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Cambarus (*Puncticambarus*) *callainus*, a new species of crayfish (Decapoda: Cambaridae) from the Big Sandy River basin in Kentucky, Virginia, and West Virginia, USA

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Abstract

Cambarus (Puncticambarus) callainus, new species, is a stream-dwelling crayfish endemic to the Big Sandy River basin in Kentucky, Virginia, and West Virginia. Within the basin, *C. callainus* occurs in the Levisa, Tug, and Russell fork watersheds. The new species is morphologically and genetically most similar to *Cambarus veteranus,* which is endemic to the Upper Guyandotte River basin of West Virginia. The new species can be differentiated from *C. veteranus* by its more lanceolate rostrum (width less than 50% length), slightly more obtuse suborbital angle, and less well-defined lateral impression at the base of the chelae.

Key words: *Cambarus*, Decapoda, Big Sandy River, conservation status, new species

Introduction

In 1914, Walter Faxon described *Cambarus veteranus* from specimens collected in 1900 by W. P. Hay from the Guyandotte River basin (Faxon 1914). Later, Arnold Ortmann (1931), in his description of crayfishes of the Cumberland Plateau, extended the range of *C. veteranus* to include the Elk and Greenbrier Rivers of the Kanawha River basin, and four streams in the Tennessee River basin in Anderson, Blount, Monroe, and Polk counties. It is likely Ortmann's extralimital records were members of the subgenus *Hiaticambarus* as currently understood. Ortmann (1931:113) stated, "… all of my specimens have the outer finger of the chelae bearded on the inner base …", a character state commonly found in the members of *Hiaticambarus* (Hobbs 1969). Horton H. Hobbs Jr. (1989) was the first to report *C. veteranus* from the Big Sandy basin (these latter populations are now considered the species herein described), and also stated the "limits of [its] range [are] very indefinite and poorly known." That same year Ray Jezerinac was completing a statewide survey of West Virginia crayfishes. In the publication that followed, Jezerinac *et al.* (1995) did not document *C. veteranus* in the Big Sandy River basin of West Virginia, but did procure 49 specimens of *C. veteranus* from 17 sites in the state. Jezerinac *et al.* (1995) ultimately concluded that extractive industries were causing *C. veteranus* to decline across its range in West Virginia, and restricted the species distribution to the Guyandotte River and Crane Creek (Bluestone River) in Wyoming, Logan, and Mercer counties, West Virginia and Russell Fork (Big Sandy basin) in Buchanan and Dickenson counties, Virginia. Finally, Taylor & Schuster (2004), in their statewide treatment of Kentucky crayfishes, indicated the distribution of *C. veteranus* to be the Guyandotte and Bluestone basins of West Virginia, and the upper portions of the Big Sandy basin in Kentucky and Virginia.

Survey work for *C. veteranus* conducted in Virginia (Thoma 2009) and Kentucky (Thoma 2010) found several disjunct populations and range-wide declines in both states. At the onset of this work, it was assumed that *C. veteranus* had been extirpated in West Virginia (e.g., Jones *et al.* 2010). While completing a statewide treatment of West Virginia's crayfish, Z. J. Loughman and S. A. Welsh rediscovered *C. veteranus* in the Guyandotte River basin,

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and documented the first occurrence of the species in West Virginia's Tug Fork and Dry Fork, both tributaries of the larger Big Sandy River basin (Loughman 2013). Genetic analysis of specimens from the Guyandotte and Big Sandy watersheds indicated there was genetic divergence between these populations (Fetzner & Thoma 2011). In addition, morphologic analysis by both R. F. Thoma and Z. J. Loughman indicated that differences existed between populations that were of taxonomic significance. These analyses represent the first time specimens from both the Big Sandy and Guyandotte River systems were examined together in a single analysis, thus highlighting the differences between them and ultimately resulting in the formal description of the Big Sandy population as a new species contained herein.

Materials and methods

All specimen morphometric measurements were taken with digital calipers to the nearest 0.1 mm and followed those used by Cooper (2006). The following abbreviations are used below: TCL, for total carapace length; PCL, for postorbital carapace length.

Specimen and tissue collection. Specimens were captured either by seine or by hand when turning rocks in streams. All specimens were kept alive on ice until a single leg (or other muscle tissue) could be taken as a tissue sample for DNA analysis (which typically occurred 6 – 12 hours post capture), at which point, the specimens were then preserved in 80% ethanol. Sampled legs were cut into 2 – 3 mm pieces and then placed directly into 1 mL of sterile Cell Lysis Buffer (10 mM Tris, 100 mM EDTA, 2% SDS, pH 8.0) that also contained 10 µL of Proteinase K (20 mg/mL stock). Samples were then stored at ambient temperature until the DNA extraction could be completed.

DNA extraction, amplification and sequencing. DNA was extracted using the high salt precipitation method described in detail by Fetzner and Crandall (2003). PCR amplifications of the mitochondrial DNA (mtDNA) cytochrome c oxidase subunit I gene (COI; EC 1.9.3.1) were conducted in a total volume of 25 µL. Each PCR reaction contained the following components: 1X PCR buffer, 3 mM magnesium chloride, 1.25 mM each dNTP, 1 µM each primer, 0.6 units of GoTaq Hotstart DNA polymerase (Promega), and 300 ng of sample DNA. PCR cycling conditions included an initial denaturation step of 2:00 min at 95°C followed by 50 cycles performed at 95°C for 0:30 sec, 50°C for 0:30 sec, and 72°C for 1:30 min. A final extension at 72°C for 10:00 min was conducted, followed by a final soak at 4°C until samples could be processed further (usually overnight). Primers used in the reaction were the standard set of Folmer et al. (1994) primers but a universal primer sequence was added to the 5' end of the Forward and Reverse COI primer (T7 and T3, respectively). These non-degenerate, non-homologous 5' tails (in bold) are then used to sequence all resulting PCR products. Primer sequences used were: HybLCO 5'-**TAATACGACTCACTATAGGG**GGTCAACAAATCA TAAAGATATTGG-3' and HybHCO 5'-**ATTAACCCTCACTAAAG**TAAACTTCAGGGTG ACCAAAAATCA-3'. The PCR reactions were checked for amplification products in the correct size range (~700 bp) by electrophoresis through a 1% agarose gel (run at 140 volts for 20 min in TAE buffer). Viable PCR products were then cleaned and purified using MultiScreen $PCR_{\mu 96}$ plates (Millipore) in preparation for DNA sequencing.

