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Insights on the evolution and conservation of Appalachian burrowing crayfishes, with the description of a new species of *Cambarus* Erichson, 1846 (Decapoda: Astacidea: Cambaridae)

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ABSTRACT

A new species of crayfish, *Cambarus nyx* n. sp., is described from the Kentucky/Tennessee border in the Dissected Appalachian Plateau sub-ecoregion of the US. Of the recognized species of *Cambarus* Erichson, 1846, it is morphologically most similar to *Cambarus deweesae* Bouchard & Etnier, 1979, found primarily in the Southern Limestone/Dolomite Valleys and Low Rolling Hills sub-ecoregion. *Cambarus nyx* n. sp., is distinguishable from other recognized congeners, except for *C. deweesae*, by the strongly serrated lateral margin of the propodi of the chelae, closed areola, two rows of palmar tubercles, truncate rostrum with convergent margins, and Form I male (MI) gonopod having a central projection shorter than the mesial process. The new species differs from *C. deweesae* in coloration (blue vs. red, respectively) and size at maturity only. There is considerable genetic, morphometric, and color variation among populations herein considered as members of the new species. The species has a limited range and highlights once more the need for increased conservation efforts for North American crayfishes. Genetic analysis of *C. nyx* n. sp. sheds light on the evolutionary process occurring in the crayfishes of the Appalachian Mountains after the origin of *Cambarus*.

KEY WORDS: Appalachian uplift, Crustacea, evolutionary significant units (ESUs), Miocene, Pliocene

INTRODUCTION

Primary burrowers in the crayfish genus *Cambarus* Erichson, 1846 are disproportionately threatened by loss of habitat and climate change as they are habitat specialists, restricted to environments with a shallow, accessible water table (Eversole & Welch, 2013). Such habitats are also frequently isolated in distribution on the landscape. Primary burrowers made up 15% of described North American crayfish species in 2006, yet they account for 32% of species ranked as critically imperiled (Welch & Eversole, 2006). Effective conservation policies and management actions aimed at the protection of burrowing crayfishes have been impeded by a lack of basic information on their taxonomic diversity and distribution. The fossorial lifestyle of burrowers,

coupled with fewer informative morphological characters, has led to an underestimate of their taxonomic diversity. An integrative approach, combining morphology, geography, ecology, and genetic variation, is a promising strategy to prioritize conservation efforts aimed at protecting crayfish diversity.

Molecular tools are effective at resolving taxonomic uncertainties in morphologically ambiguous species complexes of burrowing crayfishes. In a recent survey Hurt *et al.* (2019) used molecular-based species delimitation analyses to identify 11 genetically distinct taxonomic units among the surveyed populations of 15 burrowing crayfish populations they had morphologically identified as *C. deweesae*. These populations included Kentucky *C. deweesae* Bouchard & Etnier, 1979,

populations reported by Taylor & Schuster (2004). Amongst the resulting clades, phylogenetic reconstructions resulted in one monophyletic clade that included all individuals sampled from Whitley County, Kentucky and Campbell County, Tennessee (100% Bayesian posterior probability and maximum likelihood, ML, bootstrap support). The average pairwise Kimura-two-parameter (K2P) distance between *C. deweesae* specimens sampled at the type locality in Anderson County, Tennessee) and the populations in Whitley and Campbell counties was 9.7%. Although criteria for taxonomic delimitation based on nucleotide divergence at the mitochondrial COI gene has not been established, this K2P genetic distance estimate far surpassed distance estimates used to differentiate sister-species in related crayfish groups (Mathews et al., 2008; Dillman et al., 2010; Johnson et al., 2021). Based on the findings of Hurt et al. (2019), geographic separation, distinct morphological character states and coloration, *C. aff. deweesae* from Whitley and Campbell counties warrant species-level description. At a finer-scale, results from Hurt et al. (2019) also showed evidence of divergence between the two populations within this clade corresponding to a possible geologic barrier created by a continuous Taylor Mountain/King Mountain ridge. The Campbell and Whitley counties populations occur east and west of Taylor Mountain, respectively; individuals from these two sites were reciprocally monophyletic in phylogenetic reconstructions and the average K2P distance between these populations was 3.2%.

Here we describe a new burrowing species of *Cambarus* using morphological characters, distinguishing color patterns, and molecular sequence analysis. *Cambarus nyx* n. sp. Is known from six populations in Campbell County, Tennessee and Whitley County, Kentucky. We also define evolutionary significant units (ESUs) within *C. nyx* n. sp. That correspond to geographic barriers and warrant consideration for protective status. Our analysis builds on recent studies that demonstrate the utility of color and color pattern for identifying taxonomic boundaries (Schuster, 2020). Our results demonstrate the fine-scale endemism that characterizes North American burrowing crayfishes and the need for an integrative approach for understanding crayfish diversity.

MATERIALS AND METHODS

Collections

Specimens were collected from the area of Campbell County, Tennessee and Whitley County, Kentucky by first searching for suitable burrowing crayfish habitat, i.e. areas around waterbodies such as streams, ditches, wetlands, and fields with aquatic vegetation growing in them. The presence of rushes (Juncaceae) was a strong indicator frequently associated with burrowing crayfish colonies. An effort was made to sample crayfish colonies widely separated from each other. Once a colony was selected, its extent was estimated and those parts of the colony best suited for collecting were identified. Burrows with the greatest and most recent activity at the burrow entrance were then excavated to the water table or 15 cm, whichever was least. Water was added to bring the water level up to the excavation bottom if water was not encountered. Water in the burrow then was pumped by hand and if a crayfish emerged it was captured. An effort was made to capture at least five specimens

and create the least amount of disturbance as possible. Tissue samples were collected in the field by extracting gill filaments and placing them in individual labeled vials containing 95% ethyl alcohol. Locality information for the six sites from which tissue was collected is given in Table 1.

Molecular methods and analysis

DNA was extracted using the EZNA Tissue DNA Kit (Omega Biotek, Norcross, Georgia, USA) using the manufacturer's instructions. Two mitochondrial genes (16S and COI) were selected for sequencing; these genes have been shown to be informative for both species-level and population-level phylogenetic reconstructions in related groups (Mathews et al., 2008, Hurt et al., 2019). Primer sequences and conditions for PCR amplification follow Hurt et al. (2019). 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 (manufacturer, etc.). Sequence chromatograms were imported and visualized using SEQUENCHER v. 5.2 (Gene Codes Corporation, city, etc.). Sequences were aligned using the Clustal W multiple alignment method as implemented in Bioedit 7.2.5 (Hall 1999) Alignments were refined by eye and protein-coding genes were examined for stop codons.

