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Extensive regional endemism and cryptic diversity in the Tennessee and Kentucky, USA populations of the burrowing crayfish *Cambarus deweesae* (Bouchard & Etnier, 1979) (Decapoda: Astacidea: Cambaridae) as revealed by molecular genetics

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ABSTRACT

The southeastern United States is a global hotspot for crayfish biodiversity, with more than 300 described species in the region. Some of this diversity is unfortunately being threatened by anthropogenic activities and nearly one fifth of the North American crayfish species are currently threatened with extinction. Efforts to protect crayfish species have been hindered by a lack of information regarding their taxonomy, distribution, and conservation status. Here we target populations of the burrowing valley flame crayfish, *Cambarus deweesae* (Bouchard & Etnier 1979) for molecular taxonomy investigation. This species was originally known from the Clinch and Emory subdrainages in eastern Tennessee but it is currently listed as state endangered. The reporting of additional populations in Tennessee and Kentucky, however, has led to uncertainty about its conservation status. We analyzed sequence data from three mitochondrial genes (COI, 12S rRNA, and 16S rRNA) and from one nuclear gene (GAPDH) to decipher taxonomic questions regarding 15 crayfish populations, including 13 populations that are morphologically similar to *C. deweesae* (type locality *sensu stricto*). Combined analysis of all four genes demonstrated reciprocal monophyly for 14 out of 15 populations surveyed. Species delimitation methods, including GMYC and ABGD, identified between 11 and 13 new distinct genetic entities based on sequence divergence at the mitochondrial COI gene. Molecular results are combined with information on morphology and distribution in order to resolve taxonomic uncertainties within *C. deweesae* and its close relatives. The study highlights the need for fine-scale investigations into the phylogeography of North American burrowing crayfishes.

Key Words: conservation, morphology, mtDNA, phylogeny, species delimitation

INTRODUCTION

Molecular tools are increasingly being used to resolve taxonomic challenges posed by cryptic species. Morphologically similar, yet genetically differentiated taxa appear to be more common in some groups. Specifically, recent molecular studies in the crayfish genus *Cambarus* Erichson, 1846 has led to a dramatic increase in the number of described taxa, many of which were originally defined based solely on morphological criteria (Mathews *et al.*, 2008; Helms *et al.*, 2015; Schuster & Taylor, 2016). Approximately 20% of the described species of *Cambarus* are primary burrowers that exhibit semi-terrestrial lifestyles forming complex burrow systems (Guiasu, 2009). Burrowing crayfishes have been understudied with respect to their ecology, morphology, and genetic diversity, in part due to the greater effort required to sample and observe these species. Inaccurate species identification impedes efforts to conserve, study, and manage biodiversity. This is unfortunate in the case

of primary burrowing crayfishes as they are disproportionately threatened by loss of habitat and other anthropogenic-induced changes. Burrowing crayfishes currently comprise 15% of total crayfish species diversity, while representing 32% of the critically imperiled crayfish species (Welch & Eversole, 2006). Information regarding the genetic structure of morphologically ambiguous populations is urgently needed in order to effectively address the conservation needs of this group.

The conservation status of the valley flame crayfish, *Cambarus deweesae* (Bouchard & Etnier, 1979), has been under review based on a recent discovery of additional populations outside of its known range. This species is described as a primary burrower, known to construct complex burrows in fields composed of hydric soils where the water table is close to the surface (Taylor & Schuster, 2004). The type locality of *C. deweesae* is in Anderson County, Tennessee and its distribution were originally thought to be restricted to the Clinch and Emory subdrainages. The species was listed as state endangered in 2000 based on this limited range. Reports of collections in central and southeastern Kentucky (Taylor & Schuster, 2004), however, led to a reevaluation of its species status (Taylor *et al.*, 2007). *Cambarus deweesae* was subsequently reaffirmed as state endangered by the Tennessee Fish and Wildlife Commission in 2017. These newly discovered, isolated populations were initially assigned to *C. deweesae* based on similarities in morphology and habitat, even if the different populations exhibit three basic color variations, uniform red, brown with varying degrees of orange, and uniform blue. Additional populations morphologically similar to *C. deweesae* were also discovered in eastern Tennessee, yet exhibit marked coloration differences from *C. deweesae* at the type locality (RFT & DIW, unpublished data) (Fig. 1). Given the prevalence of cryptic taxa within *Cambarus* and the conservation needs of this group, these populations warrant further taxonomic investigation.

We describe phylogenetic data and morphological variation from three populations of *C. deweesae* and nine populations that are morphologically similar to *C. deweesae*, all located in Tennessee and Kentucky. We used DNA sequence data from multiple mitochondrial genes and a single nuclear gene to generate a phylogenetic hypothesis of the relationship between populations. Multiple molecular-based species delimitation methods were then used to identify genetically unique populations that warrant further investigation using morphological and ecological criteria. These results are discussed in the context of their conservation and management implications.

MATERIALS AND METHODS

Collections

Crayfish were collected from 15 sites in Tennessee (11 sites) and Kentucky (4 sites) (Table 1, Fig. 2). These collection sites included 12 populations that were morphologically similar to *C. deweesae*, one population of *C. striatus* from the type locality in Davidson County, Tennessee, and two populations of *C. cymatilis* from Bradley County, Tennessee. Active burrows were located and then excavated using hands and shovels. The chimneys were removed and a large diameter hole, approximately 30–60 cm, was opened to the level of standing water (water table), usually 25–50 cm below ground surface. Any lose soil or debris blocking the burrows entrance was then removed by hand, followed by agitating the water within the burrow for a few seconds, after which the collector watched for sweeping antennae. If there was no visible response from the crayfish, excavation continued until the specimen was collected or the collector abandoned the burrow. Tissue samples were collected in the field by preserving a single walking leg in 100% ethanol. All samples for DNA extraction were stored at 4°C.