Sequencing reactions were conducted in a total volume of 10 µL using the Big Dye 3.1 Cycle Sequencing Kit from Applied Biosystems. Each PCR sequencing reaction was conducted at 1/16 the standard volume and contained 0.5 µL of Big Dye ready reaction mix, 2.1 µL 5x buffer, 2.0 µL of purified PCR product, 1 µL of primer (from 10 µM stock), and 6.4 µL water. The cycle sequencing protocol followed the manufacturer's recommendations. Each cycle of the amplification process included denaturation at 96°C for 20 sec, annealing at 50°C for 25 sec, and extension at 60°C for 4 min. When cycling was completed, samples were held at 4°C until analysis. After the amplification step, the sequencing products were purified using Sephadex G_{50} fine columns in a 96 well plate format to remove any unincorporated dye-labeled nucleotides. The samples were then dried down in a vacuum centrifuge for 30 min, re-suspended in 35 µL formamide and overlaid with a drop of mineral oil before running on a Applied Biosystems 3730xl DNA Analyzer. Sequences obtained from the automated sequencer were initially corrected and aligned using the program Sequencher, version 5.0.1 (GeneCodes Corp., Inc.) and then adjusted, as necessary, by eye.

Genetic data analysis. After alignment in Sequencher, the COI barcode sequence data were checked for indels and also translated into the corresponding amino acids using Mesquite v1.7.6 (Maddison & Maddison 2011) to verify the presence of an open reading frame (i.e., no stop codons), and to avoid incorporating mtDNA nuclear pseudogenes (=numts) in the analysis (Song et al. 2008). The data were then imported and analyzed using PAUP*

v4.0b10 (Swofford 2003) via PaupUp v1.0.3.1 (Calendini & Martin 2005) in order to output distance matrices. For phylogenetic analyses of haplotype relationships, and for higher level systematic relationships among related species in the genus *Cambarus*, models of DNA sequence evolution were tested for their fit to the data. Eighty-eight different models (11 substitution schemes) of DNA sequence evolution were tested using jMODELTEST v2.1.5 (Darriba *et al*. 2012). Both maximum likelihood (ML) and Bayesian inference (BI) optimality criteria were used to estimate phylogenies using raxmlGUI v1.3 (Silvestro & Michalak 2012), which includes RAxML v7.4.2 (Stamatakis 2006), and MrBayes v.3.2.2 (Ronquist & Huelsenbeck 2003), respectively. For RAxML, the GTR+G model was selected for use over the only other alternative model (GTR+G+I), following the author's suggestion that the GTR+G+I model may cause problems with the resulting model parameter optimization. The RAxML analyses used a combined ML topology search with the rapid bootstrap (n=1000) setting, in order to determine nodal support.

Bayesian analyses were performed with MrBayes using two independent runs with one cold chain and three hot chains. The program was run for 5 x 10^6 generations, with sampling every 1000 generations. Split frequencies below 0.01 were used to check for convergence, and the first 25% of trees were discarded as burn-in. The two independent runs were then combined after the deletion of burn-in and a majority rule consensus tree was created with nodal confidence for the trees assessed using node posterior probabilities.

Systematics

Cambarus (*Puncticambarus*) *callainus* Thoma, Loughman and Fetzner, new species

Figs. 1–2, 4, Table 1

Cambarus veteranus.—Hobbs, 1955:330 [in part]; Taylor & Schuster, 2004:124, Figs. 95-96 [in part].
Cambarus (*Puncticambarus*) *veteranus*.—Hobbs, 1969:102, Figs. 7, 18b [in part]; 1974:22, Fig. 78 [in part]; Hobbs, 1989:27, Fig. 105 [in part]; Jezerinac *et al*., 1995:165, Fig. 80 [in part].

Diagnosis. Body and eyes pigmented. Rostrum moderately excavated and lanceolate in shape. Rostral margins not thickened, convergent throughout length to acumen. Acumen with prominent dorsally deflected spiniform tubercle at terminus. Areola 3.3–5.7 ($\bar{x}$ = 3.1, *n* = 80, *SD* = 4.2) times as long as wide with 6–12 (usually 9) punctations across narrowest point. Single prominent cervical spine always present. Mandibular, branchiostegal, and orbital regions of carapace with well-developed spherical tubercles. Postorbital ridges short, terminating in truncated (rarely spiniform) tubercle on adults; spiniform tubercle on juveniles and subadults. Suborbital angle weakly obtuse. Antennal scale widest at middle, 0.9–5.6 ($\bar{x}$ = 2.4, *n* = 80, *SD* = 2.1) times as long as wide. Total carapace length (TCL) 2.1–3.6 ($\bar{x}$ = 2.2, *n* = 80, *SD* = 0.2) times longer than width. Form I and II males possessing hook on ischium of third pereiopods only; hook gently curved at apex, overarching basioischial joint in form I males, not reaching basioischial joint in form II males; hooks not opposed by tubercle on basis. Mesial surface of chelae with single row of 7–13 ($\bar{x}$ = 9.7, *n* = 80, *SD* = 1.3) tubercles. Dorsal longitudinal ridge of dactyl defined by well-developed setiferous punctations. Dorsomedian ridge of fixed finger of propodus pronounced. Weakly developed lateral impression ventral to the junction of the dactyl with the propodus. Dactyl and fixed finger with sharp corneous subterminal tips. Form I male palm length 72.8–99.9% ($\bar{x}$ = 79.4%, *n* = 28, *SD* = 5.6%) of palm width, form I male palm length 41.9–62.3% ($\bar{x}$ = 46.4%, *n* = 28, *SD* = 4.5%) of total propodus length; female palm length 31.5–41.9% ($\bar{x}$ = 48.0, *n* = 44, *SD* =3.3%) of total propodus length. First pleopod of form I male with short terminal elements. Central projection not tapering distally; recurved > 90° to main shaft of gonopod, with distinct subapical notch. Mesial process directed 90° to shaft, bent cephalolaterally; inflated cephalically, tapering to distinct caudal point at or slightly beyond terminal end of central projection. Annulus ventralis immovable; distinctly asymmetrical posteriorly; cephalic portion with median trough leading to strongly sculptured central fossa; exaggerated "S" bend in sinus terminating at caudal edge.

Description of holotypic male, form I. (Figs. 1A–C, F–H, J–K, Table 1). Body compressed dorsoventrally (Fig. 1C); carapace posterior to cervical groove wider than abdomen. Carapace depth less than carapace width. Total carapace length 46.3 mm; post orbital carapace length 36.4 mm. Areola 2.5 times longer than wide, with 7 punctations across narrowest part (Fig. 1J); length of areola 36.1% of TCL, 46.0% of PCL. Rostrum moderately excavated, more so anteriorly than posteriorly; margins slightly thickened, convergent throughout and continuous to base of acumen; floor of rostrum with numerous punctations. Rostrum 1.4 times longer than base width. Margins

of acumen contiguous with basiorostral line, ending in dorsally deflected corneous tip (Fig. 1C). Postorbital ridges well developed, short and terminating in weak cephalic tubercles on right and distinct spine on left. Suborbital angle weakly obtuse, lacking tubercle (Fig. 1C). Cervical spine present. Mandibular, branchiostegal, and orbital regions of carapace punctate with small tubercles; greatest tubercle density in hepatic region.

