659	-86.6842	CR
F. forceps					
Battle Creek	3	F1, F2	35.0856	-85.7424	TN
F. durelli					
Green River	3	D1	35.2889	-87.7625	TN

amplified using polymerase chain reactions (PCR) for Sanger sequencing. Primer sequences used in the PCR reactions were LCO1490(5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198(5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994). Thermocycler conditions for PCR were as follows: initial denaturation step of 5 min at 95 °C followed by 35 cycles of 15 s at 95 °C, 15 s at 54 °C, and 60 s at 72°C, and a final extension of 10 min at 72 °C. Sanger sequencing was performed on an ABI 3730 automated sequencer platform (MCLAB, San Francisco, CA, USA). Sequence chromatograms were visually inspected and trimmed using SEQUENCHER 5.2 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequence alignment was implemented using ClustalW with default parameters (Thompson *et al.*, 1994). The final alignment was examined

for indels and stop codons in the translated sequences while in the corrected reading frame using the software BioEdit version 7.2.5 (Hall, 1999).

Library preparation and sequencing

Library preparation for GBS followed the protocol described in Elshire *et al.*, (2011). Genomic DNA was extracted using the same protocol used for mitochondrial sequencing. Total nucleic acid extracts were quantified in a 96-well plate using Quantifluor dsDNA System (Promega, Madison, WI, USA) and diluted to a standard concentration of 5 ng μl^{-1}. Standardized DNA was digested using the restriction enzyme ApeKI, and barcoded adaptors were added in a ligation reaction prior to pooling the library. The resulting library was cleaned using the Qiaquick PCR (Qiagen, Germantown, MD, USA) purification kit to remove excess adaptors and was then PCR amplified using primers specific to the barcoded adaptors. The amplified library was cleaned using the AxyPrep Mag PCR Clean-up kit (Axygen, Union City, CA, USA), and the distribution of fragment lengths was inspected using a BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). The subsequent library was sequenced with a 75-bp single-end chemistry utilizing an Illumina NexSeq 500 (Illumina, San Diego, CA, USA).

Mitochondrial sequence analyses

Phylogenetic reconstructions for mitochondrial COI haplotypes were performed using both Bayesian and maximum likelihood (ML) optimization criteria. Maximum likelihood analyses were performed using the program RAxML (Stamatakis, 2014) on the CIPRES server (version 3.3) (Miller *et al.*, 2010) under the GTR+G model. The tree with the best ML score was retained, and nodal support was assessed by non-parametric bootstrap (1,000 replications). Bayesian reconstructions were performed using MrBayes (version 3.2.7a; Huelsenbeck & Ronquist, 2001), also on the CIPRES server. The best-fit model of substitution was selected separately for each codon position using the BIC criteria as performed by Modeltest in MEGA X (version 10.1.5) (Posada & Crandall, 1998; Kumar *et al.*, 2018). The Markov chain Monte Carlo (MCMC) algorithm ran for 10,000,000 generations. Every 1,000 generations were sampled after a fractional burn-in of 0.25. A majority rule consensus tree was retained as the final topology. A minimum spanning haplotype network (MSN) was generated using the R package *pegas* (Paradis, 2010) as implemented in RStudio v4.0.2. Haplotype sequences were trimmed to an equal length of 537 bp for the MSN analysis. Average Kimura-2-parameter (K2P) (Kimura, 1980) pairwise genetic distances were estimated between sites and between clades identified in phylogenetic reconstructions from mitochondrial haplotypes, using the program MEGA X.

Bioinformatics and filtering for genomic SNPs

Raw sequence reads were filtered for quality (phred < 33 were discarded) and de-multiplexed using the *process_radtags* module in the STACKS bioinformatic pipeline (v. 2.4.1) (Catchen *et al.*, 2011). Individuals with fewer than 5×10^5 reads were removed. The *denovo* pipeline in STACKS was used to assemble tags and generate SNP datasets for downstream analyses. Parameters for locus assembly in the *ustacks* module included a maximum of three nucleotide differences between reads and a minimum of three reads per locus. For the catalog assembly step in the *cstacks* module, a maximum of three nucleotide differences was allowed between alleles and a minimum depth of three reads was required per locus. The three individuals with the highest quality score from each site were retained for the final catalog. The *populations* module was used to generate input datasets for downstream analyses and estimate summary statistics with parameters (p, number of populations a locus must be present in; r, percentage of individuals per population a locus must be present in; R, percentage of individuals a locus must be present in regardless of the population designation). We used three different parameter sets for quality filtering of SNP genotypes as follows: p = 2, r = 0.70 (p2r70) one SNP per locus; p = 1, r = 0.70 (p1r70) one SNP per locus; and R = 0.70 (R70) multiple SNPs per locus.

Genomic SNP analyses

Population-genetics summary statistics including observed heterozygosity (H_o), expected heterozygosity (H_e), and pairwise measures of population differentiation F_{ST} and Φ_{ST} were performed using the *populations* module in the STACKS pipeline using the (p = 2, r = 0.70) dataset. Wright's F_{ST} measures the deviation from Hardy-Weinberg equilibrium due to population structure and is based solely on allele frequencies (Weir & Cockerham, 1984). By contrast, Φ_{ST} is an AMOVA-based statistic that incorporates both allele frequencies as well as information regarding the genetic distance between haplotypes (Excoffier, 1992).

Geographic partitioning of SNP genotypes among populations was investigated with Discriminant Analysis of Principal Components (DAPC) and Principal Component Analysis (PCA), using the R package *adegenet* (Jombart, 2008). These two methods provide complementary information of displaying population clusters and population structure across a graphical framework. DAPC maximizes differences between a priori groups identified using Bayesian Information Criterion (BIC) (Jombart & Ahmed, 2011) with a specified number of principal components identified with the *optima.a.score* function. By contrast, PCA accounts for individual variation and does not include a priori information regarding an individual's population of origin. To maximize the total number of loci and examine structure among individuals, both analyses used the p1r70 dataset. Population structure was examined twice for both DAPC and PCA using a dataset that included only *F. placidus* populations and a dataset with all five populations to assess the genetic relationship of populations morphologically identified as *F. placidus* with and with ought influence from *F. shoupi*.