Molecular methods and sequence analyses

DNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) using a modified protocol that preserved both genomic DNA and RNA. We selected four loci for sequencing; these included three mitochondrial genes (COI, 16S, and 12S) and a single nuclear glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. We also generated sequences from part of the nuclear gene 28S; however, this gene was invariable across nearly all populations and was therefore not included in the final analyses. Primer sequences and amplification strategies are summarized in Table 2. Conditions for polymerase chain reactions (PCR) were the same for all primer sets: 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. This program ended with a final extension of 10 min at 72°C.

PCR products were cleaned prior to cycle sequencing reactions by exonuclease I/shrimp alkaline phosphatase (New England Biolabs, Ipswich, MA, USA) and used for bi-directional Sanger sequencing on an ABI 3730 automated sequencer (MCLAB, South San Francisco, CA, USA). Sequence chromatograms were imported and visualized using SEQUENCHER vers. 5.2 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were initially aligned using the software MAFFT (Katoh *et al.*, 2005). Alignments were refined by eye and protein-coding genes were examined for stop codons using the software Bioedit vers. 7.2.5 (Hall, 1999). Alignments were unambiguous for protein coding genes COI and GAPDH. Heterozygous sites in the nuclear gene GAPDH were identified as double peaks in both forward and reverse chromatograms. To identify pseudogenes, we examined the sequences for indels and translated sequences to search for stop codons. Sequence alignments were then imported into MEGA 10.0.4 (Kumar *et al.*, 2018) and Kimura-2-parameter (K2P) genetic distances (Kimura, 1980) were used to calculate within and between population sequence divergence estimates.

Phylogenetic analyses

Phylogenetic reconstructions were estimated using both maximum likelihood and Bayesian optimality criteria. Maximum-likelihood analyses were performed using the software RAxML (Stamatakis, 2014) on the CIPRES Science Gateway (Miller *et al.*, 2010) under the GTR+G model. Each gene was initially analyzed independently. Individual gene trees were compared in order to identify well-supported topological conflicts between phylogenies based on different genes. The concatenated alignment was partitioned by loci allowing for gene specific rates of substitution and nucleotide composition. We retained the tree with the best ML score and nodal support was estimated through 1,000 bootstrap replications.

Bayesian phylogenetic reconstructions were performed using MrBayes 3.2.1 (Huelsenbeck & Ronquist, 2001) also on the CIPRES Science Gateway. The best model of substitution was selected by Modeltest (Posada & Crandall, 1998) as performed by MEGA 10.0.4 (Kumar *et al.*, 2016) using the Bayesian information criterion (BIC). The concatenated analysis was partitioned by locus and the best-fit model of evolution was applied to each partition. If the best-fit model was not available in MrBayes then the next most complex model was selected as per the author's suggestion. The Markov chain Monte Carlo (MCMC) algorithm ran for 10,000,000 generations, sampling every 1,000 generations. Two independent runs were performed and the resulting trees were combined after the deletion of a burnin (first 1,000 trees). A majority-rule consensus tree was generated and nodal support was estimated by posterior probabilities.

Species delimitation

We implemented two methods to test species boundaries based on our COI dataset. The Automatic Barcode Gap