Phylogenetic reconstructions were estimated using ML 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. 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) as well as on the CIPRES Science Gateway (Miller et al., 2010) (<https://www.phylo.org/index.php/>). 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 (https://en.wikipedia.org/wiki/Bayesian_information_criterion). 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 (https://mrbayes.sourceforge.net/mrbayes3.2_manual.pdf) then the next most complex model was selected as per the author's suggestion. The Markov chain Monte Carlo (MCMC) (https://en.wikipedia.org/wiki/Markov_chain_Monte_Carlo) 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. Pairwise genetic distances between populations of *C. nyx* n. sp. and *C. deweesae* sensu stricto (type locality) and between populations of *C. nyx* on either side of Taylor Mountain were estimated using the K2P method as implemented in MEGA X (Kumar et al., 2016). The K2P model was selected for pairwise distance estimates as it allows for cross-study comparisons; the K2P model is the most commonly used model for taxonomic identification and species delimitation based on the mitochondrial COI gene (Collins et al., 2012).

Table 1. Locality information and GenBank accession numbers for genetic material used in this study.

Species	Site ID	Site description	Latitude	Longitude	Number specimens	GeneBank Acc. COI/16S
<i>Cambarus nyx</i> n. sp.	MUC	Mulberry Cr. east of Taylor Mountain	36.69958°N	-84.06448°W	3	OQ127514/
						OQ146756,
						OQ127515/
	CUR	Cumberland R. flood plain east of Taylor Mountain.	36.725161°N	-84.129022°W	2	OQ146757,
						OQ127516/
						OQ146758
	PAR	Patterson Cr., C. <i>nyx</i> east of Taylor Mountain, type locality	36.69958°N	-84.06448°W	3	OQ127513/
						OQ146755,
						OQ127512/
	CRC	Crooked Cr. west of Taylor Mountain	36.57364°N	-84.18118°W	3	OQ146754
						MK773685/
						MK773684/
	LEC	Little Elk Cr. west of Taylor Mountain	36.529038°N	-84.196308°W	2	MK773683/
						MK773684/
						MK773765
WOF	Wolf Cr. west of Taylor Mountain	36.659694°N	-84.14188°W	2	MK773766,	
					MK773683/	
					MK773765	
WOF	Wolf Cr. west of Taylor Mountain	36.659694°N	-84.14188°W	2	MK773767,	
					MK773684/	
					MK773766,	
WOF	Wolf Cr. west of Taylor Mountain	36.659694°N	-84.14188°W	2	MK773658/	
					MK773740,	
					MK773657/	
WOF	Wolf Cr. west of Taylor Mountain	36.659694°N	-84.14188°W	2	MK773739,	
					MK773656/	
					MK773738	
WOF	Wolf Cr. west of Taylor Mountain	36.659694°N	-84.14188°W	2	OQ127511/	
					OQ146753,	
					OQ127510/	
WOF	Wolf Cr. west of Taylor Mountain	36.659694°N	-84.14188°W	2	OQ146752	
					OQ127509/	
					OQ146751,	
WOF	Wolf Cr. west of Taylor Mountain	36.659694°N	-84.14188°W	2	OQ127508/	
					OQ146750	
					OQ146750	

Table 1. Continued

Species	Site ID	Site description	Latitude	Longitude	Number specimens	GeneBank Acc. COI/16S
<i>C. deweesae</i>	POC	Poplar Cr., type locality	36.021364°N	-84.311821°W	3	MK773678/ MK773760, MK773677/ MK773759, MK773676/ MK773758
<i>C. cymatilis</i>	CYM	Bradley County, Georgia	35.107373°N	-84.776790°W	3	MK773652/ MK773735, MK773651/No seq., MK773650/ MK773734.1

Morphological methods

Morphometric measurements were taken from 33 adult individuals of *C. nyx* **n. sp.** from Campbell County, Tennessee, and Whitley County, Kentucky (13 MI, 4 Form II males (MII), and 16 females). Measurements were taken from mature individuals only (those > 25 mm total carapace length (TCL)). The following morphometric characters were measured using hand-held digital calipers (nearest 0.01 mm, L, length, W, width, D, depth); total carapace L (TCL), W (CW), and D (CD), anterior W; abdominal L (AL) and W (AW); areola L and W; chelae L, W, and D; dactyl L; palm L; opposable propodus L and W. The following morphometric distances were measured using an ocular micrometer: post orbital ridge W; rostrum L, rostrum W at base, midpoint, and acumen angle; antennal scale L and W; gonopod L and W at umbo; central projection L and W, mesial process L and W; annulus ventralis L and W, and L of associated denticles. The following meristic characters were recorded from the chelae: number of palmar tubercle rows, number of tubercles per row, number of tubercles and their size on the opposable margins of the chelae dactyl and propodus, dactyl/propodus dorsal ridge development, lateral impression development. Number of serrations on outer edge of propodus; carapace: number of cervical spines and/or tubercles. Number of hepatic spines and/or tubercles, post orbital ridge spine or tubercle, number of areola punctations at narrowest point, estimated angle in degrees at acumen base, rostrum excavation; gonopod: presence/absence of subapical notch on central projection; extent of the overhang of the central projection of the mesial process estimated as less than, equal to, or greater than by projecting a vertical line from the tip of the mesial process to the central projection. Post orbital carapace length was calculated by subtracting rostrum length from total carapace length.

Twenty-nine morphometric ratios were calculated as follows: carapace TCL/W, W/D, TCL/D TCL/chelae L, TCL/abdomen L, rostrum L/eye Di (diameter), L/W at base; eye Di/rostrum W at eyes; areola L/TCL, TCL/areola L, areola L/W, W/L; abdomen L/TCL, TCL/abdomen W; abdomen L/W, W/L; antennal scale L/W; chelae L/TCL, L/W, W/L, W/D, chelae L/palm L, palm L/chelae W, palm L/dactyl L and vice versa, opposable propodus L(OPL)/palm L, OPL/W, opposable propodus denticule L/OPL, opposable dactyl denticules L/opposable dactyl L (ODL). Post orbital carapace length (PCL) was calculated from TCL-rostrum L.