Partitioning of genetic variation was also investigated using the Bayesian assignment method implemented in the program STRUCTURE 2.3.1 (Pritchard *et al.*, 2000). Tests for Hardy-Weinberg equilibrium (HWE) for each of the five populations were performed with the p1r70 dataset in PLINK 2.0 (Purcell *et al.*, 2007); loci deviating from HWE ($P < 0.01$) were excluded from further analyses. We performed 10 independent runs for each value of K = 2–10 populations; MCMC parameters for each run included 500,000 MCMC generations, with a burn-in of 100,000 generations. The optimal number for K

was assessed using the ΔK (Evanno *et al.*, 2005) method implemented in the program STRUCTURE HARVESTER (Earl & VonHoldt, 2012). Bar plots for K = 2–5 were generated in the program Clumpak (Kopelman *et al.*, 2015).

Species delimitation

We performed multiple species delimitation analyses to assess congruency of results across methods as suggested by Carstens *et al.*, (2013). Four different single-locus delimitation methods were applied to our mitochondrial sequence data set as follows: 1) General Mixed Yule Coalescent (GMYC) (Pons *et al.*, 2006), Poisson Tree Processes using both, 2) Bayesian (bPTP-B) and 3) maximum likelihood (bPTP-ML) criteria (Zhang *et al.*, 2013), and 4) Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.*, 2012). For the GMYC analysis, a Bayesian ultrametric tree was generated using the program Beast 2.6.0 (Bouckaert *et al.*, 2014) utilizing the GTR+G model of evolution; default parameters were used except for the selection of a strict clock model and coalescent model of constant population size. The MCMC chain was set for 100,000,000 generations sampling every 10,000 generations, with a burn-in of 50,000. The resulting 10,000 trees were summarized in TREE ANNOTATOR, a function of Beast 2.6.0, to create a maximum clade credibility tree with common ancestor heights. The resulting topology was used as input for the GMYC analysis, performed on the Exelixis Lab server (www.species.h-its.org/gmyc/; Fujisawa & Barraclough, 2013). The GMYC method recognizes species boundaries by identifying recurring shifts in topology patterns as the threshold for speciation events. The bPTP method is based on the phylogenetic species concept (Eldredge & Cracraft, 1980; Cracraft, 1983) and estimates the number of lineages in the topology as a function of time. The inflection point that marks an increase in the number of trees was used as the boundary of intra and interspecific branching. For the bPTP method, both maximum likelihood and Baysian based estimates were analyzed independently, and the mitochondrial COI haplotype-based ML phylogeny was used as input topology (https://species.h-its.org/gmyc/ ; Zhang *et al.*, 2013). The MCMC chain was set for 100,000 generations with a burn-in of 10,000 trees and sampled every 100 generations. Two related species, the surgeon crayfish, *Faxonius forceps* (Faxon, 1884), and the saddle crayfish, *Faxonius durelli* (Bouchard & Bouchard, 1995), were included in the analysis as undefined outgroups. The ABGD method identifies gaps in the frequency distribution of pairwise sequence distances to identify the boundary between intraspecific and interspecific distance values. For this analysis, we applied the K2P model of substitution on all mitochondrial COI haplotypes and the ABGD analysis was performed on the ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/) as described in Puillandre *et al.*, (2012).

Coalescent-based estimates of the species delimitation, species-tree reconstruction and divergence times were performed using the Bayesian Phylogeny and Phylogeography method (BPP v.4.2) (Yang, 2015) using the R70 SNP dataset. First, an unguided species delimitation analysis (A11 model) was performed using the following run parameters: two independent MCMC chains, 500,000 generations, a burn-in of 10,000 generations, and a sample frequency of 10. Parameter settings for the inverse gamma distribution used were theta prior (3, 0.04) and tau prior (3, 0.2). The BPP A01 model was then used to estimate a majority rule consensus species tree using species groups recovered from the A11 analysis. The A01 model was run for 100,000 generations with a burn-in of 1,000. Finally, divergence times were estimated (A00 model) on the species tree produced with the A01 analysis; the tree with the highest posterior probability was used as the input topology. Divergence times were estimated without introgression. Parameter settings for the theta prior, tau prior, and phi prior were set to 3 0.04, 3 0.2, and 1 1, respectively. MCMC chains were run for 1,000,000 generations with a burn-in of 8,000, default fine-tuning parameters, and default heredity parameters. The R package BPPr (Angelis & Dos Reis, 2015) was used to convert samples from the MCMC chain in the A00 analysis to mean divergence time in years with generation time set to the default of 1. The default generation time was used since accurate life history information is currently unavailable, though generation time is potentially higher in large-bodied crayfishes. The mutation rate calibration was set at $\mu = 2.64 \times 10^{9}$; this estimate is based on mutation rate calibrations for genome-wide sequence data in sister-species pairs of *Alpheus* snapping shrimp that span the Isthmus of Panama (Silliman *et al.*, 2021).

RESULTS

Mitochondrial haplotype analysis

Mitochondrial COI sequences were generated for 93 individuals from 36 *F. placidus* sites and three *F. shoupi* sites (Fig. 3). Three *F. forceps* and three *F. durelli* individuals were also included as outgroups for phylogenetic reconstructions. Fifty-three unique haplotypes were identified across the 39 sampled locations. Haplotypes were trimmed from 602 bp to equal lengths of 537 bp for MSN analysis, reducing unique haplotypes from 53 to 25. The MSN showed eight haplotype groups identical to clades identified by phylogenetic reconstruction. Major haplotype groups were separated by a minimum of six mutational steps between the next haplotype group (Fig. 4).