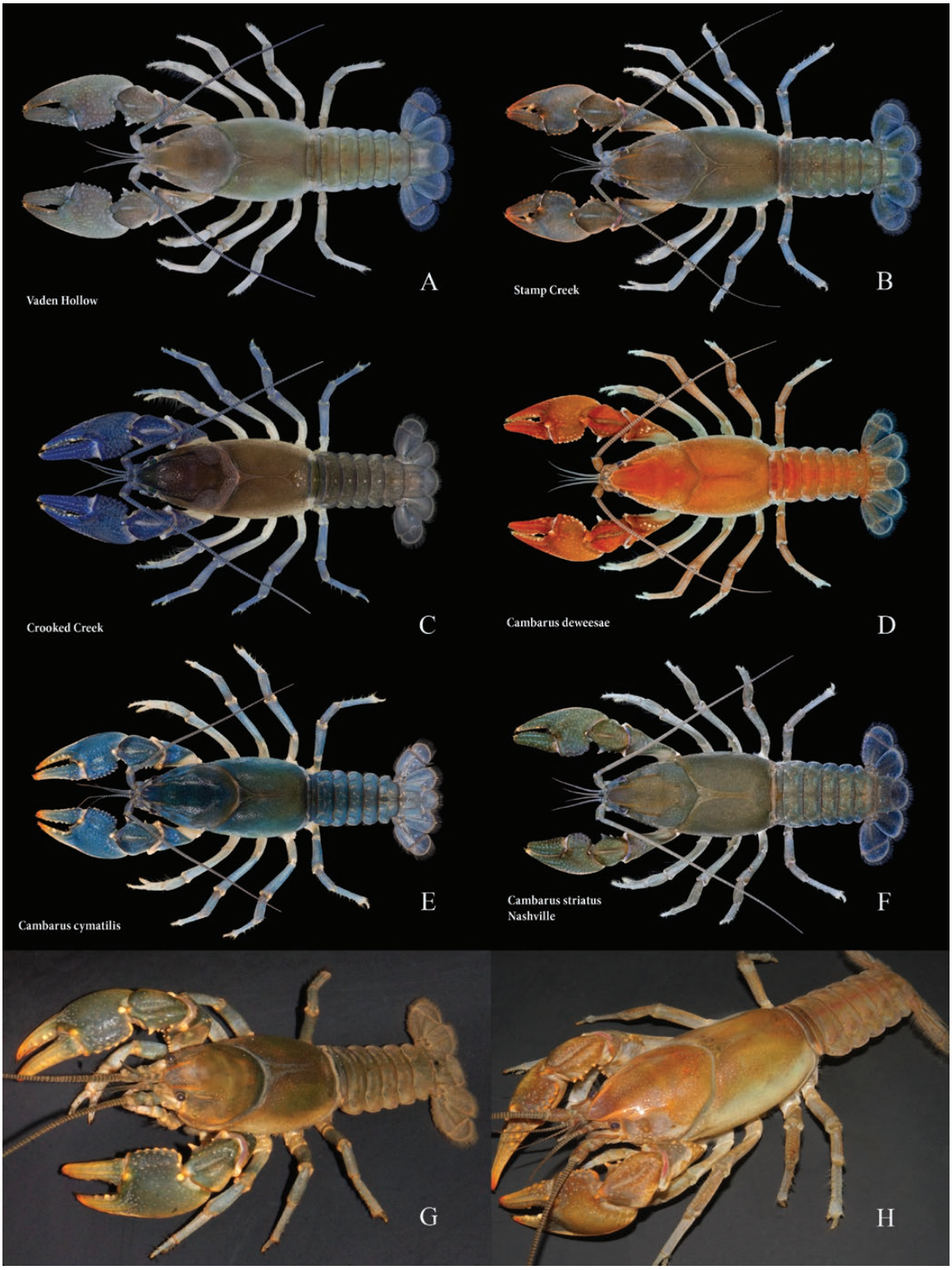

Figure 1. Variation in color among sampled populations of *Cambarus deweesae* in Tennessee and Kentucky: Vaden Hollow (**A**), Stamp Creek (**B**), Crooked Creek (**C**), TriCounty (**D**), *C. cymatilis* (**E**), *C. striatus* (**F**), Dripping Spring (**G**), Roaring Paunch (**H**). This figure is available in color at the *Journal of Crustacean Biology* online.

Discovery (ABGD) (Puillandre *et al.*, 2012) method was first used to preliminarily identify species based on sequence divergence using COI sequences from all populations excluding outgroup sequences from *C. cymatillis*. This method ranks pairwise genetic distance values from smallest to largest in order to identify a gap in the distribution of distances. ABGD then recursively applies this threshold value to the remaining groups in order to obtain finer partitions until no further partitioning can be performed. The ABGD method was performed using default parameters (P_{min} = 0.001, P_{max} = 0.100,

Table 1. Taxa, locations, and sample sizes for *Cambarus* crayfishes collected in Tennessee and Kentucky. Site codes correspond to location identifiers on the map in Figure 1.

Taxa	Site description	Latitude	Longitude	Sample size	Site code
C. deweesae	Tri County	36° 1' 15.60"	–84° 18' 43.632"	3	1
C. deweesae	Lee Baptist Ch.	35° 57' 24.59"	–84° 32' 26.38"	3	2
C. deweesae	Coal Creek	36° 13' 27.12"	–84° 9' 24.01"	3	3
C. cf. deweesae	Beaver Creek	36° 5' 30.23"	–83° 54' 32.65"	4	4
C. cf. deweesae	Stamp Creek	35° 46' 0.62"	–84° 32' 18.02"	3	5
C. cf. deweesae	Whitley County	36° 41' 58.49"	–84° 3' 52.14"	3	6
C. cf. deweesae	Crooked Creek	36° 34' 18.73"	–84° 10' 44.29"	3	7
C. cf. deweesae	Roaring Paunch	36° 35' 24.94"	–84° 26' 17.23"	3	8
C. cf. deweesae	Metcalfe County	37° 6' 1.08"	–85° 38' 4.02"	3	9
C. cf. deweesae	Nelson County	37° 45' 49.82"	–85° 40' 41.84"	3	10
C. cf. deweesae	Vaden Hollow	35° 37' 43.97"	–84° 7' 12.65"	4	11
C. cf. deweesae	Dripping Spring	37° 29' 47.04"	–84° 28' 1.416"	1	12
C. striatus	Shelby Bottoms	36° 10' 1.93"	–86° 43' 32.40"	2	13
C. cymatilis	Bradley County 1	35° 6' 27.32"	–84° 46' 38.21"	3	14
C. cymatilis	Bradley County 2	35° 9' 15.55"	–84° 50' 10.46"	3	15