RESULTS

Molecular results

Both Bayesian and ML phylogenetic reconstructions resulted in a monophyletic clade that included all sampled populations herein identified as *C. nyx* (100% posterior probability and bootstrap support (Fig. 1)). Populations of *C. nyx* were separated from *C. deweesae* sensu stricto by an average of 9.7% K2P genetic distance. Within *C. nyx* **n. sp.**, populations separated by Taylor Mountain were reciprocally monophyletic in both Bayesian and ML analyses with high posterior probability and bootstrap support. The average K2P genetic distance between populations separated by Taylor Mountain was 3.4%, similar to estimates from Hurt *et al.* (2019).

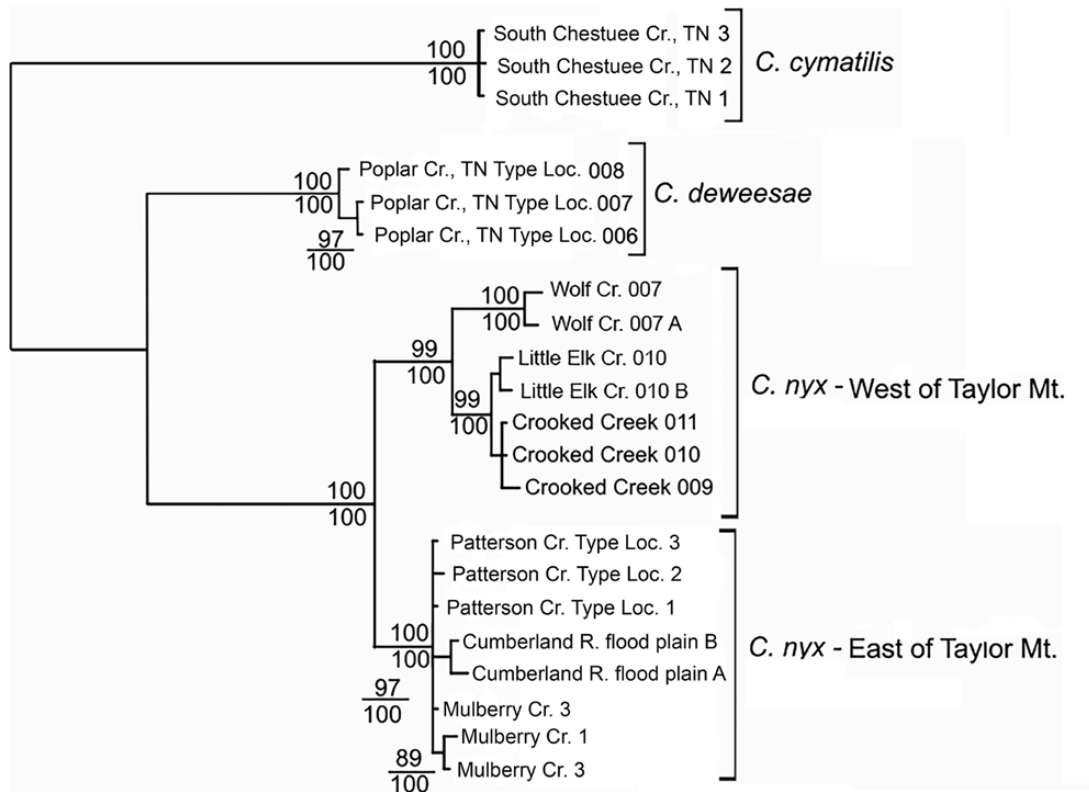


Figure 1. Bayesian phylogenetic tree from concatenated COI/16S nucleotide sequence from *Cambarus nyx* n. sp. ($N = 15$) and *C. deweesae* (type locality, $N = 3$). Sequences from *C. cymatilis* were used as an outgroup. Individual identifications used in genetic analysis correspond to labels listed in Table 3. Numbers at nodes indicate posterior probabilities (above branch) and bootstrap support from ML analysis (below branch). Only posterior probabilities or bootstrap values > 70 are shown.

SYSTEMATICS

Family Cambaridae Hobbs, 1942

Genus *Cambarus* Erichson 1846

***Cambarus nyx* n. sp.**

(Figs. 2–4, Table 2)

Cambarus deweesae Taylor & Schuster, 2004: 77 (in part); Hurt et al., 2019: 1.

Type material: Holotype: OSUMC 10886, allotype: OSUMC 10887, morphotype: OSUMC 10888.

Type locality: Kentucky, Whitley County, ditch to Patterson Creek of Cumberland River downhill from cemetery adjacent Kentucky Rt. 904 and just east of Powers Cemetery Rd., 2.14 km NW of Nevisdale, 9.64 km SE of Williamsburg (36.69958°, -84.06448°). It appears that in this area the population is comprised of individuals that previously left their burrows, which are located in a seep uphill, and found themselves at the bottom of the hill where they established new burrows in a suboptimal habitat. The hillside, though a cemetery, has an extensive area of seepage (a more optimal burrower habitat) with burrows that likely form the main population of the colony.

Disposition of the type material: The holotype, allotype, and morphotype are in the collection of The Ohio State University Museum of Biological Diversity Crustacean Collection, Columbus, OH, USA (OSUMC 10886, 10887, 10888

respectively). Paratypes are housed in the United States National Museum of Natural History, Crustacean Collection, Washington, D.C. (USNM 1620900), and Carnegie Museum of Natural History, Pittsburgh, Pennsylvania (CMNH 39028).