Bayesian and ML phylogenetic reconstructions for mitochondrial haplotypes resulted in nearly identical topologies; results from Bayesian analysis are shown in Figure 5 along with bootstrap support from ML analysis. Both methods identified five well-supported clades that corresponded to hydro-geographic boundaries; however, relationships at basal nodes were not well supported for haplotypes associated with topotypic *F. placidus*. Geographic distributions of each clade are as follows (Fig. 1): *F. placidus* sensu stricto: eleven sites from the Cumberland and lower Tennessee river drainages; Clade I: four sites from the upper Duck and Elk river drainages and Tennessee River (Nickajack Reservoir); Clade II: nine sites from the Duck, Elk, and lower Tennessee river drainages; Clade III: four sites from the lower Tennessee and lower Cumberland river drainages and upper Caney Fork; Clade IV: nine sites from the Stones River and Caney Fork drainages; and *F. shoupi* from the Mill Creek drainage.

Pairwise genetic distance estimates (K2P) between all monophyletic clades ranged 1.3%–10.2% and averaged 6.7% (Table 2).

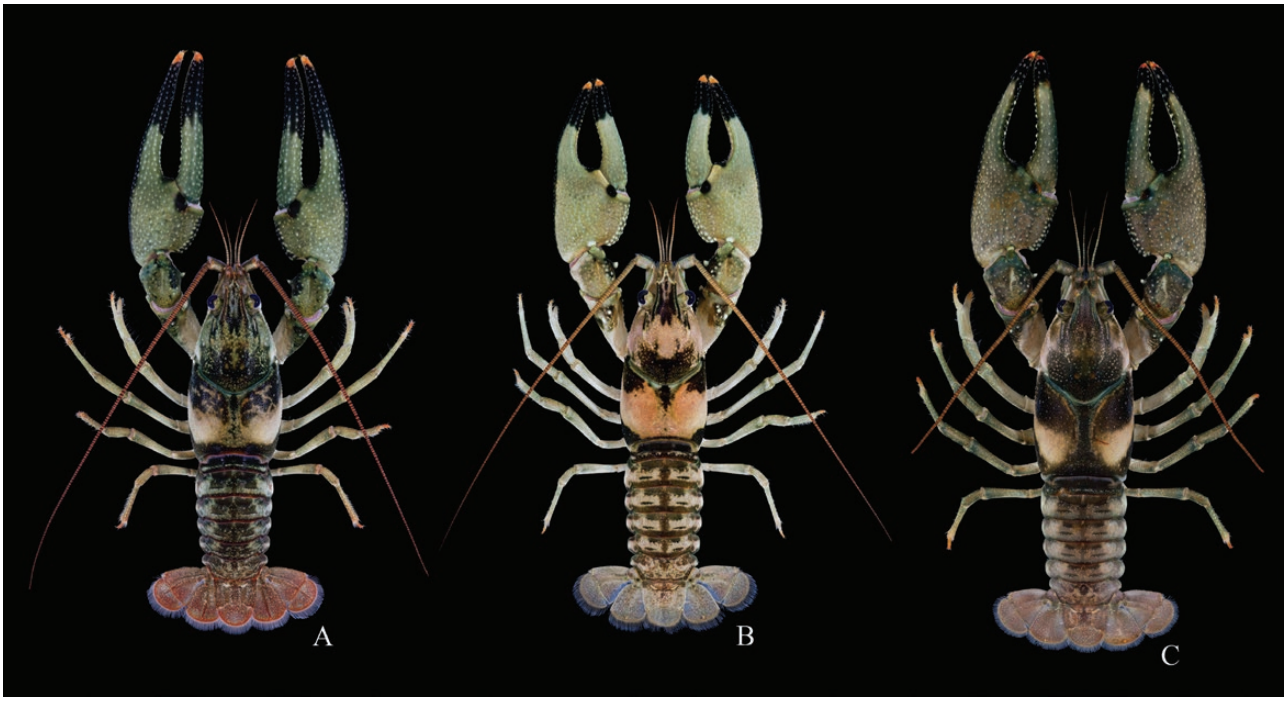

Figure 3. Dorsal view of representative males collected from Little Duck River (**A**), Passenger Creek (**B**), and Barren Fork (**C**).

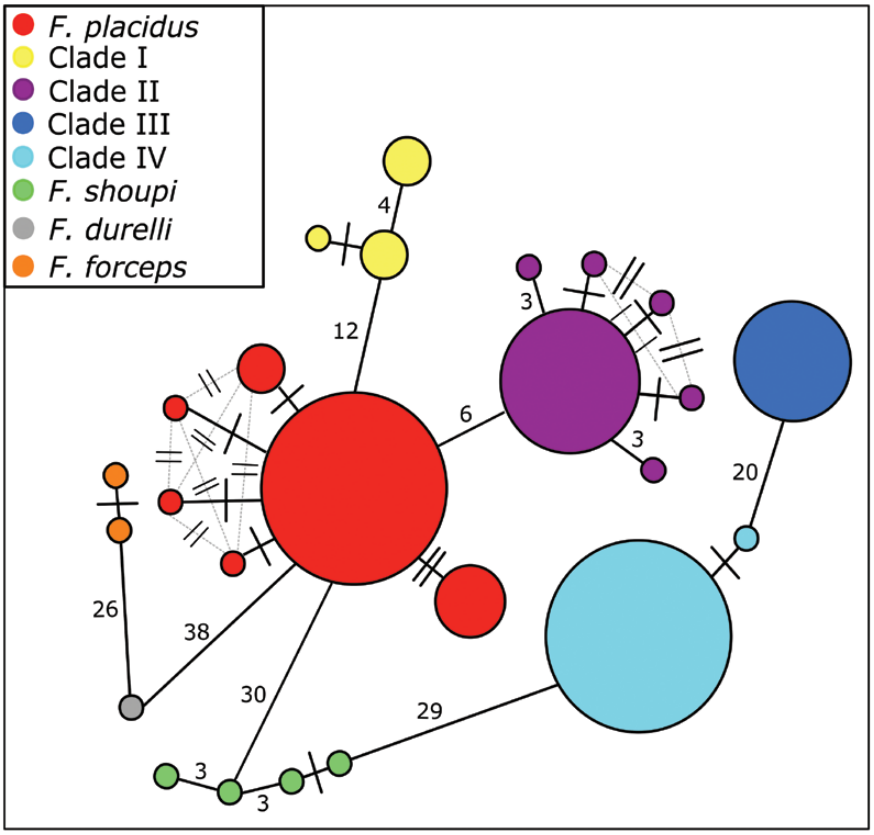

Figure 4. Minimum spanning haplotype network depicting differences among *Faxonius placidus* species clades and *F. shoupi, F. durelli*, and *F. forceps*. The size of the circle corresponds to the number of individuals per haplotype. Haplotype circles are color-coded to match (Figures 1, 8). Numbers and perpendicular dashes on lines between circles represent the number of mutational differences between haplotypes.