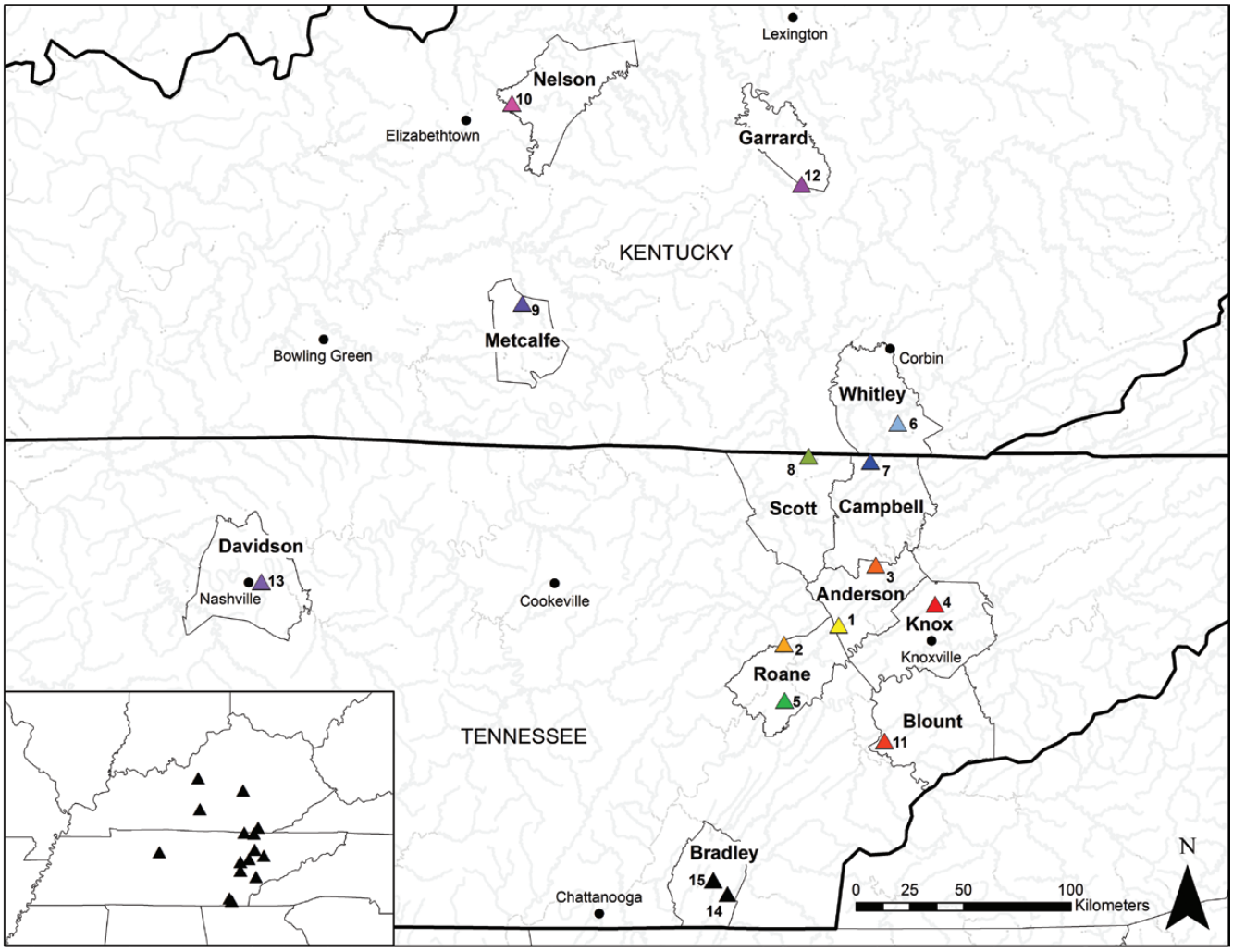

Figure 2. Locations for the 15 populations sampled. Numbers next to triangles correspond to site numbers listed in Table 1. Triangle shades or colors correspond to species delimitation in Figure 4. This figure is available in color at the *Journal of Crustacean Biology* online.

steps = 1, bins = 20) except that the relative gap width (X) was set to 1. The Kimura-2-parameter model was used to compute distances.

In the second method, we applied the general mixed Yule-Coalescent model (GMYC) to identify species boundaries from the COI dataset (Pons *et al.*, 2006). GMYC is a likelihood-based

Table 2. Primer sequences, sources, and amplification strategies used to amplify all loci in the Tennessee and Kentucky crayfishes analyzed.

Locus	Sequence 5'-3'	Source	Amplification strategy
COI			
COI-44F	5'- TGGTACTTGGGCTGGGATAG-3'	*Cambarus* alignment	Nested reaction
COI-687R	5'- AAATTTCGATCCGTCAACAA3'	*Cambarus* alignment	First amplification
COI-622R	5'-CAAAATAAATGTTGGTAGAGAATR-3'	*Cambarus* alignment	COI-44F/COI-62 Second amplification
			COI-44F/COI-687R
16S			
16sf-cray	5'- GACCGTGCKAAGGTAGCATAATC-3'	Buhay & Crandall, 2005	16Sf-cray/16S-492R
16S-492R	5'- CACACCGGTCTGAACTCAAAT-3'	*Cambarus* alignment	
12S			
12SF	5'- GAAACCAGGATTAGATACCC-3'	Mokady *et al.*,1994	12SF/12SR
12SR	5'- TTTCCCGCGAGCGACGGGCG-3'	Mokady *et al.*, 1994	
GAPDH			
G3PCq157F	5'-TGACCCCTTCATTGCTCTTGACTA-3'	Mathews *et al.*, 2008	Nested reaction
G3PCq981R	5'-ATTACACGGGTAGAATAGCCAAACTC-3'	Mathews *et al.*, 2008	First amplification
			G3PCq157F / G3PCq981R
G3P-40F	5'- CTCGACTCATGGTGTGTTCAA-3'	*Cambarus* alignment	Second amplification
G3p-809R	5'- TGTCTTGCTCAGCTGGATACC-3'	*Cambarus* alignment	G3P-40F / G3p-809R

method that identifies shifts in the branching patterns on a tree from coalescent processes (intraspecific events) to speciation events (interspecific events). GMYC uses an ultrametric tree as an input file. We calculated an ultrametric tree using Bayesian inference as performed by BEAST 2.5.1 (Bouckaert *et al.*, 2014) under a GTR+G, constant population-size coalescent, strict clock model. MCMC analyses were run for 100 million generations, sampling every 10,000 generations. A summary tree was generated using TREE ANNOTATOR. The GMYC analysis was performed on the consensus tree using the single threshold model in the R package SPLITS (Monaghan *et al.*, 2009).

Isolation by distance

We tested for a correlation between genetic distance at COI (K2P genetic distance) and geographic distance (km) for the 13 populations by means of a Mantel test (Mantel, 1967) using the R package ecodist. The two outgroup populations belonging to *C. cymatilis* were not included in this analysis. Confidence intervals were estimated using non-parametric bootstrapping with 1,000 permutations (Goslee & Urban, 2007).

Morphology methods

All specimens collected were compared for discrete morphological differences (no measurements were taken) in all body parts. Each collection was first compared to *C. deweesae* material from the Coal Creek, Lee Baptist Church, and TriCounty sites (see Table 1 for location of sites) and then to each of the other collections. A database was developed and the state of each character for each collection was entered for those character states showing potential differences. The database was then sorted to find shared and not-shared characters. The final characters selected for examination were the presence or absence of a small spine on the mesial ramus of the uropod, development of the suborbital angle (classified as obsolete, obtuse, or acute following Hobbs, 1972), number and development of tubercles on the dorsal merus of pereiopod 1 (the cheliped), number of tubercle rows and number of tubercles on the mesial margin of the chelar propodus, development and extent of tuberculation on the dorsal surface of the chelae, length of M-I central projection in comparison to the mesial process, the presence or absence of a subapical notch on the central projection, areola open or closed, and the curvature of the margins of the annulus ventralis.