Abdomen (43.61 mm) approximately 94.2% length of carapace, pleura rounded cephaloventrally, angled distoventrally. Lateral margins of terga angulate; lateral margin of second pleuron deeply furrowed. Cephalic section of telson with 2 large spines in each caudolateral corner. Proximal podomere of uropod with distal spine on mesial lobe; mesial ramus of uropod with median ridge ending distally in distomedian spine not over-reaching margin of ramus; laterodistal spine small. Distal margin of proximal segment of lateral ramus of right uropod with 15 small, immovable spines and 1 large, laterally disposed, movable spine subtended by 1 small laterally disposed immovable spine. Cephalomedian lobe of epistome subtriangular, zygoma moderately arched (Fig. 1G); cephalolateral margins thickened, forming angle at junction with endostyle (Fig. 1G). Body of epistome possessing prominent cephalomedian fovea. Antennal scale broadest in middle; lateral margin thickened, terminating in corneous spine; setiferous on inner margin. Right antennal scale 7.9 mm long, 3.2 mm wide (Fig. 1F).

TABLE 1. Morphological measurements (in mm) of type specimens of *Cambarus callainus*, new species.

	Holotype	Allotype	Morphotype
Carapace			
Total carapace length	46.30	47.55	49.65
Postorbital length	36.39	37.54	39.04
Width	25.61	24.91	27.18
Depth	19.78	20.43	21.35
Length rostrum	9.91	10.01	10.61
Length acumen	2.53	2.81	2.71
Length areola	16.73	17.63	19.15
Width areola	3.16	4.17	3.72
Antennal scale			
Length	7.94	8.10	8.00
Width	3.18	3.10	3.40
Abdomen			
Width	20.49	23.05	21.62
Cheliped			
Length mesial margin palm	15.30	13.84	16.76
Width palm	20.89	16.89	16.76
Depth palm	11.23	9.59	12.10
Length dactyl	37.09	31.74	39.60
Length opposable propodus	28.88	25.05	31.63
Gonopod			
Length	9.20	N.A.	10.3
Width	2.70		3.80
Eye Diameter	2.78	2.90	3.00

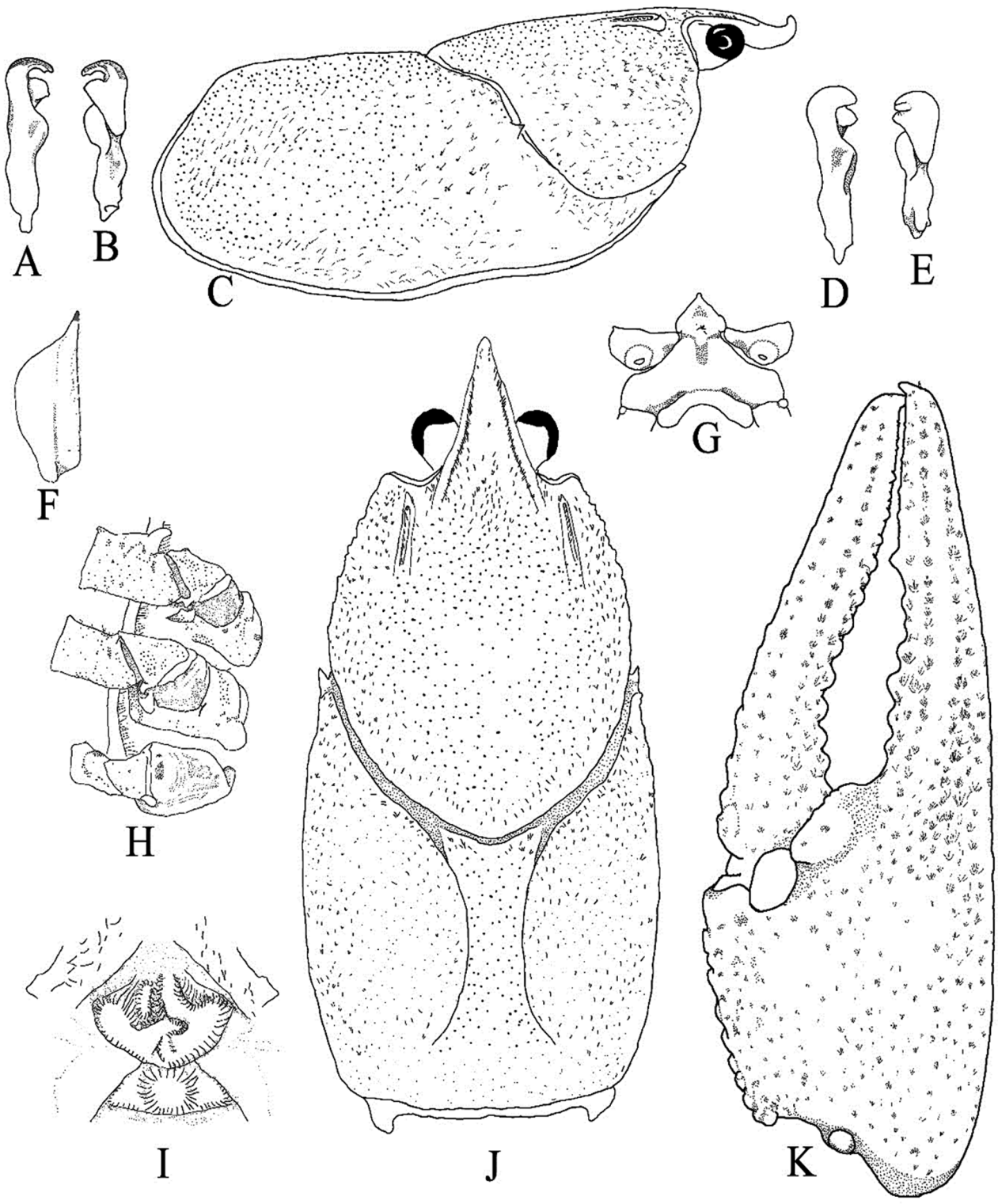

FIGURE 1. *Cambarus callainus* **n. sp.**: A. lateral (left) and B. mesial (right) view of left first gonopod of form I male; C. lateral view of carapace; D. lateral (left) and E. mesial (right) view of form II male gonopod; F. antennal scale; G. epistome; H. ventral view of third, fourth and fifth pereiopods showing ischial hook of form I male on third pereiopod; I. annulus ventralis; J. dorsal view of carapace; K. dorsal view of distal podomere of right cheliped of form I male; A–C, F–H, J–K from holotype; I from allotype; D–E from morphotype.