Additional material examined: A total of forty specimens from eight sites were examined. KENTUCKY, Whitley County: RFT-15-021 (type locality), ditch to Patterson Creek of Cumberland River downhill from cemetery, 2.14 km NW of Nevisdale, 9.64 km SE of Williamsburg, 36.69958°, -84.06448°, Roger F. Thoma (RFT), Zachary B. Thoma coll., 30 April 2015, 6 MI, 3 MII, 3 F, 2 ovigerous F, 2 juvenile M, 3 juvenile F; Eastern Kentucky University (EKU) Jar #1, 11.3 km north of Tennessee Stateline just off I75, from burrows, 36.675423°, -84.133141°, E.L. Lauder milk coll., 19 April 1991, 1 MI; EKU Jar #8, behind Wolf Creek Church/cemetery approximately 2.9 km from I75 on Kentucky Rt. 628, 36.66154°, -84.161595°, S.A. Walker coll., 6 April 1996, 1 MI, 2 F; USNM 207571, Dinkins Farm, Clear Creek, 0.8 km S.E. of Williamsburg, University of Tennessee coll., 21 May 1983, 1 MI, 1 MII, 1 F; RFT-19-003, Wolf Creek River Road 0.16 km S of Wolf Creek crossing, 1.1 km E of Mountain Ash, 36.659694°, -84.141881°, RFT, J.K. Grow (JKG) coll., 10 April 2019, 2 MI, 3 F, 1 Fjuv.; RFT-21-007, hay field adjacent Wolf Creek east of Wolf Creek River Road, 36.659806°, -84.142175°, RFT, JKG coll., 17 April 2021; RFT-21-008, wet meadow adjacent Kentucky Rt. 92 at 90° bend, 36.725161°, -84.129022°, collectors FT, JKG coll., 17 April 2021; RFT-21-009, wet meadow and ditch to Mulberry Creek

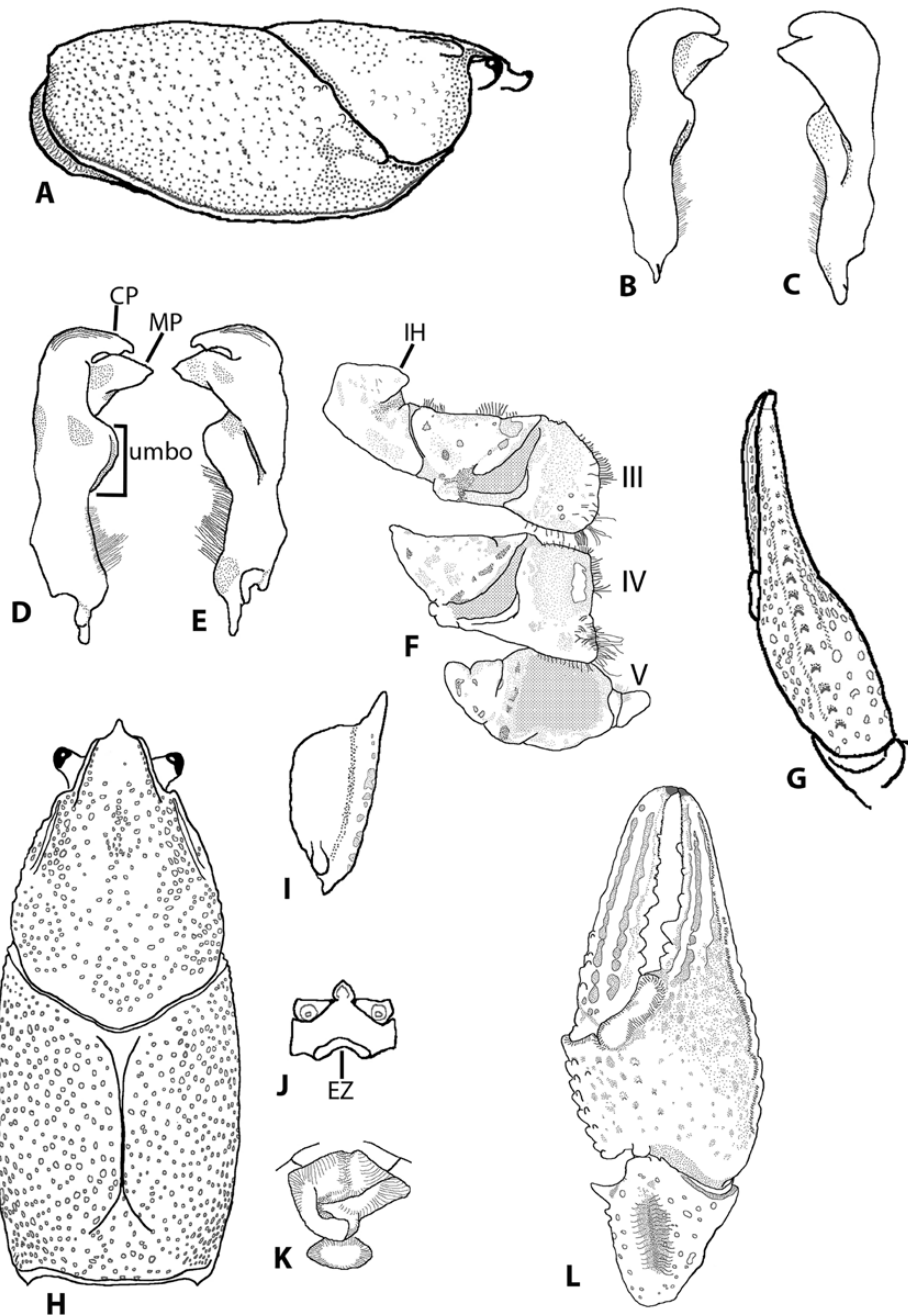


Figure 2. *Cambarus nyx* n. sp. (A, D–J, L holotype MI (OSUMC 10886); B, C from morphotype MII (OSUMC 10888), K from allotype female (OSUMC 10887). Lateral aspect of carapace (A); lateral aspect of MII gonopod (first pleopod) (B); mesial aspect of MII gonopod (first pleopod) (C); lateral aspect of MI gonopod (first pleopod) (D); mesial aspect of MI gonopod (first pleopod) (E); basal podomeres of right III thru V pereopods (F); lateral aspect of right chela (G); dorsal aspect of carapace (H); antennal scale (I); epistome (J); annulus ventralis (K); dorsal aspect of distal podomeres of right cheliped (L). central process (CP), mesial process (MP), ischial hook (IH), epistomal zygoma (EZ).

on east side of Loudon Road, 36.727351°, –84.046908°, RFT, JKG coll., 18 April 2021; TENNESSEE, Campbell Co.: OSUMC 8856, wetland field E of Crooked Creek on Wooldridge Lane, 4.9 km SW of Jellico, 2.2 km NW of Newcomb, RFT, Max A. Luehrs coll., 21 September 2012, 3 MI, 1 F, 4 Fjuv.; Tennessee Wildlife Resource Agency (TWRA) #2044, burrows in wet ditch near Crooked Creek along Old Wooldridge near junction with Wooldridge Lane, 36.57364°, –84.18118°, Carl E. Williams (CEW), Z. Harbison coll., 16 June 2016, 2 MI, 1 MII, 1 F;

TWRA #2107, burrows along Coontail Branch, off Hwy 297, 300 m. upstream of crossing on Baker Lane, 21.4 air km SW of Jellico, 36.45066°, –84.29985°, CEW coll., 18 September 2018, 5 F; RFT-21-010, pasture adjacent Little Elk Creek of Elk Creek of Crooked Creek west of Newcomb Pike (Tennessee Rt. 297), 36.529038°, –84.196308°, RFT, JKG coll., 18 April 2021.