RESULTS

A total of forty-four specimens were sequenced for four genes, including the mitochondrial genes COI (439 bp), 16S (~384bp), 12S (~371 bp), and the nuclear gene GAPDH (716 bp) (GenBank accession MK773646–MK773806); the aligned concatenated dataset was 2,010 bp in length. This alignment included six specimens from two populations of *C. cymatilis* that were used to root the trees. Examination of protein coding genes COI and GAPDH did not identify any stop codons or indels, suggesting that our primers did not amplify pseudogenes. The best models of substitution for each gene based on the BIC analysis were HKY+G for COI and 16S, T92+G for 12S, and Jukes-Cantor for GAPDH.

The COI gene had the highest average K2P sequence divergence (8.1%), followed by 16S (6.1%), 12S (6.0%), and GAPDH (0.5%). At the COI gene, the average K2P sequenced divergence between populations ranged from 1.8% (Tri County/Lee Baptist Church) to 10.7% (Dripping Spring/Coal Creek). Distances within populations averaged 0.0% (Beaver Creek, Coal Creek, LBC, Metcalfe, and Nelson) to 0.4% (Roaring Paunch and Tri-County) (Table 3). The nuclear gene GAPDH provided little resolution for this group compared to the mitochondrial datasets. Only 19 out of 716 sites in the GAPDH dataset were variable; of those, 11 sites were parsimony informative.

Phylogenetic analyses

Comparisons of individual gene trees did not identify any strongly supported conflicts between the four genes, therefore all loci were included in the concatenated analyses. Maximum likelihood and Bayesian phylogenetic reconstructions resulted in identical topologies as depicted in Figure 3. Posterior probabilities resulting from Bayesian analysis generally resulted in higher nodal-support values than bootstrap percentages generated from ML analyses. For both analyses, all individuals were reciprocally monophyletic with respect to their population, having high bootstrap and posterior probability support, with the exception of the two *C. striatus* individuals collected from Shelby Bottoms in Davidson County, Tennessee. These two individuals were paraphyletic with respect to the three individuals collected from the Metcalfe County, Kentucky and were separated by 5.9% K2P sequence divergence.

Both analyses identified the same four major clades with high support. Clade I (*C. striatus* group) is composed of individuals from the two sites in central Kentucky (Nelson and Metcalfe counties)

Table 3. Mean pairwise percentage Kimura-two-parameter inter and intrapopulation distances (bold, diagonal) between 13 sampled populations of *Cambarus* crayfishes in Tennessee and Kentucky. Outgroup populations from *C. cymatilis* were excluded from analysis.

Populations	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Beaver Creek	**0.00**												
2. Coal Creek	8.40	**0.00**											
3. Crooked Creek	10.00	10.40	**0.12**										
4. Dripping Spring	9.80	10.70	8.70	---									
5. Lee Baptist Church	7.80	2.90	10.00	9.10	**0.00**								
6. Metcalfe County	9.50	8.80	6.60	8.00	7.40	**0.00**							
7. Nelson County	8.30	9.10	9.40	9.10	8.00	5.70	**0.00**						
8. Roaring Paunch	8.30	7.10	7.30	10.30	6.90	8.70	8.60	**0.37**					
9. Stamp Creek	7.70	5.30	8.70	9.40	4.40	8.00	9.30	5.40	**0.14**				
10. *C. striatus*	9.40	8.50	7.50	8.10	7.60	3.10	5.00	8.10	8.50	**5.89**			
11. Tri County	8.30	2.70	10.20	10.20	1.80	7.60	8.30	6.30	5.60	7.80	**0.37**		
12. Vaden Hollow	2.70	7.10	9.60	9.80	6.70	7.80	7.20	7.80	6.80	7.80	6.40	**0.25**	
13. Whitley County	9.40	8.80	3.20	7.30	9.00	6.50	8.80	7.30	8.60	7.10	9.10	9.40	**0.12**

and also includes the two *C. striatus* individuals collected near the type locality in Davidson County in central Tennessee. Clade II included all individuals from Crooked Creek in Campbell County, Tennessee and all individuals from Whitley County just north of the Kentucky border. The single individual from Dripping Spring in Garrard County, Kentucky is basal to clade II; additional sampling is needed to determine if this population represents its own unique clade. Clade III included the Beaver Creek and Vaden Hollow populations in Knox and Blount counties in east Tennessee, respectively. Clade IV (*C. deweesae* group) included individuals collected from five populations in east Tennessee; the Roaring Paunch population is the basal member of Clade IV, which also includes the Lee Baptist Church, Tri County (*C. deweesae* type locality), Coal Creek, and Stamp Creek populations in east Tennessee in the region of Roane, Anderson, and Knox counties. Assuming a molecular clock and a rate of 1.4% sequence divergence per million years (based on Knowlton & Weigt, 1998), these distances indicate that the earliest divergences among Clades I-IV occurred more than seven million years ago, much earlier than has been reported for other cryptic crayfish complexes (Mathews *et al.*, 2008).

Species delimitation

The ABGD analysis of the COI dataset delimited 13 species. Each of the 13 geographic populations was designated as a unique taxonomic unit with the exception of *C. striatus* (striatus 1), which grouped with other individuals from Metcalfe County. The other *C. striatus* sample (striatus 2) was identified as its own unique entity. In the GMYC analysis of the COI dataset the likelihood of the GMYC model was significantly higher than the null model ($P < 0.001$) indicating the presence of multiple species (Fig. 4). A total of 11 clusters and 14 entities were identified. Results were identical to results from ABGD analysis except that each *C. striatus* individual was identified as its own unique genetic entity.

Isolation by Distance

Mantel tests did not show a significant correlation between geographic distance and genetic distance at COI. The Mantel *R*-value was 0.234 ($P = 0.179$) (Fig. 5).