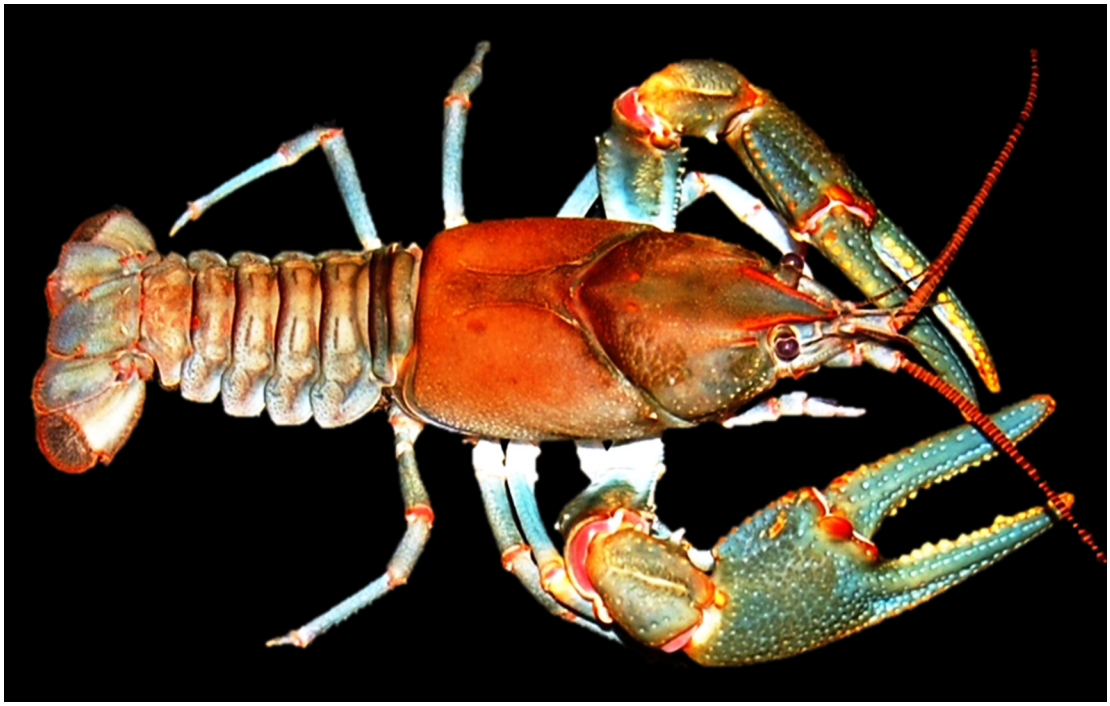

FIGURE 2. *Cambarus callainus* **n. sp.**, non-type Form I male from Dry Fork, McDowell County, West Virginia.

Mesial surface of right chela with single row of 10 tubercles (Fig. 1K). Palm length 0.8% of palm width; depth of palm 11.2 mm. Ventral surface of palm lacking subpalmar tubercles. Dorsal longitudinal ridge of dactyl weakly developed and lacking tubercles (Fig. 1K); dactyl terminating in large corneous spine. Dorsomedian ridge of fixed finger of propodus moderately pronounced. Lateral impression at the junction of fixed finger with propodus. Fixed finger of propodus with sharp, corneous tip. All measurements and counts from right chela.

Carpus with prominent dorsal furrow and 1 weak dorsomesial tubercle; remainder of surface with some punctations; mesial margin with large, procurved spine at midlength, and reduced proximal tubercle. Distodorsal surface of merus with 2 spiniform tubercles and 1 distal tubercle; ventrolateral ridge with 2 spines, distal largest; ventromesial ridge with 11 well-developed spines; ventrolateral margin of ischium with 3 small, spiniform tubercles. Carapace depth less than width. Hook on ischium of third pereiopods only (Fig. 1H.); hook gently curved at apex, overarching basioischial joint, not opposed by tubercle on basis. Form I gonopod as described in diagnosis (Fig. 1A,B); tip reaching anterior margin of caudomesial boss of third pereiopod.

Description of allotypic female. (Fig. 1I, Table 1).—Differing from holotype in following respects: carapace height less than carapace width (20.43 and 24.9 mm, respectively); TCL 47.6 mm, PCL 37.5 mm. Areola 37.1% of TCL (47.0% of PCL), 1.9 times as long as wide. Distal portion of rostrum more excavated than proximal portion; rostrum 1.3 times longer than wide. Abdomen length 43.8 mm. Mesial surface of chelae with 1 row of 10 tubercles. Palm length 13.8 mm; 81.9% of palm width (13.8 mm, 16.8 mm respectively); depth of palm 9.6 mm. Antennal scale 8.1 mm long, 3.1 mm wide. All measurements and counts from right chela. Annulus ventralis as described in diagnosis (Fig. 1I); width of postannular sclerite more than half width of annulus ventralis; first pleopods uniramous, reaching middle of postannular sclerite when abdomen flexed.

Description of morphotypic male, form II. (Fig. 1D,E, Table 1). Differing from holotype in the following respects: carapace wider than abdomen (27.18 and 21.62 mm, respectively); carapace height less than carapace width (21.4 and 27.2 mm respectively); TCL 49.7 mm and PCL 39.0 mm. Areola length 38.6% of TCL (49.1% of PCL), 2.1 times longer than wide. Rostrum margins convergent throughout length to base of acumen; rostrum ventrally deflected; rostrum 1.5 times as long as wide. Abdomen 45.4 mm long. Mesial row of tubercles on palm of left chela with 10 tubercles. Palm length 16.8 mm, 77.5% of palm width (21.6 mm). Antennal scale 8.0 mm long, 3.4 mm wide. Gonopods reaching anterior margin of 3rd pereiopod; 20.7% of TCL. Central projection curved 90° to shaft, with complete apex; bulbous in appearance (Fig. 1D,E). Mesial process tapered, bulbous, directed caudolaterally. Hook on ischium of third pereiopod small, reaching just to basioischial joint. Right chela regenerated, all measurements and counts from left chela.