Diagnosis: Pigmented with well-developed eyes. Carapace subovate in dorsal view (Fig. 2H) (TCL 2.00–2.24× CW), near

Table 2. Measurements (mm) of type material of *Cambarus nyx* n. sp.

Character	Holotype	Allotype	Morphotype
Carapace			
Height	17.41	16.29	13.60
Width	16.41	16.56	14.14
Length	35.82	34.44	30.11
Areola			
Length	15.52	15.04	12.58
Width	0	0	0
Chelae			
Length lateral margin of chela	23.40	21.13	18.50
Length mesial margin of palm	7.72	6.86	6.35
Width of palm	11.57	10.26	9.36
Thickness of palm	6.21	6.03	5.56
Tubercle rows on mesial palm: number per	2:7/5	2:6/5	2:6/4
Length of dactyl	15.84	14.37	12.34
Length opposable propodus	11.75	10.32	8.24
Width	4.76	4.41	4.12
Gape of fingers	0.90	0.70	0.50

cylindrical in cross section (CW 0.93–1.11× CD), TCL 1.40–1.88× chelae L, TCL 1.11–1.30× abdominal L, TCL 0.95–1.58× abdominal W. Abdomen W 0.34–0.54× abdominal L. Areola closed, no punctations, L 0.39–0.57× TCL (mean 43%). Suborbital angle (Fig. 2A) obsolete, no hepatic spines but some tubercles, normally 2–5 small cervical tubercles. Chelae (Fig. 2L) L 0.53–0.71× TCL, chelae W 0.47–0.58× chelae L, chelae D 0.53–0.64× chelae W, palm L 0.31–0.36× chelae L, dactyl L 1.57–2.37× palm L, strong dorsal ridges on fingers, weak lateral impression, lateral serrations (Fig. 2G) 12–21 (mean 16) formed by strong punctations with setae, palm with 2–3 rows of tubercles (one MII with 4), first row 6–8 (mean 7), second 2–6 (mean 4), third 2–3 (mean 2), mesial most row strongly developed to nearly spiniform. Opposable margin of propodus with 2 basal small tubercles followed by single enlarged tubercle, normally followed distally by 2 or 3 smaller tubercles, occasionally final almost spiniform tubercle.

Antennal scale (Fig. 2I) widest at or distal to midpoint, length 2.1–3.3× W. Rostrum margins (Fig. 2H) moderately thickened, convergent, angled distally 40–80° to midline, moderately to strongly excavated. Postorbital ridges moderately developed, lacking tubercle or spine, eye diameter 28–60% rostrum W at eye W.

Central projection (Fig. 2D) shorter than mesial process, with subapical notch. Annulus ventralis (Fig. 2K) asymmetrical, normally with curved sinistral margin and straight diagonal dextral margin running under left margin or mirror image thereof. Midpoint trough running from anterior margin to midpoint, then leading into an asymmetrically placed fossa. Sub-rhomboid post annulus ventralis sclerite.

Holotypic male (MI): Carapace subovate, TCL 2.18× W (Fig. 2H, Table 2), cylindrical in cross section, width 0.94× depth. Abdomen narrower than cephalothorax. Areola length 43.3% of total carapace L (Table 2). Rostrum from lateral aspect deflected

ventrally from dorsal view with slightly converging margins, moderately thickened, margins converging distally at 45° angles to acumen, distal tip upturned, not reaching distal margin of penultimate podomere of antennular peduncle, upper surface of rostrum slightly concave with few punctations basally. Subrostral ridge present. Postorbital ridge distinct with longitudinal sulcus, surface directed laterally, lacking distal spine or tubercle. Suborbital angle obsolete. Branchiostegal spine represented by small, pointed tubercle. No cervical spines but 2 small tubercles at lateral midpoint, small, isolated tubercle ventral to midpoint, and 8 small tubercles just posterior of branchiostegal spine. Hepatic region with few scattered small tubercles. Remainder of carapace with shallow punctations dorsally and laterally. Abdomen slightly shorter than carapace, pleura rounded. Cephalic section of telson lacking spines in caudolateral corners. Proximal podomere of lateral uropod with small spine on distolateral margin of mesial lobe; mesial ramus of uropod with weak median rib ending distally with short distomedian spine not overreaching margin of ramus, laterodistal spine of ramus small.

Cephalomedian lobe of epistome (Fig. 2J) broadly lancelet-like, pointed, proximal margins raised, tip forming slight point, ventral surface not setiferous, main body of epistome lacking shallow fovea; epistomal zygotoma arched (Fig. 2EZ). Ventral surface of antennal peduncle lacking setae, proximal podomere lacking acute spine at base of distal third. Antennal scale (Fig. 2I) 3.0× as long as broad, broadest at distal third, straight in lateral view, mesial border convex, with short setae on distal third of mesial margin; distal spine strong, reaching to distal midpoint of antennular peduncle and distal tip of rostrum, directed slightly lateral.