Morphological considerations

Very little variation exists in the overall gross morphology of the body and abdomen among the 13 populations examined. Variation within populations for the character states examined could not be assessed as only one, or at most, two collections were available for most of the populations used in our genetic analysis. Examination of the rostrum and antennal scale yielded no usable traits. All antennal scales displayed the same shape, narrow with a straight mesial edge. The rostra showed some small differences but the within-population variation for some collections was significant enough to make those differences unusable. Distinguishing character states within our limited material was nevertheless observed. Variations in gonopod structure exists but was not used as not all populations had M-I specimens available for examination of this structure. As a result, only suborbital angles and tubercle ornamentation of the chelae and meri were used to separate the populations herein considered. Supplementary material Table S1 provides a summary of the nine character states that display differences between the populations examined.

DISCUSSION

Molecular tools are increasingly being used to uncover biodiversity that may not be evident using morphologically-based taxonomic criteria. Certain taxonomic groups harbor a greater number of cryptic species. Several recent studies have shown that North American crayfishes are much more diverse than previously thought (Larson *et al.*, 2016; Matthews *et al.*, 2008). We postulate that among crayfishes, primary burrowers may be particularly likely to exhibit regional endemism as it is thought opportunities for dispersal are limited, making gene-flow between populations unlikely. The results herein support the contention that a burrowing, semi-terrestrial lifestyle does create barriers to dispersal. Our multi-gene phylogenetic analyses combined with species delimitation suggest that populations currently considered a single burrowing-crayfish species, *C. deweesae*, include morphologically cryptic species diversity. Multiple lines of evidence, including subtle morphological and/or color differences, geographic distances, and molecular distances suggest that these populations, once considered a single panmictic species, represent multiple distinct species groups. These results have implications for conservation and management planning as several of these proposed species are very narrowly distributed and may warrant a higher protective status.

Species delimitation methods utilizing mitochondrial sequence data can provide evidence for proposing species boundaries. The mitochondrial gene COI is used most frequently for this purpose; the vast number of available COI sequences gives this gene added comparative value. Surveys of the enormous inventory of COI sequences have revealed that sequence variability

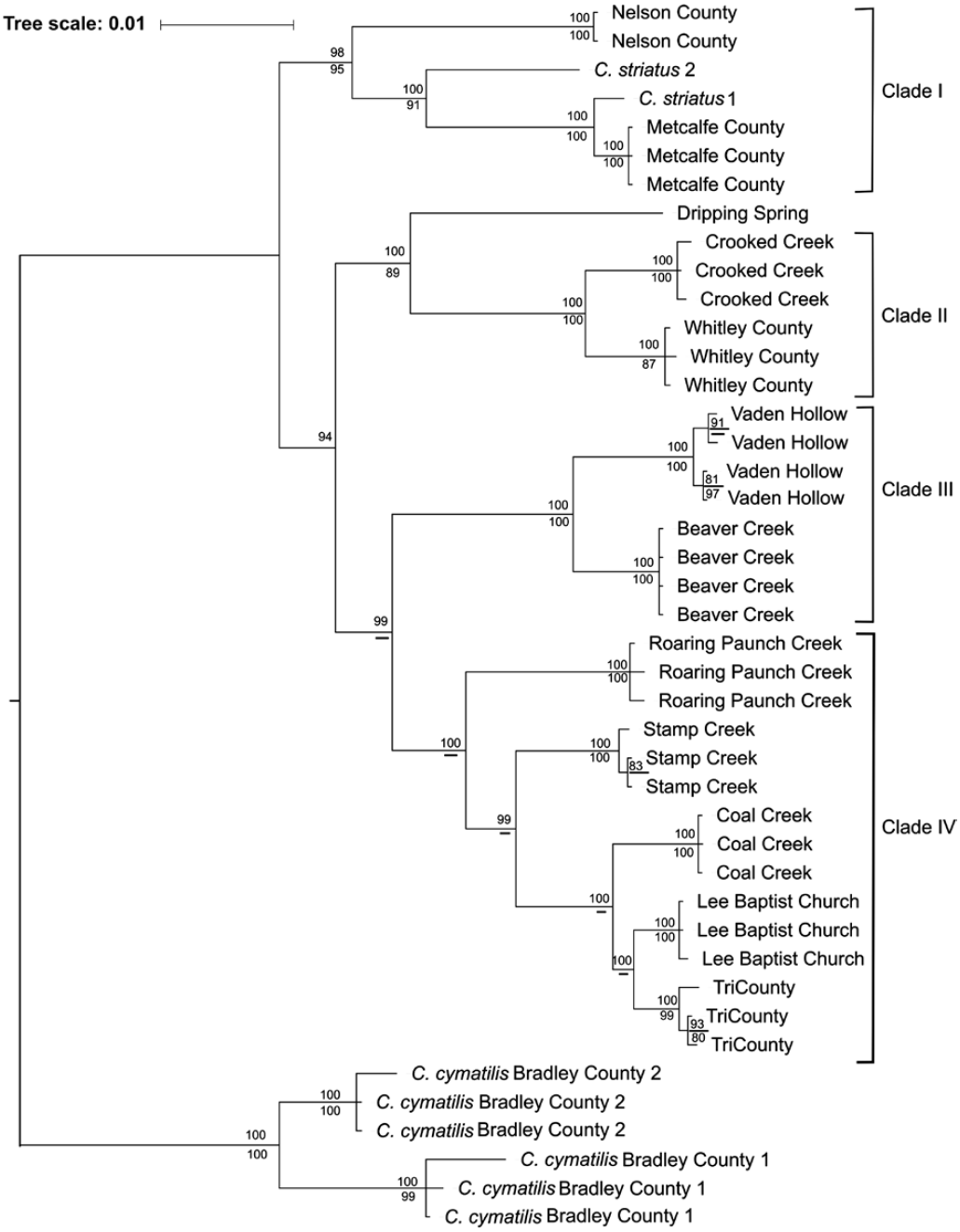

Figure 3. Bayesian tree of the combined COI/16S/12s/GAPDH genes from crayfishes collected from the 15 populations listed in Table 1. Numbers above nodes indicate Bayesian posterior probabilities. Numbers below nodes indicate bootstrap support from the maximum likelihood analysis. Only probability and bootstrap values are noted.