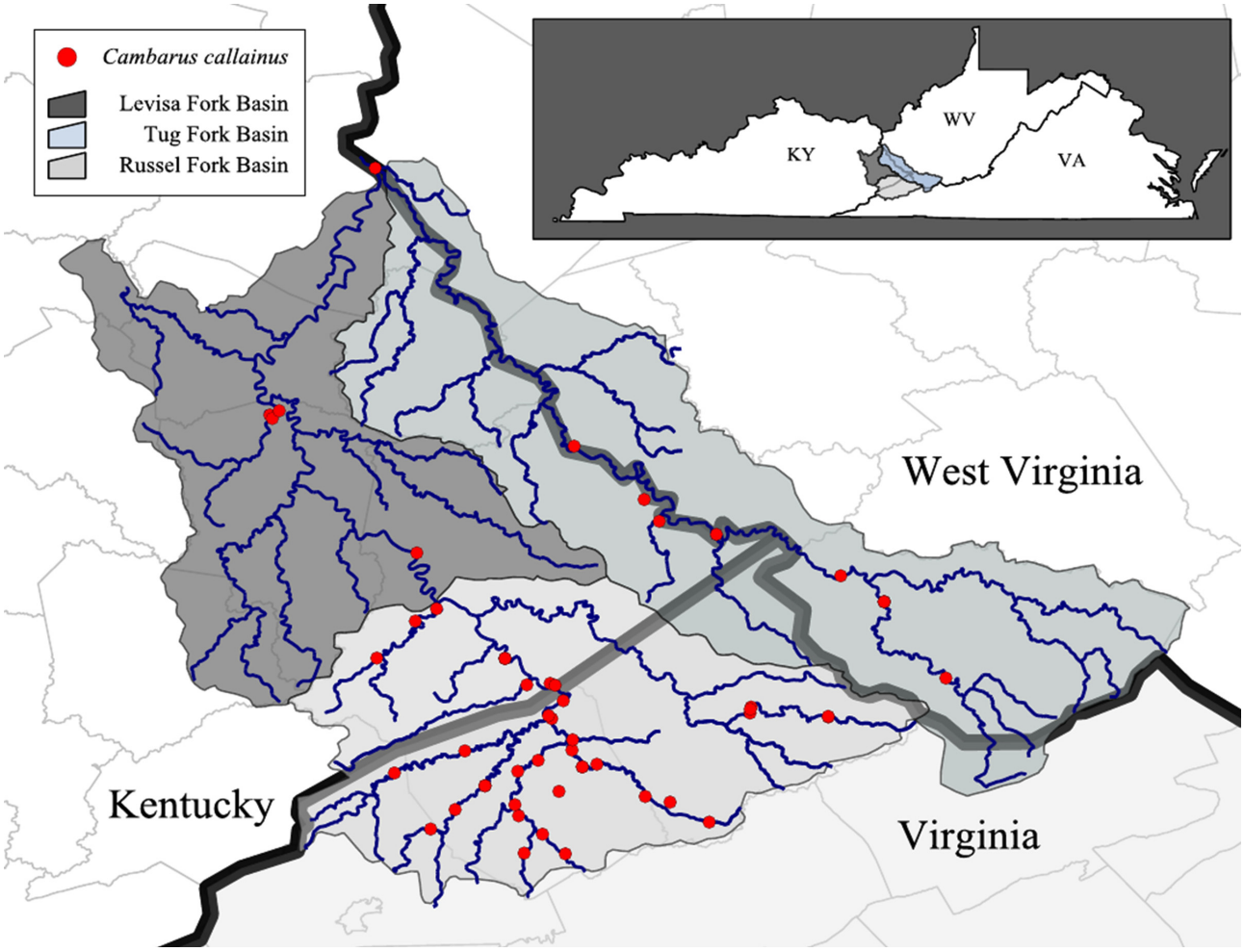

FIGURE 3. Known range of *Cambarus callainus* **n. sp.** in Kentucky, Virginia and West Virginia, with red dots indicating sampling localities for the specimens listed in the specimens examined section (below).

Size. Form I male (n = 28) TCL ranges in size from 37.4–53.6 mm (PCL 30.3–41.4 mm) with a mean TCL of 36.0 mm. Form II male (n = 8) mean TCL is 42.5 and ranges in size from 37.2–49.7 mm (PCL 29.9–39.0 mm). Non–ovigerous female (n = 44) TCL mean is 41.9 mm and ranges from 32.4–52.4 mm (PCL 26.1–43.7 mm). Ovigerous female (n = 2) TCL ranges in size from 42.0–46.0 mm (PCL 34.9–38.2 mm) with a mean of 44.0 mm. The largest specimen examined was a form I male with TCL of 53.6 mm (PCL 44.5 mm).

Color. Carapace ground color brown to brown-green; posterior margin of carapace dark. Hepatic and antennal region of carapace punctuated with white or cream tubercles. Postorbital ridge, and edges of rostrum and acumen crimson red. Cephalic section of carapace immediately anterior to and including cervical groove chestnut brown to green-brown; mandibular abductor scars ranging from brown to chestnut brown. Lateral margin of antennal scale red; body of antennal scale slate grey to blue-grey. Antennal flagellum and antennules crimson red; dorsal surface of lamellae red; ventral surface red to olivaceous. Dorsal surface of chelae usually aqua, occasionally green-blue to blue. Mesial row of dactyl tubercles orange, orange-cream or cream. Denticles on opposable surfaces of fingers yellow, white, or cream. Ventral surface of chelae red-brown or cream. Dorsal surface of carpus aqua or blue-green; occasionally green–brown; region adjacent to, and including furrow, brown-green to blue-brown; carpus spine white. Merus blue-brown, blue-grey, or olivaceous brown. Podomeres of pereiopods light blue, blue–green, or aqua; joints of pereiopod podomeres pink. Dorsal and dorsolateral surface of abdomen aqua, blue-grey, or blue; anterior region of abdomen same as dorsal cephalic section surface; tergal margins crimson red. Uropods aqua, green-blue or blue-grey, with olivaceous tint; margins gray to brown. Ventral surface of abdomen and carapace cream. Dorsal ridge of form I gonopod central projection amber; body of central projection, gonopod, and mesial process tan. Form II gonopod and all associated processes cream. Cephalic portion of annulus ventralis pink to pink-cream; ridge of fossa pink; caudal region of annulus ventralis ranges from pink to cream colored.