Third maxilliped reaching distal margin of penultimate antennal peduncle, all segments with moderately long setae of moderate density, ischiopodite with 2 rows of flexible setae on ventrolateral and ventromesial margins; mesial margin of

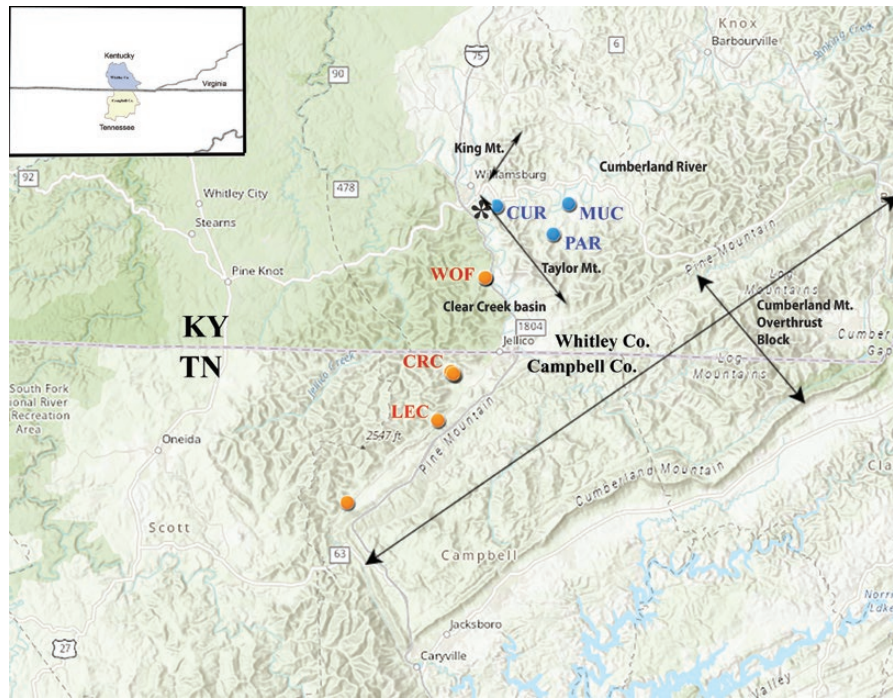


Figure 3. Known locations of *Cambarus nyx* n. sp.: eastern population (upstream Taylor Mountain), blue stars; western population (Clear Creek), orange dots; *, USNM collection. General position of the Cumberland Overthrust Block, Taylor Mountain, and King Mountain indicated by black lines with arrows. Upper left insert shows general range of *C. nyx* n. sp. within the Kentucky-Tennessee-Virginia border area, Whitley County, Kentucky in blue, Campbell County, Tennessee in yellow. Labeled points represent sites from which genetic material was examined and correlate with Table 1.



Figure 4. *Cambarus nyx* n. sp. (left) and *Cambarus deweesae* (right). Photo by C. Williams.

ischium with row of 21 spines, most distal largest, spines decreasing in size moving proximally.

Right chela (Fig. 2L), L 0.65 × TCL; W 0.49 × chela L; palm L 0.33 × chela L; dactyl L 2.05 × palm L. Dorsal surface of palm studded with punctations, mesial margin with 2 rows of tubercles, 7 in mesial most row, 5 in second row, moderate lateral impression at base of fixed finger, ventral surface punctate, not forming impression; lateral margin of chela with 11 deep punctations forming serrated edge, deepest proximally, decreasing in depth distally; mesial ventral palm surface smooth, 2 small tubercles on distal articular rim opposite base of dactyl; small

subpalmar tubercle. Opposable margin of fixed finger with row of 2 small (one enlarged, one small), and pointed tubercle progressing from base (considerable gap between fourth and fifth tubercles). Opposable margin of dactyl with row of 7 tubercles, first 3 smaller than fourth, fifth through seventh very small; single row of minute denticles extending distally from third distal most tubercle; mesial surface (outer edge) of dactyl with row of 4 tubercles basally. Dorsomedian longitudinal ridges on both fingers well developed.

Carpus of cheliped (Fig. 2L) with distinct furrow dorsally, punctations both mesial and lateral; mesial surface with distal

Table 3. Mean pairwise percentage Kimura-two-parameter distances between *C. nyx*, n. sp. Populations west of Taylor Mountain (CRC, LEC, and WOC), east of Taylor Mountain (MUC, CUR, PAR), and for *C. deweesae* sensu stricto (POC). Outgroup populations from *C. cymatilis* were excluded from analysis. Mean within group population distances are shown in bold font on the diagonal. See Table 1 for localities associated with site ids.

		<i>C. nyx</i> n. sp.			
		CRC, LEC	WOC	MUC, CUR, PAR	POC
<i>C. nyx</i> n. sp.	CRC, LEC	0.004			
	WOC	0.025	0.006		
	MUC, CUR, PAR	0.034	0.041	0.004	
<i>C. deweesae</i>	POC	0.100	0.100	0.092	0.004

large spiniform tubercle subtended by small tubercle and additional small tubercle proximally; 2 very small tubercles dorsal to mesial spiniform tubercles, ventral surface with tubercle on distal articular rim. Merus with row of 4 dorsomesial tubercles on distal half, ventrolateral row of 3 tubercles with gap between second and third moving from distal end, ventromesial row of 10 spiniform tubercles, distal most directed distally. Ischium and proximal segments lacking tubercles. Ischium of third pereopod (Fig. 2F) with hook extending proximally over basioischial articulation (Fig. 2F), not opposed by small tubercle on basis. Coxa of fourth pereopod (Fig. 2F) with moderately developed, setiferous, vertically disposed, caudomesial boss on anterior positioned ridge.

First pleopods contiguous at base, not reaching coxa of third pereopod; MI central projection (Fig. 2D, E) shorter than mesial process with weak subapical notch, angled caudally at approximately 90° to main shaft; mesial process larger than central projection, laterally compressed, tapered to tip, directed caudally at angle of 90° to main shaft; caudal knob absent.

Allotypic female: Except secondary sexual characters, differing most notably from holotype by: carapace width 1.01× carapace depth; carapace L 1.20× abdomen L; chela L 0.61× carapace L; antennal scale L 2.47× width; opposable margin of dactyl with 5 tubercles distal to largest (forth) tubercle, denticles on distomesial surface of dactyl not extending as far proximally but extending further on propodus distomesial surface. Rostrum excavation deeper, eye diameter 0.38× rostrum W at eye, rostrum L 4.17× eye diameter. Fifteen serrations on lateral margin of chela. Annulus ventralis (Fig. 2K) as in diagnosis.

Morphotypic male, MII: Differing from holotype in following respects: carapace length 2.21× depth, width 1.04× depth; chela length 0.61× carapace length, depth 0.59× width, width 0.51× length, palm length 0.77× opposable propodus length, lateral margin with fifteen serrations; abdomen length 2.92× width; rostrum deeply excavate; one cervical tubercle; antennal scale length 2.07× width. Secondary sexual characteristics as in diagnosis.