within species is much lower than divergence estimates between species, a pattern referred to as the "barcoding gap" (Čandek & Kuntner, 2015). Based on observations at COI within and between North American birds, Hebert *et al.* (2004) proposed the 10X rule where a threshold of interspecific genetic divergence is equal to 10× the average K2P intraspecific distance. The average K2P intrapopulation distance across all populations we sampled (excluding outgroup sequences) was 0.13%. Ten times this value would give a minimum interspecific genetic distance of 1.30%. This threshold is low for interspecific distances compared to other studies in decapod crustaceans (Silva *et al.*, 2011). The average K2P pairwise genetic distance between populations in our study was 7.78%. Populations at Lee Baptist Church and Tri-County, both near the type locality, had the smallest divergence (K2P = 1.76%). The largest genetic distances were between Coal Creek and Dripping Spring population (K2P = 10.65%).

Molecular-based species delimitation should ideally make use of a wide range of analytical methods and species inferences should be limited to cases where there is congruence among results (Carstens *et al.*, 2013). The GMYC and ABGD species delimitation methods were in general agreement with the implications drawn from the 10X method regarding the genetic distinctiveness of the sampled populations. Both ABGD and GMYC delimited each collection site as a unique taxonomic unit. The sole disagreement between these methods was in the placement of the two *C. striatus* individuals; the large genetic distance between these

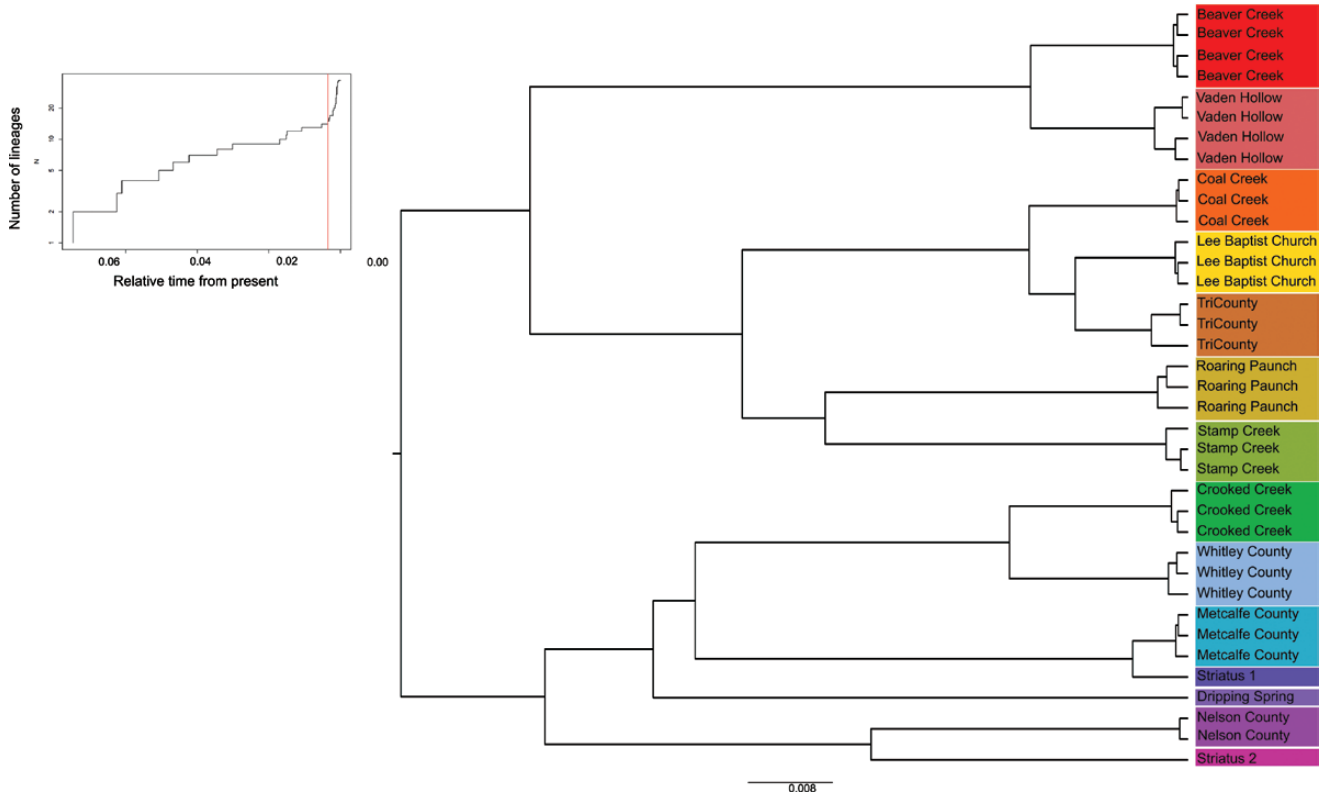

Figure 4. Phylogeny resulting from GMYC analysis of COI sequence dataset. The fourteen distinct genetic entities identified by GMYC are each represented by a different shade or color. The inset shows a lineage through time plot based on the ultrametric tree obtained for COI. The transition from interspecies to intraspecies branching events is designated by a red line. This figure is available in color at the *Journal of Crustacean Biology* online.