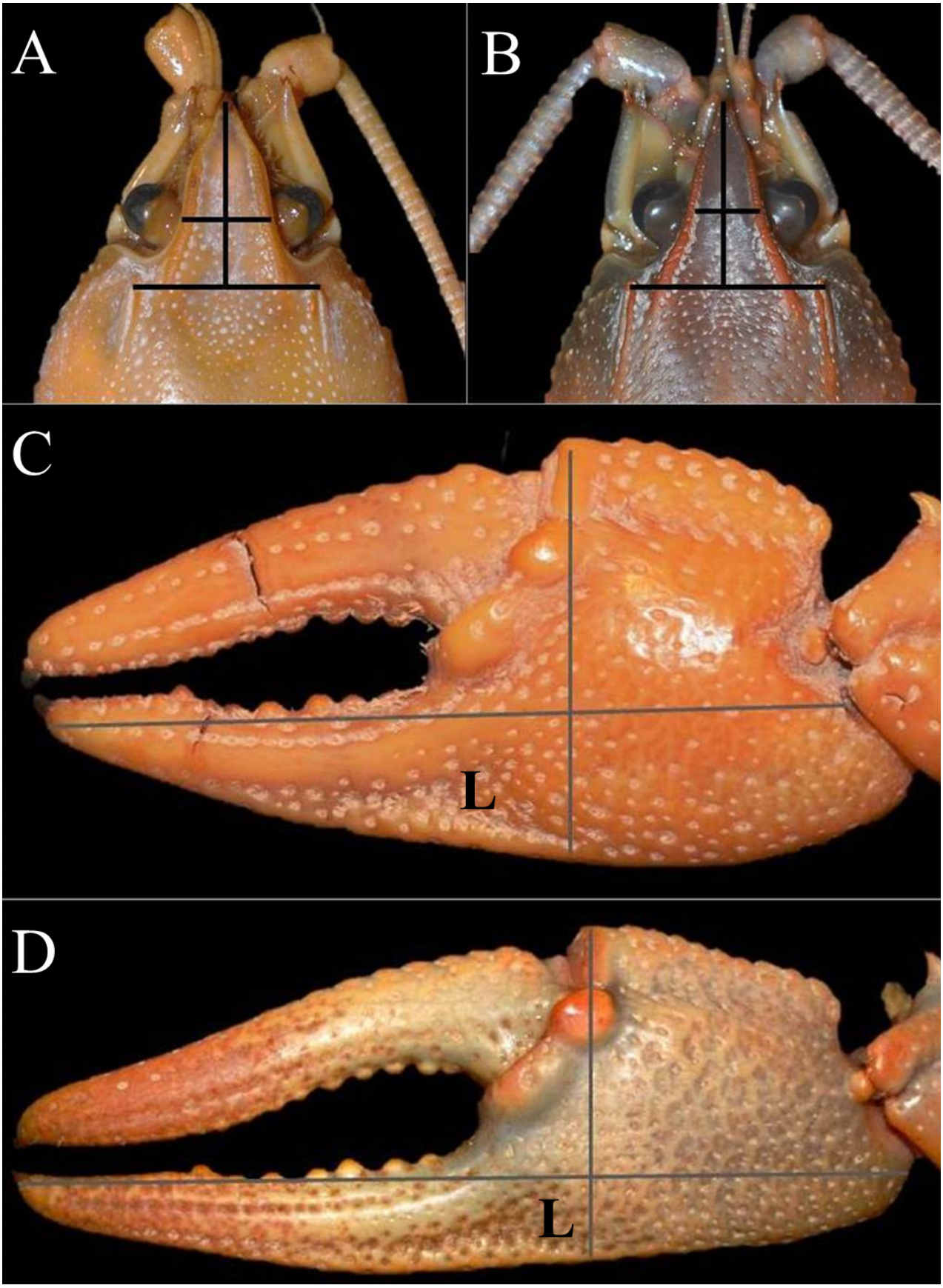

FIGURE 4. Morphological differences between *Cambarus veteranus* and *Cambarus callainus* **n. sp.**, respectively in A–B. the rostrum and C–D. the chelae. L indicates the lateral impression.

TABLE 2. Average pairwise sequence divergences among river drainages calculated using the COI gene sequences both within (along diagonal) and among populations. Uncorrected p-distances (lower diagonal) and HKY+G distances (upper diagonal).

	Russell	Levisa/Tug	Guyandotte
C. callainus—Russell Fork	0.36%	2.43%	5.67%
C. callainus—Levisa/Tug	2.08%	0.48%	6.11%
C. veteranus—Guyandotte	4.16%	4.36%	0.51%

Type locality. Russell Fork adjacent to VA Rt. 605 at county line of Dickenson and Buchanan counties, 3.79 air km NW of Davenport, 14.2 air km SE of Haysi, VA, 37.11954 N, -82.17185 W. At this site, Russell Fork is six meters wide, with 20-m long riffles immediately upstream of a 10-m wide, and 50-m long pool. Stream substrates consist of slab boulders with additional cobble, gravel, and silt substrates present. The stream riparian corridor consists of mesophytic forests dominated by various hardwoods and eastern hemlocks. Gradient in this area is approximately three m/km.

Disposition of types. The holotype, allotype, and morphotype are deposited in the National Museum of Natural History (USNM), Smithsonian Institution, Washington, D. C. (catalogue numbers USNM 1251657, 1251658, 1251659, respectively). Paratypes are deposited in the following museums and collections: Carnegie Museum of Natural History, Pittsburgh, PA (CMNH 38390.1 – 38390.8; 1 MI, 2 MII, 5 F), and Cleveland Museum of Natural History (CvMNH 1116).

Range and specimens examined. *Cambarus callainus* (Fig. 2) is endemic to the Levisa Fork, Tug Fork, and Russell Fork watersheds in the upper Big Sandy River basin of Kentucky, Virginia, and West Virginia (Fig. 3). Kentucky populations occur in Floyd, Johnson, and Pike counties, with animals collected from the mainstem of both Levisa and Russell Forks, Knox Creek, Shelby Creek, Blackberry Creek, and the Tug Fork mainstem. Virginia counties harboring *C. callainus* include Buchanan, Dickenson, and Wise, with populations occurring in Russell Fork and its tributaries. In Virginia's Levisa Fork basin, only Dismal Creek harbors the species. In West Virginia, populations have only been recorded from McDowell County in the Tug Fork mainstem and Dry Fork, a major tributary to the latter. In lower reaches of the Big Sandy basin, stream gradient becomes lower and water velocity subsequently decreases, resulting in stream substrates dominated by sands and fines. In these situations, *C. callainus* is replaced by *Cambarus theepiensis* Loughman *et al.* (2013).

All of the following collections, with the exception of the previously discussed type series, are either housed in the Ohio State University Museum of Biological Diversity Crustacean Collection, denoted with the prefix OSU, or the West Liberty University Astacology Collection, denoted with the prefix WLU. Abbreviations are defined as follows: CR = creek; I = interstate; KY = Kentucky state highway; mi = miles; RD = road; US = U. S. route; VA = Virginia state highway; WV = West Virginia state highway; Rd = road; F = female; OF = ovigerous female; IM = Form I male; IIM = form II male; JV = juvenile.

A total of 292 specimens were examined from 34 collections. **KENTUCKY**: *Pike Co*. (1.) OSU 7400, Levisa Fork downstream of KY Rt. 3051 bridge, adjacent to KY Rt. 3 at Auxier, 14 September 2009, 1 F, R. F. Thoma. *Floyd Co*. (2.) OSU 7362, Shelby Creek adjacent to old US Rt. 23 / Collins Hwy, 10 July 2009, 1 IM, 3 IIM, 6 F, 2 JV, R. F. Thoma. (3.) OSU 7403, Levisa Fork between US Rt. 23 and shopping mall, 15 Sept. 2009, 1 F, R. F. Thoma, D. L. Baxley. (4.) OSU 7404, Shelby Creek at Sookeys Cr. Rd. bridge crossing, 15 Sept. 2009, 1 IM, 1 F, R. F. Thoma, D. L. Baxley. (5.) OSU 7407, Long Fork of Shelby Creek downstream of KY Rt. 1469 crossing adjacent to Old Long Fork Rd., 15 September 2009, 1 IM, 1 IIM, 1 F, 1 JV, R. F. Thoma, D. L. Baxley. (6.) OSU 7414, Russell Fork at Camp Fork Rd. in Draffin, 16 September 2009, 1 IM, 1 IIM, 4 F, R. F. Thoma. (7.) OSU 7415, Elkhorn Creek in Elkhorn City adjacent to KY 197, 16 September 2009, 1 F, R. F. Thoma. (8.) OSU 7419, Russell Fork at campground in Breaks Interstate Park at Potter Flats, 16 September 2009, 3 II M, 11 F, 6 JV, R. F. Thoma. (9.) OSU 7422, Knox Creek upstream of confluence with Tug Fork at intersection of Woodman CR. RD. and unnamed rd., 17 September 2009, 1 IM, 1 IIM, 5 F, R. F. Thoma. (10.) OSU 7426, Peter Creek in Freeburn adjacent to KY 194, 17 September 2009, 1 IIM, 1 F, 1 JV, R. F. Thoma. **VIRGINIA**: *Buchanan Co*.(11.) OSU 6342, Russell Fork at park in Council adjacent VA Rt. 80, 17 Aug. 2006, 1 F, 3 JV., R. F. Thoma, M. Puckett. (12.) OSU 6827, Dismal Creek at intersection of VA 690/638, 24 July 2007, 1 IM, 2 F, 6 JV, R. F. Thoma, M. Puckett. (13.) OSU 6830, Dismal Creek at VA SSR 638, 24 July 2007, 1 IM, 2 F, R. F. Thoma, M. Puckett. (14.) OSU 6832, Russell Fork adjacent VA SSR 612 downstream of intersection with VA SSR 611 at Bartlick, 24 July 2007, 1 IM, 1 IIM, 1 OF, 1 JV., R. F. Thoma, M. Puckett. (15.) OSU 6879, Dismal Creek at handicapped fishing access site