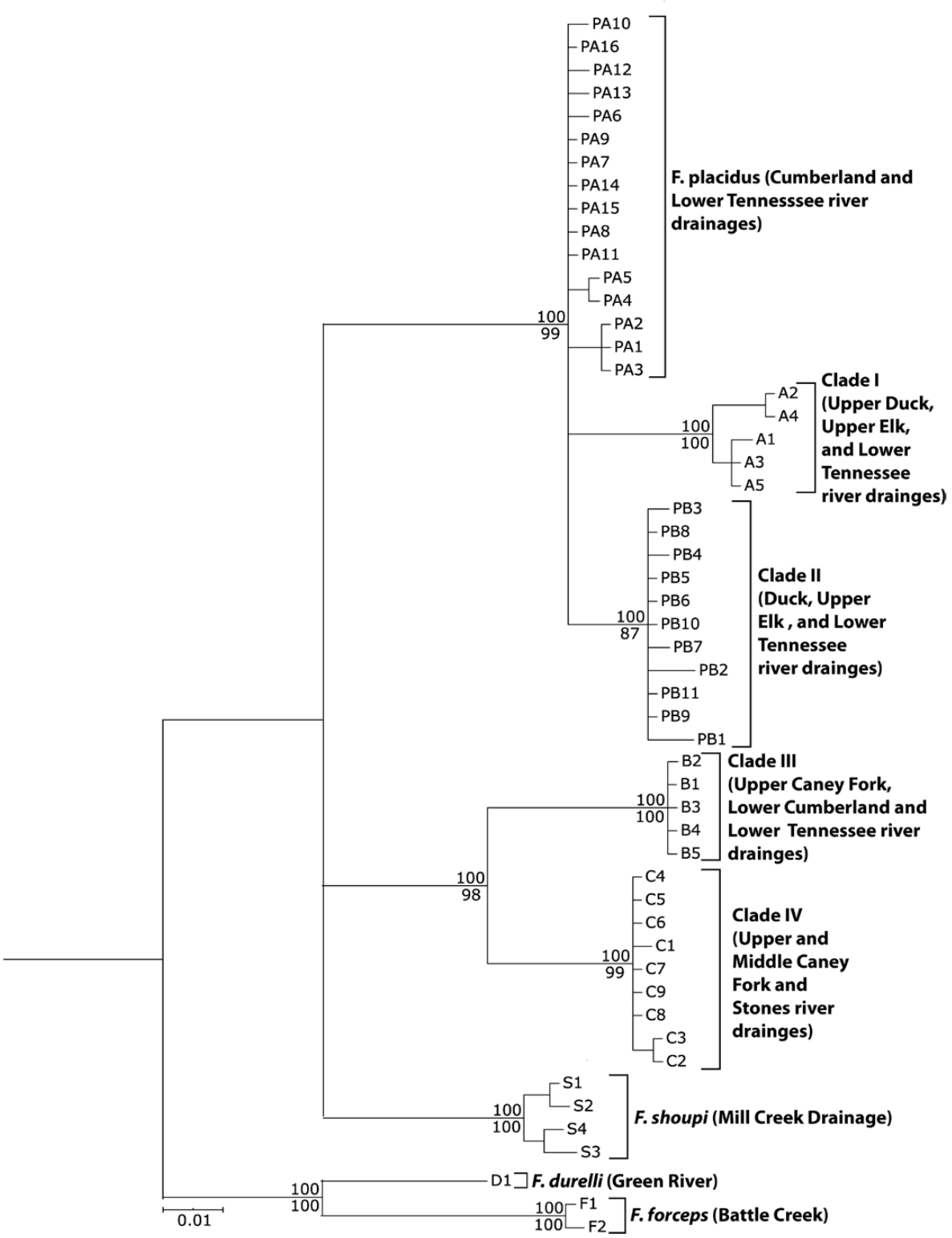

Figure 5. Bayesian phylogenetic tree representing 36 *Faxonius placidus* sites and 3 *F. shoupi* sites in the Cumberland and Lower Tennessee River drainages. Haplotypes at tips correspond to haplotypes listed in Table 1. Numbers at nodes indicate statistical support (85%) for each clade as Bayesian posterior probabilities (above line) and bootstrap support for the same clade from maximum likelihood (below line).

Average K2P genetic distance estimates between the major clades identified in phylogenetic reconstructions ranged from 1.3% (*F. placidus*/ Clade II) to 6.9% (*F. shoupi*/ Clade I) (Table 2). Pairwise genetic distances within each clade were less than 0.06%.

Genotyping by sequencing

A total of 78 individuals from four populations of *F. placidus* and 25 *F. shoupi* individuals were included for genome-wide SNP genotyping. Initial filtering of raw sequence reads recovered

Table 2. Mean pairwise Kimura-2-parameter (K2P) genetic distances between monophyletic clades of *Faxonius placidus*, *F. shoupi*, *F. forceps*, and *F. durelli* in our mitochondrial phylogenetic analysis (Fig 5). Within clade average K2P distances are in bold. *S*, number of sites per clade; *N*, number of individuals per clade; *H*, number of haplotypes per clade.