Etymology: The species is named after the Greek Goddess of the night Nyx to highlight the species habit of appearing on the surface only at night, and its blue coloration. Nyx is frequently depicted clothed in blue at night. The common name of Midnight Blue Burrower is proposed.

Range: *Cambarus nyx* n. sp. is found on alluvial sediments of the Clear Fork Valley west of Taylor Mountain (Crooked, Wolf, and Little Elk creeks) and Cumberland River basin east of Taylor

Mountain (Patterson Creek, Mulberry Creek, Cumberland River flood plain) in Whitley County, Kentucky and Campbell County, Tennessee within the Dissected Appalachian Plateau Sub-Ecoregion (Fig. 3). The distribution of the new species is very narrow, being limited to alluvial sediments primarily of the Clear Fork basin. It has not yet been found north of the Cumberland River. The alluvial sediments are of Quaternary Period age and are derived from surrounding Middle Pennsylvanian bedrock.

Color notes: Some populations of *C. nyx* n. sp. are a monochromatic dark blue, especially in the north of its range, whereas some can have a brownish blue carapace and abdomen, mostly in the south of the range (Fig. 4).

Considerable overlap exists in coloration. Immature and younger individuals display less intense blue coloration and background browns show through over the body and chelae throughout the range. Mature males and females have chelae that are always blue, but can have an all blue, partly blue, or mostly brown carapace. In general, a brown color is most prevalent on the abdomen, with an all-blue abdomen being infrequent.

Variation: For such a narrowly distributed species, considerable intraspecific variation has been observed. Coloration is variable (see above). On the chelae the number of palmar tubercle rows varies from two to four with the first row varying from six to eight and the second row displaying two to seven. The serrations on the lateral chelae margin vary from moderately to strongly serrate and the number of serrations varies from twelve to twenty-one. Surprisingly, there is no sexual variation in the width of the abdomen and only small variation in the size of the chelae. The angle of the rostral edges to the acumen can vary from 40 to 80°. Genetic variation of the two groups examined (east and west of Taylor Mountain) is 0.033032 for the Kimura-two-parameter interpopulation distance. We have not identified any morphological variation that is restricted to either genetic population.

Size: The largest individual observed was a female with a 40.6 mm total carapace length.

Life history: All specimens were collected in April, May, June, and September. MI, MII, and females were observed in all four months. Two ovigerous females were recorded in April.

Crayfish associate: *Lacunicambarus thomai* (Jezerinac, 1993) has been collected with *C. nyx* n. sp.

Remarks: The new species is most closely related to an undescribed burrowing species found in Metcalfe County,

Kentucky that was in turn found most closely related to *Cambarus striatus* Hay 1902, from the Nashville, Tennessee area (type locality of *C. striatus*) by Hurt *et al.* (2019). Hurt *et al.* (2019), recovered Clade II, containing the new species herein described, along with Metcalfe and Nelson County material plus Clade I containing Nashville *C. striatus* material. All of Clades I and II are from areas west of Pine Mountain and Walden Ridge in the Cumberland River basin. Clade III and most of Clade IV (containing *C. deweesae* sensu stricto) are from the Tennessee River basin east of Walden Ridge. The one exception is an undescribed species from the Roaring Paunch basin of the South Fork Cumberland River. The Roaring Paunch population is geographically closest to *C. nyx* n. sp. but genetically closest to the Stamp Creek population of *C. cf. deweesae*.

Cambarus nyx n. sp. displays the typical chelae ascribed to the former subgenus *Depressicambarus* (Hobbs, 1969). There is a wide range of variation within the *Depressicambarus* group in the shape of the central projection of the MI gonopod from strongly to mildly recurved and long, thin, sickle shaped to short and stout (Bouchard, 1978). This variation occurs between and within species. We use the term stout central projection herein to refer to a MI central projection that is approximately 1.1× or less the width of the gonopod main shaft at the umbo (term follows Glon *et al.*, 2019) and always recurved at 90° or less. *Cambarus nyx* n. sp. belongs to the group of species within that former subgenus having a short, stout central projection recurved at 90° that is always shorter than the mesial process and equal to or not noticeably overhanging the gonopod umbo. The new species will key out as *C. deweesae* (couplet 23' in Thoma, 2016: 669). Other species similar to *C. nyx* n. sp. with a stout MI central projection are *C. harti* Hobbs 1981, *C. truncatus* Hobbs 1981, *C. obstipus* Hall 1959, and *C. cymatilis* Hobbs 1970. Distinguishing *C. nyx* n. sp. and *C. deweesae* is best by using life colors. Individual of *C. deweesae* are all red, while *C. nyx* n. sp. are all blue (Fig. 4).

Bouchard (1978) assessed the members of the former subgenus *Depressicambarus*, Hobbs 1969, redescribing the type population of *Cambarus striatus* and providing information on the many populations found in Tennessee, Alabama, and Mississippi. Bouchard did not report on the Kentucky populations other than to state the species was recorded from that state. He also did not include any specimens from the area within the range of *C. nyx* n. sp. At the time of Bouchard's publication, *Depressicambarus* encompassed eleven species. Several species within the taxon were illustrated by Bouchard as, in some populations, having a MI central projection shorter than the mesial process, as in the case of *C. cymatilis*, which was illustrated having the central projection extending just to the umbo (Bouchard, 1978: fig. 3b) and having a stout gonopod (the umbo is found on the anterior margin of the male gonopod just below the junction of the mesial process when the main shaft is flexed perpendicular to the body axis (Fig. 2D, E)). Hobbs (1981) reported on five of the species Bouchard discussed (*C. englishi* Hobbs & Hall, 1972, *C. halli* Hobbs, 1968, *C. cymatilis* Hobbs, 1970, *C. latimanus* Le Conte, 1856, *C. striatus*) and described four new species of the then subgenus from Georgia. Some of the illustrations of *C. latimanus* and *C. striatus* in Hobbs (1981) display MI central projections that are equal in length to the mesial process, whereas all other species are illustrated with central projections shorter than their associated mesial processes. *Cambarus cymati-*

lis, *C. strigosus* Hobbs, 1981, *C. harti* Hobbs, 1981, and *C. truncatus* Hobbs, 1981, are illustrated with stout gonopods similar to *C. nyx* n. sp. Bouchard & Etnier (1979) described *Cambarus deweesae*, and Taylor & Schuster (2004) reported the species from numerous localities in Kentucky, all specimens having a stout central projection shorter than the mesial process. Hurt *et al.* (2019) reported the potential presence of seven new species of crayfishes among the specimens of *C. deweesae* populations in Tennessee and Kentucky that were studied. These authors restricted *C. deweesae* to the Valley and Ridge physiographic province in Roane and Anderson counties, Tennessee, and obtained Kimura-two-parameter interpopulation distances of 9.2 to 10 % between *C. deweesae* and *C. nyx* n. sp.