adjacent VA 638, 25 Sept. 2007, 1 IIM, 1 F, 1 JV., R. F. Thoma, J. A. Thoma. (16.) OSU 6942, Dismal Creek at intersection of VA 690/638, 3 June 2008, 1 IM, 1 F, 1 JV, R. F. Thoma. (17.) OSU 7051, Dismal Creek adjacent VA 638, 5.54 mi. ENE of Vansant, 15 Oct. 2008, 1 IM, 1 IIM, 1 F, R. F. Thoma. *Dickenson Co*. (18.) OSU 6349, Russell Fork downstream bridge and pipeline at Martha Gap (VA Rt. 80 & VA Rt. 722), 17 Aug. 2006, 4 IM, 11 F, 3 JV. R. F. Thoma, M. Puckett. (19.) OSU 6791, Russell Fork at intersection of VA Rt. 810 / 605, 24 May 2007, 1 IM, 1 IIM, 1 F, R. F. Thoma. (20.) OSU 6795, McClure River at baseball field at VA Rt. 63 and Caney Creek confluence, 24 May 2007, 1 IM, 1 IIM, 4 F, 3 JV, R. F. Thoma. (21.) OSU 6796, Pound River adjacent VA Rt. 754, 24 May 2007, 2 IM, 2F, 3 JV., R. F. Thoma. (22.) OSU 6798, Pound River adjacent VA Rt. 754, first pullout north of fenced horse track, 27 Sept. 3007, 1 IIM, 2 F, 10 JV, R. F. Thoma, J. A. Thoma. (23.) OSU 6888, Lick Creek at ford upstream of 90 degree bend in stream on VA SSR 670, 26 Sept. 2006, 1 IM, 1 IIM, 1 F, 2 JV., R. F. Thoma, J. A. Thoma. (24.) OSU 6897, McClure River at VA Rt. 83 / 63 behind Rescue Squad building, 26 Sept. 2006, 1 IIM, 3 F, R. F. Thoma, J. A. Thoma. (25.) OSU 6901, Cranes Nest River upstream VA Rt. 83 bridge adjacent to VA 649, 27 Sept. 2007, 1 IM, 4 IIM, 7 F, 3 JV., R. F. Thoma, J. A. Thoma. (26.) OSU 6902, Cranes Nest River at VA 637 bridge 1.0 mi. east of Darwin, 27 Sept. 2007, 2 IM, 1 IIM, 1 F, 5 JV, R. F. Thoma, J. A. Thoma. (27.) OSU 6958, Cranes Nest River downstream of VA 83 bridge 2.0 mi. SE Clintwood, 15 Oct. 2008, 3 IM, 10 F, 1 JV. R. F. Thoma. (28.) OSU 6989, Russell Fork adjacent to VA SSR 605, 15 October 2008, 2 IM, 1 F, 2 JV, R. F. Thoma. (29.) OSU 7000, Lick Creek at intersection of VA 670 / VA 661 at Counts, 15 July 2008, 2 JV, R. F. Thoma, V. M. Thoma. (30.) OSU 7002, Prater Creek at Haysi Community Library in Haysi, 16 July 2008, 2 F, 3 JV, R. F. Thoma, V. M. Thoma. (31.) OSU 7054, McClure River downstream VA 738 bridge 1.45 mi. NE of Clinchco, 15 Oct. 2008, 2 IM, 2 IIM, 5 F, 1 JV., R. F. Thoma. **WEST VIRGINIA**: *McDowell Co*. (32.) WLU 3000, Dry Fork adjacent to WV 80 1.2 mi S of Avondale, 10 July 2009, 1 IIM, 2 JV, D. A. Foltz, M. I. McKinney, Z. J. Loughman, S. A. Welsh. (33.) WLU 3001, Tug Fork at Horse Creek confluence adjacent to WV1, 12 May 2011., 3 IM, 1 IIM, 6 F, D. A. Foltz, Z. J. Loughman, K. McGill, N. Taylor, K. Skalican. (34.) WLU 3002, Dry Fork in War, 19 March 2014, 3 IM, 2 IIM, 6 F, Z. J. Loughman, M. Lucero, L. Sadecky, N. Sadecky.

Conservation status. *Cambarus callainus* should be listed as endangered (E) using American Fisheries Society criteria (Taylor *et al*. 2007), and assigned G2 ranking using Master (1991) global conservation criteria for conservation listing due to (1.) its limited range and (2.) the documented loss of populations. *Cambarus callainus* should be listed as endangered (E) using the International Union for the Conservation of Nature (IUCN 2001) criteria due to (1.) its narrow distribution, and (2.) the reduction in range, caused by (3.) anthropogenic stressors, specifically coal mining activities (Thoma 2009, 2010; Loughman 2013). *Cambarus callainus* appears to have been lost from over 70% of its historic range in the past half century (Thoma 2009, 2010; Loughman 2013).

Habitat and life history notes. The senior author (RFT) has previously studied both the life and natural history of both Virginia and Kentucky *C. callainus* populations in 2007 and 2008, respectively. Slab shaped boulders provided optimal conditions for the species during all months of the year (Thoma, personal observation). *Cambarus callainus* populations decreased as gradient, pollution, and fines (gravel, sand, silt) increased, and were stable when these habitat attributes were not in flux (Thoma 2009, 2010). *Cambarus callainus* rarely inhabited pool areas, displaying marked preference for fast flowing runs and riffles (Thoma 2009, 2010; Loughman 2013). In Kentucky and Virginia, the most abundant populations were found in streams with riffles approximately 4.5 m wide and gradients of 4.7 m/km.