	S	*N*	*H*	1	2	3	4	5	6	7	8
1. *F. placidus*	11	25	16	**0.002**							
2. Clade I	4	6	5	0.024	**0.005**						
3. Clade II	10	19	11	0.013	0.034	**0.004**					
4. Clade III	6	11	5	0.061	0.066	0.064	**0.000**				
5. Clade IV	10	23	9	0.063	0.060	0.069	0.040	**0.001**			
6. *F. shoupi*	3	4	4	0.059	0.069	0.067	0.064	0.067	**0.006**		
7. *F. durelli*	1	3	1	0.073	0.076	0.076	0.090	0.081	0.088	**0.000**	
8. *F. forceps*	1	3	2	0.090	0.081	0.092	0.080	0.080	0.102	0.049	**0.001**

Table 3. Average pairwise F_{ST} (below shaded diagonal) and Φ_{ST} (above shaded diagonal) for four populations of *Faxonius placidus* (SKCR, Sinking Creek; BFRV, Barren Fork; LDRV, Little Duck River; PGCR, Passenger Creek), and a single population of *F. shoupi* (MLCR, Mill Creek).

	SKCR	MLCR	BFRV	LDRV	PGCR
SKCR	-	0.088	0.096	0.133	0.104
MLCR	0.072	-	0.060	0.202	0.117
BFRV	0.082	0.050	-	0.121	0.108
LDRV	0.111	0.159	0.101	-	0.127
PGCR	0.083	0.096	0.092	0.104	-

1,237,974 genotyped loci with an average coverage of 12.2 per loci. The average number of sites per locus across sequenced individuals was 82.2. The final p1r70 dataset included 1,246 SNPs (3,522 loci) after filtering for Hardy Weinberg Equilibrium (Supplementary material Table S1). The final p2r70 dataset used for estimates of within-population genetic variation (H_o, H_e, and π) and pairwise population differentiation analyses (F_{ST} and Φ_{ST}) recovered 452 SNPs (852 loci). The R70 dataset used in species delimitation and divergence estimation analyses recovered 618 SNPs (313 loci).

Variation within populations

Estimates of within-population genetic variation were similar across the five surveyed populations; the average expected heterozygosity (H_e) and observed heterozygosity (H_o) across sites variable within populations were 0.098 and 0.083, respectively (Supplementary material Table S2). Estimates of genetic variation were greatest for the BFRV population, with the exception that π for all sites was greatest in SKCR. The PGCR population had the lowest estimates of heterozygosity for all sites.

Divergence between clades

Pairwise population differentiation estimates based on SNP genotype frequencies were consistent with results from COI phylogenetic analyses suggesting isolation among the mitochondrial clades (Table 3). Estimates of pairwise F_{ST} ranged from 0.050 (BFRV/MLCR) to 0.159 (LDRV/MLCR).

The DAPC analysis (Fig. 6A) of combined *F. shoupi* and *F. placidus* genotypes suggested an optimal number of five genetic clusters based on BIC criteria (K = 5; Supplementary material Fig. S4) and optima-a-score suggested retention of 7 PCs (Supplementary material Fig. S4). Membership probabilities assigned all individuals to their respective collection sites with high probability (Fig. 6B). The DAPC scatter plot demonstrated overlap between BFRV and *F. shoupi*. Genetic clusters representing PGCR, LDRV, and SKCR showed no overlap (Fig. 6A). Results from the PCA analysis were consistent with DAPC. The scatter plot of PC1 vs. PC2 spatially isolated all populations except *F. shoupi* and BFRV individuals (Supplementary material Fig. S5). For Bayesian assignment tests, an optimal number of K = 4 genetic populations were determined by the ΔK method (Fig. 7, Supplementary material Table S3). Three populations SKCR, LDRV, and PGCR were each assigned to unique clusters; *F. shoupi* MLCR and BFRV were grouped into a single cluster.

Species delimitation

Both single locus mtDNA (GMYC, ABGD, bPTP) and multi-locus SNP (BPP) species delimitation analyses identified a minimum of five congruent taxonomic groups corresponding to *F. placidus*, *F. shoupi*, and clades I, II, and IV (Fig. 8). The GMYC analysis identified eight genetically distinct groups ($P < 0.05$). The two bPTP analyses differed in the optimal number of distinct entities. The bPTP-ML method identified eight genetic entities (0.16–0.90), whereas bPTP-Bayesian analysis provided a range of optimal taxonomic groups (7–35) with an acceptance rate of r = 0.60. The ABGD analysis differed from GMYC and bPTP results by combining *F. placidus* and Clade II as a single genetic entity. The multi-locus species delimitation method (BPP) identified each population as a separate entity with high posterior probabilities ($P > 80$).