Within those members of *Cambarus* with a stout central projection, *C. nyx* n. sp. and *C. deweesae* can be distinguished by the strong serrations on the lateral margins of their respective chelae. In life, *C. nyx* n. sp. is mostly blue, especially the chelae, whereas other members of the *C. deweesae* complex are dark brown, red, or a mix of browns, reds, and blues, but never with all blue chelae. So far, only coloration in life and genetic makeup can be used reliably to separate *C. nyx* n. sp. from *C. deweesae*. There are subtle differences in body and chelae robustness between the two later species but individuals of the two cannot be reliably identified using these characters. The one morphological character that shows some difference between the two species is the relationship of the chela palm with the length of the chela, dactyl, and opposable propodus (*C. nyx* n. sp. means 0.31, 0.45, 0.6; *C. deweesae* 0.34, 0.52, 0.74, respectively). The palm is generally shorter in relation to the three previous characters but there is some overlap. The dactyl and opposable propodus in *C. nyx* n. sp. (means 0.69, 0.49, respectively) are slightly longer in relation to those of *C. deweesae* (means 0.66, 0.46, respectively) when compared to chela length.

DISCUSSION

Cambarus nyx n. sp., is a narrowly distributed species and that makes it a species of conservation concern. Its total known distributional range is slightly more than 93.5 km², though the range will certainly be found to be larger, especially in the upstream reaches of the Cumberland R. downstream of Pine Mountain. For now, the species. Should be considered "Vulnerable" following Taylor *et al.* (2007) and IUCN (2001) Red List criteria. It should be monitored in both Kentucky and Tennessee. Any further efforts to Channelize streams or drain wetland areas within range of the new species that would impact groundwater supply should be prevented. In addition, populations of *C. nyx* n. sp. separated by Taylor Mountain should be designated as separate ESUs as defined by Moritz *et al.*, 1994, based on their phylogenetic distinctiveness and substantial genetic distance. This would ensure that historically isolated populations retain their unique evolutionary potential.

The evolution of *Cambarus nyx* n. sp., in relation to *C. deweesae* illustrates what will likely be found to be common among the burrowing Appalachian crayfishes. The large mean pairwise percentage K2P distances between *C. deweesae* and *C. nyx* n. sp. (Table 3) indicates a long period of separation between the two species. An approximate 110 million year period of

quiescence existed in the Central Appalachian area from 125 million mya to 16 mya when a significant uplift event began (Poag & Sevon, 1989). It is possible that a widespread ancestor of *C. deweesae* and *C. nyx n. sp.* existed in the ancient Central Appalachians prior to 16 mya. This ancestral population may have experienced isolation after the +13 mya point as the intervening terrain grew more dissected and developed greater relief. The two species and their close relatives (Hurt *et al.*, 2019) clearly have an aversion to areas of high relief (RFT, personal observation) unlike *C. dubius* sensu lato. Which is known to advance up gradient to the very tops of ridges and mountains if suitable wet habitats exist. At a later unknown date, the Taylor Mountain/King Mountain area must have risen and created an isolating landform between the east and west populations of *C. nyx n. sp.*

One question is whether the two *C. nyx n. sp.* populations are a result of dispersal or vicariance. In the southern portion of the range there is some interdigitation at Cane Gap between an unnamed tributary of Cane Creek and Patterson Creek that could possibly have served as a dispersal route to the Cumberland basin (upstream Taylor Mountain) from the Clear Creek basin. Cane Gap has a relief of 61 m over a 600 m length (approximate 1:1.5 slope) which may have been traversable by ancestral populations of *C. nyx n. sp.*

The safeguarding of *C. nyx n. sp.* presents an important reality in the conservation of North American crayfishes. Taylor *et al.*, (2006), discussed the discovery of undescribed crayfish diversity in “neglected” habitats, i.e. habitat types that have been little examined by collectors. In general, these habitat types are small in area and few in number. This is especially true for burrowing species that inhabit groundwater seeps in the Appalachian hills and mountains. Within the dissected topography, seep habitats can be found in river valleys that are isolated from each other by high ridges, mountains, or narrow bedrock gorges. Such areas function as island habitats. Many such isolated valleys harbor atypical populations in need of examination (RFT, unpublished data). Glon *et al.* (2022) has documented an additional undescribed species occupying the same valley as *C. nyx n. sp.*

Taylor *et al.* (2007) reported on the conservation status of the crayfishes of the United States and Canadian and found that 48.4% of the total number of species, or 363 species) are in some state of conservation need (extinct, endangered, threatened, or vulnerable), further stating that the primary cause of imperilment was limited natural range. An examination of the conservation status of species described since Taylor *et al.* (2007), was conducted using distributional range, describing author’s assessment of the species’ conservation status, the primary literature, the American Crayfish Atlas (<https://americancrayfishatlas.web.illinois.edu/?page=home>), and/or the personal experience of RFT (13 burrowing species senior or junior authorship). The following observations were made: 57 species have been described since Taylor *et al.* (2007). Forty of those species (70.1%) are estimated endangered or threatened primarily because of narrow range distributions (as used by Taylor *et al.*, 2007) and 14 (24.6%) are estimated stable having wide distributions. The range size of three species is uncertain, which prevents an assessment. Our assessment is a broad and cursory assessment that will likely change but it is apparent the major-

ity of the newly discovered and described species have limited ranges and as such will be more vulnerable to endangerment. There are yet many species of crayfishes to be described in North America and many of these display a limited range. As these rare species are added to the list it will become more evident that the fauna is in critical need of conservation attention if extinctions are to be avoided.

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