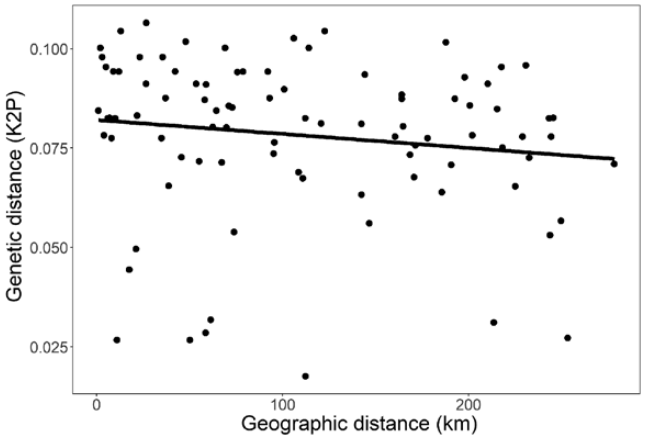

Figure 5. Results from the Mantel test showing the correlation between COI K2P genetic distances and geographic distances.

two individuals warrants further investigation of genetic variation within this population. The genetic diversity across populations we observed may merit new taxonomic descriptions, but should be evaluated in the context of other factors such as ecological exchangeability, zoogeography, and morphology.

Regarding morphological characters, several caveats need to be considered in the discussion of distinguishing character states. First and foremost, there are very few collections for some of these populations and the range of variation is little known. It is possible that character state integrations will be found that will cloud their usefulness for making comparisons. There were no first-form male specimens for two of the populations so gonopod structure could not be effectively evaluated. It is possible that inclusion of gonopod characteristics could result in major revisions in our concept of population separation. For example, one population (Vaden Hollow) has a gonopod that is distinctly different from all other forms except the type locality of *C. striatus* (Supplementary material Table S1). Furthermore, the differences between strongly, moderately, and weakly developed characters is, as yet, not well defined. The question of how much variation exists within populations is also poorly defined, thus conclusions about morphological differences between populations cannot be drawn. From the material at hand the most promising characters are presently the extent and development of tubercles on the propodus and merus of the chelipeds. One population, Dripping Spring, strikingly differs from all other populations in two character states, openness of the areola and the number of tubercle rows on the mesial palm of the chelae. No other population or species examined in this study has an open areola or only one row of tubercles on the mesial palm. Both genetic and morphological evidence clearly point to a separate-species status. A formal description should be undertaken if the zoogeography of the population supports an isolated and morphologically unique status. Our second possibility of a separate species is the Crooked Creek population. Genetic and zoogeographic evidence indicate full-species status but additional morphological evidence is needed, primarily for the other populations, before reaching a conclusion.

Our current understanding of morphological variation between the sampled populations is a hinderance to drawing further conclusions about the separate-species status for the groups. Bouchard (1978) reported on the variation among the known members of the subgenus *Depressicambarus*. His work focused in part on the character states found in first-form male gonopods, and he documented extensive variation in gonopod structure in the case of *C. striatus*. We were not able to employ gonopod structure since

not all collections had first-form males available. The tuberculation of the first pereiopod (cheliped) was the most helpful in distinguishing populations. Every character observed was shared by two or more of the populations except for the open areola and the single row of palm tubercles found in the Dripping Spring population (Supplementary material Table S1). This population does not appear to warrant to be morphologically considered a member of the *C. deweesae* (Clade IV) or *C. striatus* (Clade I) groups but would be best considered a potential relative of *C. dubius*. The Dripping Spring population was included in this study because it possesses a gonopod similar to that found in C. *deweesae*. Aside from this exception, the remaining populations shared most of the character states at some level. Considering the body and abdomen (pleon), the similarities are very high and differences were subtle and not quantified. This pattern of evolutionary divergent yet morphologically cryptic species diversity has been found in other species of burrowing crayfishes (Schultz *et al.*, 2009). We propose that the evolutionary constraints exerted by a burrowing lifestyle have placed strong limitations on body form.

One unexpected finding was the placement of *C. striatus* within the other *deweesae*-like populations and the large divergence separating individuals within the same population. Bouchard (1978) described a new species (*C. pyronotus*) from Florida, splitting it from *C. striatus* but did not conclude that the remaining variation observed in what he considered *C. striatus* warranted species-specific recognition. More recent phylogenetic analyses based on mitochondrial and nuclear sequence data has demonstrated non-monophyly of *C. striatus* populations and have suggested that this broadly distributed taxon is actually a diverse species complex (Breinholt *et al.*, 2012). Our results support this finding, and suggest that morphological conservatism may be common in burrowing crayfishes. The morphological variation initially thought to be non-significant may actually be indicative of a large genetic separation. Fine-scale sampling and genetic analysis spanning the known distribution of *C. striatus* is needed to clarify the degree of cryptic diversity in this lineage and the relationships between populations within this species complex.

Our results are highly relevant to the management and conservation of crayfish biodiversity in this region. *Cambarus deweesae* was originally known from only four locations in Anderson and Roane counties, Tennessee and was listed as state-endangered in 2000 due to this limited distribution (Tennessee Department of State, 2000). The subsequent discovery of a number of populations in the Cumberland Plateau region in Tennessee and Kentucky led to the listing of this species to "currently stable" by the American Fisheries Society (Taylor, 2007), although its status as state-endangered was reaffirmed by the Tennessee Fish and Wildlife commission in 2017 (TWRA, 2018). Our study suggests that these *C. deweesae* populations do not represent a single widespread, panmictic species, but rather genetically distinct populations, each of which exhibits a limited distribution. Short range endemics are particularly vulnerable to extinction as they frequently possess narrow habitat tolerances and demonstrate poor dispersal abilities (Burnham & Dawkins, 2013). Anthropogenic activities pose a threat to the persistence of burrowing-crayfish populations (Welch & Eversole, 2006; Loughman *et al.*, 2012). These threats primarily include conversion of open space and wetland habitats to hard surfaces, dewatering of inhabited areas, and loss of saturated soils due to groundwater alterations. Elevation of the conservation status of *C. deweesae* should be considered in order to preserve this structured genetic variation. Our study also points to the urgent need for further geographic sampling of other burrowing species such as *C. cymatilis* and *C. striatus* across their currently known distribution.

SUPPLEMENTARY MATERIAL

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

Supplementary material Table S1. Summary of morphological differences (nine character states) between 13 sampled populations of *Cambarus* crayfishes in Tennessee and Kentucky.

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