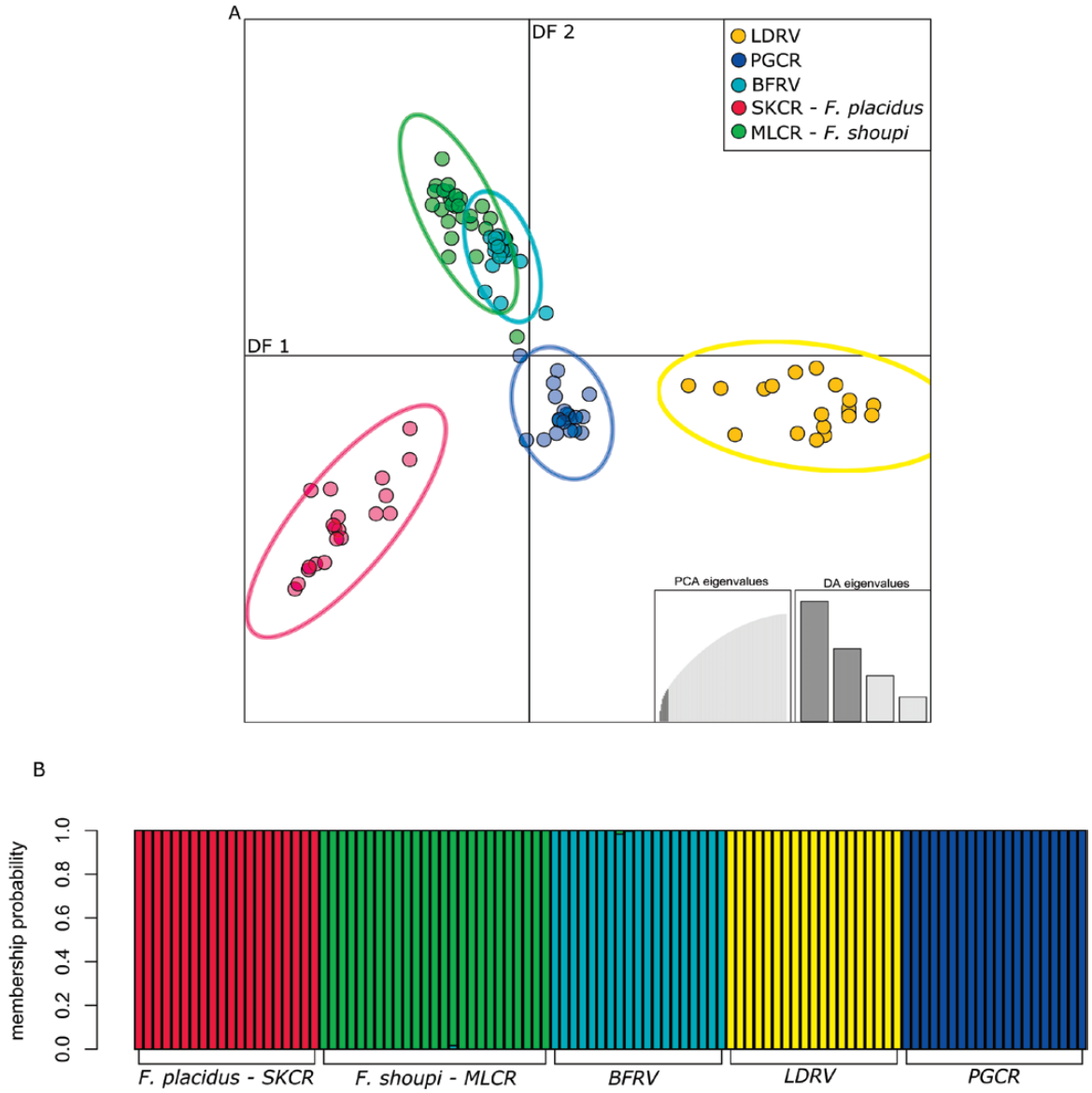

Figure 6. Scatter plot of DF1 vs. DF2 from discriminate analysis of principal components analysis (**A**) and cluster membership probabilities of individuals color coded to match individuals to their respective clusters (**B**). Scatter plot and cluster membership correspond to sites found in Table 1.

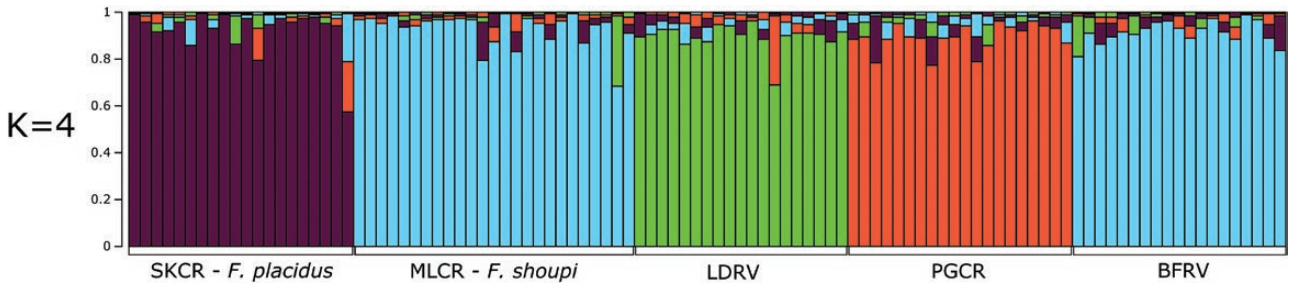

Figure 7. Bayesian probability assignment plot results using SNP data implemented in the analysis STRUCTURE. The number of populations was set to K = 4 determined by ΔK in STRUCTURE HARVESTER. Blocks and species correspond to sites listed in Table 1.

Divergence time estimates

Species tree reconstruction using the BPP A01 model recovered *F. placidus* from SKCR as a supported basal lineage to all other clades with limited support that included (LDRV, PGCR, BFRV, and MLCR) (Fig. 9). Divergence time estimates from the A00 analysis were scaffolded onto the best-fit species tree produced by the A01 model and calculated as Ka (Fig. 9). Divergence time estimates ranged from ~80,000 to 427,000 years before

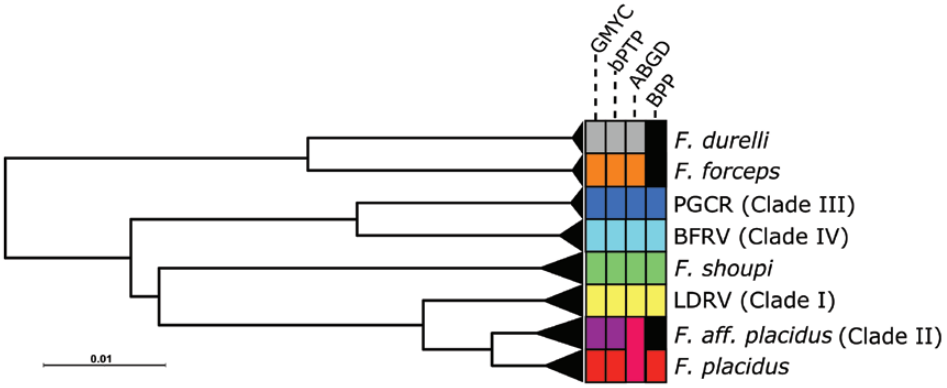

Figure 8. An ultrametric COI based phylogeny produced in Beast2 used to scaffold the results of four species delimitation analyses. Tips of the tree are collapsed with adjacent colored blocks representing results of GMYC, bPTP, ABGD, and BPP. Corresponding identifiers are left of colored blocks. Solid column blocks represent groups identified as the same species for specific analyses. Black blocks represent clades not used in the BPP analysis.

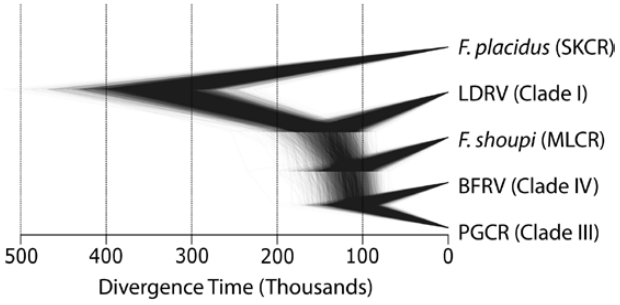

Figure 9. DensiTree plot of an ultra-metric phylogeny from the A01 and A00 BPP analyses. The tree displays overlapping sampled topologies from the A00 Bayesian multispecies coalescent analysis. Names in parentheses correspond to sample sites listed in Table 1; 95% confidence intervals for divergence time estimates are stated in the text.

present (ybp). The divergence time estimate separating *F. placidus* from SKCR from all other clades was 344,014 ybp (95% CI: 266,324–426,781 ybp). The next clade, LDRV, was separated by the subsequent cladistic grouping (MLCK) at 141,983 ybp (95% CI: 101,864–185,467). Estimated divergence time of *F. shoupi* (MLCR) from BFRV was 118,948 ybp (95% CI: 84,733–154,487). The youngest clades, PGCR and BFRV, were separated 110,899 ybp (95% CI: 79,685–144,003).

DISCUSSION

Our results demonstrate that a combination of mitochondrial sequence data followed by genome-wide SNP genotyping was effective for clarifying the presence of taxonomic diversity within a species complex. This approach is well suited for phylogeographic surveys that span a large number of collection sites, where population-level genotyping of all sites may be unfeasible. Analysis based on both mitochondrial sequence data and genomic SNP genotypes identified five major clades within morphologically defined populations of *F. placidus* and provided evidence for the existence of three additional lineages. Genetically unique clades identified here warrant species-level description following further examination of phenotypic differences and have implications for establishing conservation priorities.

Morphology

The morphological characters and geographic distribution that define *F. placidus* have been poorly understood since its initial description. *Faxonius placidus* was originally described from three geographically distant localities: Lebanon, Tennessee; Quincy, Illinois; and an unknown locality in Texas (Hagen, 1870). Several discrepancies in this description have been noted. Specifically, a second species was included as syntypes along with *F. placidus* (MCZ 170; Ortmann, 1931: 80). The identification of *F. placidus* in Texas and Quincy, Illinois is unlikely because the range of *F. placidus* does not extend west of the confluence of the Ohio and Mississippi Rivers (Taylor *et al.*, 2015). Error in syntypes and the range estimation of *F. placidus* led Hobbs (1989) to designate Lebanon, Tennessee as the type locality; however, this designation is considered erroneous because a lectotype has not yet been identified (Poly & Wetzel, 2003). We consider herein Sinking Creek (SKCR) as the type locality following Hobbs (1989), as this is the only waterbody within the historic center of Lebanon, Tennessee. The SKCR population sampled here represents the only site of the three original locales where *F. placidus* could be positively identified from available syntypes.

Results from our molecular analyses provide a new interpretation of earlier morphology-based descriptions of *F. placidus*, adding to numerous studies demonstrating that comparative gonopod morphology alone is not sufficient for evolutionary reconstructions (Taylor & Hardman, 2002; Breinholt *et al.*, 2012). *Faxonius placidus* was previously included in the subgenus *Procericambarus,* based on its gonopod structure. The central projection and mesial process of the gonopod of *F. placidus* lack distinct sculpturing, limiting its usefulness for taxonomic identification (Hobbs, 1989; Bouchard & Bouchard, 1995). This simplified gonopodial structure is shared with other species placed in subgenus *Procericambarus* (e.g., *F. durelli* and *F. forceps*). All *Faxonius* subgenera designations, including *Procericambarus,* were more recently removed due to molecular phylogenetic evidence that refuted the monophyly of these groups (Crandall & De Grave, 2017).

The reliance on gonopod morphology has also confounded our understanding of the evolutionary relationships among *F. placidus* and its congeners. Molecular results presented here are consistent with Hurt *et al.* (2022) indicating that BFRV is more closely related to *F. shoupi* than to other *F. placidus* populations, so that *F. placidus* is paraphyletic as currently defined. *Faxonius shoupi* was originally considered to be a distant relative of *F. placidus* based on exaggerated differences in gonopod morphology, despite similarities in the chelae and cephalothoracic region (Taylor & Hardman, 2002; Bizwell & Mattingly, 2010). *Faxonius shoupi* possesses a similar color pattern to some *F. placidus* populations. Most notably, the saddle coloration of *F. shoupi* resembles the pattern observed in the BFRV; both *F. shoupi* and BFRV have a solid or mottled hourglass-shaped saddle pattern on the cephalothorax with the secondary saddle extending laterally in a diagonal direction (Figs. 2B, 3C). These two groups can be distinguished by differences in the morphologies of the gonopod and chela and by the presence/absence of prominent black bands on the propodus and dactyl of the chelae.

Molecular-based species delimitation

Mitochondrial markers serve as an effective discovery tool for identifying genetically distinct, morphologically cryptic populations that warrant further investigation (Bloom *et al.*, 2019; Fetzner & Taylor, 2018; Perkins *et al.*, 2019). Our phylogenetic reconstructions based on COI barcodes identified three monophyletic clades that were validated with additional genome-wide SNP genotyping. Many molecular-based taxonomic investigations in crayfishes have relied solely on mitochondrial markers for species delimitation. These studies often provide estimates of genetic distances between unique clades to support decisions regarding species delimitation; however, the threshold of genetic distances has been variable across taxonomic studies. Listed are recent studies that employ uncorrected *p*-distances and morphology with results that are comparable to our study: 1.9% (*Faxonius roberti* Fetzner & Taylor, 2018/ *F. wagneri* Fetzner & Taylor, 2018), 2.5% (*Cambarus johni* Cooper, 2006/ *C. franklini* Perkins, Williams & Russ, 2019 [Perkins *et al.*, 2019]), 4.71% (*C. hazardi* Loughman, Henkanaththegedara, Fetzner & Thoma, 2017 [Loughman *et al.*, 2017]/ *C. guenteri* Loughman, Henkanaththegedara, Fetzner & Thoma, 2017 [Loughman *et al.*, 2017], and 6.3% (*Faxonius barrenensis* Rhoades, 1944/ *Faxonius bellator* Bloom, Blanton, Schuster, and Blanton, 2019 [Bloom *et al.*, 2019]). Mathews *et al.* (2008) reported K2P genetic distances for mitochondrial COI sequences ranging from 2.8% to 4.0% to support species delimitation among populations of the virile crayfish (*Faxonius virilis* Hagen, 1870) in Massachusetts and Kansas. Pairwise K2P genetic distance estimates separating monophyletic clades identified in our study ranged from 2.4% to 6.7%, falling within the range of estimates used to differentiate species in closely related crayfish groups.

Despite the many recent advances in DNA sequencing technology, population-level datasets needed to examine genome-wide patterns of differentiation require substantially greater investment than surveys of mitochondrial barcodes. For widely distributed taxonomic groups, exhaustive, population-level sampling across the entire range of a species may be unfeasible; targeted population-level genetic surveys, guided by mitochondrial barcodes or other independent datasets (e.g. morphological variation, color pattern differences, biogeographic barriers) can effectively identify evolutionarily-distinct taxonomic lineages. We used SNP genotyping as an independent validation tool to test taxonomic hypotheses generated using mitochondrial barcodes. Analyses of SNP genotypes were congruent with hypotheses based on phylogenetic reconstruction of COI barcodes. It is possible that mtDNA surveys can fail to reveal genetically distinct lineages that may be present in nuclear DNA, especially in cases of historical introgression (Barnes *et al.*, 2022; Couch & Hayes, 2022)

Our study uses a conservative approach to molecular-based species delimitation, as individual methods may classify population structure as species-level differences (Padula *et al.*, 2016; Sukumaran & Knowles, 2017). The identification of lineages requires concordance among multiple delimitation analyses, in addition to other lines of evidence (Padial *et al.*, 2010). We employed four single-locus delimitation methods as well as a multi-locus, coalescent-based approach for identifying unique taxonomic clades. All five methods suggest the Little Duck River (Clade I), Passenger Creek (Clade III), and Barren Fork drainages (Clade IV) are genetically distinct from *F. placidus* at Sinking Creek (type locality). In addition, the evolutionary relationships among *F. aff. placidus* (Clade II) compared to Clade I and *F. placidus* warrant future investigation because this clade lacked congruence among results from single locus mitochondrial delimitation methods and was not included in SNP genotyping validation approach. These populations likely represent evolutionarily distinct species that warrant further examination of color patterns and morphological features to improve identification in the field and the verification of museum specimens. Patterns of genetic variation observed in this study can also be used to differentiate phylogenetically informative morphological characters from traits that reflect selection and homoplasy.

Divergence of these lineages may have occurred following erosional periods of the Nashville Basin, a large depression that formed from erosion of the former Nashville Dome. These geologic events resulted in the separation of the eastern and western Highland Rims which has greatly influenced biogeography of fishes in these ecoregions (Starnes & Etnier, 1986). Many species that occur in both the eastern and western Highland Rim are absent from the Nashville Basin (e.g., Starnes & Etnier, 1986; Simmons, 2021; Near *et al.*, 2023). These geologic processes may have been responsible for similar patterns of divergence observed in where Little Duck River (Clade I), Passenger Creek (Clade III) and Barren Fork (Clade IV) are contained in the eastern and western Highland Rim, whereas Mill Creek *F. shoupi* and topotype *F. placidus* are contained exclusively in the Nashville Basin.

Further investigations are needed to address potential introductions of *F. placidus* and the putative new taxa identified herein. Records of *F. placidus* outside of their historical range in the middle and upper portions of the Tennessee River are considered to be the result of introductions based on the known distribution of this species; specimens from these drainages are cataloged as introduced in the TWRA and Tennessee Valley Authority (TVA) research collections. Supporting evidence

for introductions in the upper and middle portions of the Tennessee River includes the presence of many other introduced crayfish that are in some areas syntopic: *Procambarus acutus* (Girard, 1852), *P. clarkii* (Girard, 1852), *Faxonius juvenilis* (Hagen, 1870), and *F. virilis*. Additionally, erroneous identification of *F. placidus* may have led to false conclusions regarding the species' natural range. Numerous records of *F. placidus* in tributaries of the Tennessee River in Alabama may be incorrectly identified and actually be *F. forceps* instead of *F. placidus* (PLH *et al.*, unpublished data).

Future directions

Results from our study highlight the need for additional fine-scale sampling and molecular surveys of understudied crayfish groups. Our survey of *F. placidus* was restricted to portions of the Tennessee and Cumberland river drainages, and it is likely that expanded surveys across the broader distribution of this species will reveal additional cryptic lineages. Furthermore, disjunct populations of Clade III should be surveyed at a finer scale using genome-wide markers. Additional studies are also needed to examine potential introduced populations of *F. placidus* in the upper and middle Tennessee river drainages. The three newly identified clades will be examined further using an in-depth examination of morphological characteristics to establish diagnostic traits needed for the descriptions of these putative species.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Table. Bioinformatic summary of four different SNP filtering parameters.

S2 Table. Within population summary statistics.

S3 Table. Results of Evanno's Delta K method used to determine the optimal number of populations based on Bayesian assignment tests.

S4 Figure. Plot of Bayesian Information Criteria and a-score optimization results used to determine the optimal number of clusters for the DAPC analysis.

S5 Figure. Scatter plot of PC1 vs. PC2 from principal component analysis of SNP genotypes.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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