First form males and females were observed cohabitating beneath slab rocks in July, which was followed by egg extrusion in July through September (Thoma 2009, 2010). Females carrying eggs were observed in July, August, and October; females carrying instars were observed in September, October, and March (Thoma 2009, 2010; Loughman 2014). Two ovigerous females measuring 42 and 46 mm carapace length (collected on 6 Aug 2006 and 24 July 2007, respectively) contained a total of 90 and 142 eggs, respectively. One female (52 mm CL) with 13 instars was collected on 17 Sept 2009. First form males were observed year round, but were most common in October (Thoma 2009, 2010). Thoma (2009) also reported "Numerous freshly molted individuals were observed in May at several sites."

Crayfish associates. *Cambarus* (*P.*) *callainus* has been collected with *Cambarus* (*Jugicambarus*) *dubius* Faxon 1884, *Cambarus* (*Cambarus*) *hatfieldi* Loughman *et al.* 2013, *Cambarus* (*P.*) *theepiensis* Loughman *et al.* 2013, and *Orconectes* (*Procericambarus*) *cristavarius* Taylor 2000.

Variation. Ontogenetic variation in rostrum morphology and tubercle shape exists in *C. callainus.* Adult rostral margins are subparallel, with the anterior terminus of the margin distinct from the origin of the acumen while juvenile rostra are weakly convergent and grade directly into the acumen without a distinct demarcation. Overall carapace architecture in juveniles is more spinose compared to adult individuals. Geographic variation exists in both chelae and rostral morphology across all *C. callainus* adult demographics along a southwest/

northeast gradient. Animals from the southwestern portion of the *C. callainus* range, including Shelby Creek and Cranes Nest River populations, have consistently narrower, more elongate rostrums, as well as narrower palms and more elongate chelae. Populations in the northeastern portion of the range, including all western Virginia populations and Levisa Fork populations, have wider rostrums and chelae, relative to southwestern populations. Russell Fork mainstem populations are morphologically intermediate to both aforementioned populations.

Relationships and comparisons. *Cambarus callainus* is placed in the subgenus *Puncticambarus* based on its elongate chelae, its broad, densely punctate areola that is 2.1 to 6.2 times as long as broad, and the presence of a subapical notch on the form I male gonopod (Hobbs 1969; Cooper 2001). Among described members of the subgenus, *C. callainus* is most similar to *C. veteranus*. It can be distinguished morphologically from the latter species by its narrower, more elongate rostrum (Fig. 4B), which is 44–48% as wide as long when compared to the broader rostrum of *C. veteranus,* which is 50–56% as wide as long (Fig. 4A). *Cambarus callainus* also has more elongate, narrower chelae when compared to *C. veteranus* (Fig. 4C-D). *Cambarus callainus* chelae are subrectangular and more elongate in profile than *C. veteranus* (Fig. C-D). In addition to chelae shape, *C. callainus* chelae lack a deep, well pronounced lateral impression at the base of the immovable finger (Fig 4D); *C. veteranus* always maintains a well pronounced lateral impression (Fig 4C).

As with its sister species *C. veteranus*, *C. callainus* is easily differentiated from all other members of the subgenus by its lack of a second dorsal-mesial row of tubercles on the chelae. *Cambarus callainus* can be differentiated by sympatric *Cambarus bartonii cavatus* Hay, 1902 and *Cambarus hatfieldi* both morphologically and through comparison of color. Both *C. b. cavatus* and *C. hatfieldi* have thickened rostral margins with a 90° angle at the acumen base, and lack cervical spines. In *Cambarus callainus*, the rostrum is lanceolate and has well pronounced cervical spines. Both *C. b. cavatus* and *C. hatfieldi* have blunted, subrectangular chelae compared to *C. callainus* elongate chelae. Regarding color, *C. callainus* chelae are aqua, blue–green, or blue-grey, compared to both *C. b. cavatus* and *C. hatfieldi* which both possess olivaceous–brown to green–brown chelae. Sympatric *C. theepiensis* lack cervical spines, which *C. callainus* possess, and have two dorsal rows of tubercles on the mesial margin of the chelae; *C. callainus* maintains a single dorsal row of palmar tubercles.

Etymology. Callainus: Latin for Bluish–green or turquoise–green in reference to the color of chelae in freshly molted individuals.

Common name. Prior to the description contained herein, the common name of *C. veteranus* was the Big Sandy Crayfish since the majority of the species' range was encompassed within the headwaters of the Big Sandy River basin. With the description herein of the Big Sandy population as *C. callainus*, it is proposed that the name Big Sandy Crayfish be allied to *C. callainus*, and the new name, Guyandotte River Crayfish, be applied to *C. veteranus*, given the entirety of the *C. veteranus* range, as currently delimited, falls within the Guyandotte River system, and no populations persist in the Big Sandy River basin.

Phylogenetics. The twenty-three unique COI haplotypes discovered by Fetzner *et al.* (In Prep) among sampled populations of *C. callainus*, that were collected from various localities from throughout the Levisa, Russell and Tug Fork drainages were included in the phylogenetic analysis, along with sequences generated for *C. veteranus* (Upper Guyandotte), *C. acuminatus* Faxon 1884, *C. robustus* Girard 1852, *C. georgiae* Hobbs 1981, and *C. rusticiformis* Rhoades 1944. Unique haplotype sequences generated as part of this study for the above species have been deposited in GENBANK under accession numbers (KM979401–KM979430).

The Bayesian phylogenetic tree (Fig. 5) suggests that, of the species included in the analysis, *C. veteranus* was the most closely related to *C. callainus*, which is not surprising, given that the new species has long been classified as *C. veteranus*. Genetically, the new species was distinct from the sampled population of *C. veteranus* from the Guyandotte River drainage, differing on average by 4.26% (range 4.16 – 4.36%) uncorrected sequence divergence. The best fit model, determined by jMODELTEST and using the BIC criterion, was the HKY+G model with the following parameters: base=(0.2581 0.1399 0.2161) nst=2 tratio=7.2521 rates=gamma shape=0.1650 ncat=4 and pinvar=0. Genetic distances generated based on this model resulted in an average divergence of 5.89% (range 5.67 – 6.11%) between these two species (Table 2). The *C. callainus* haplotypes appeared to form two completely separate groups according to the watershed from which they were sampled (=haplogroups), with the exception of a single individual. The Levisa/Tug drainages grouped together with a total of 12 unique haplotypes, while the Russell Fork drainage and its associated tributaries formed another group with 11 unique haplotypes (Fig. 5). The average uncorrected sequence divergence between the two *C. callainus* haplogroups was 2.08%, or 2.43% when using HKY+G corrected distances. In contrast, within major river drainages, the average sequence divergences among haplotypes were low (Russell = 0.36%, Levisa/Tug = 0.48%, Table 2). Both the high level of sequence divergence, along with the morphological differences described above, suggest that these populations are distinct from *C. veteranus* and supports the view that they be described as a separate species.

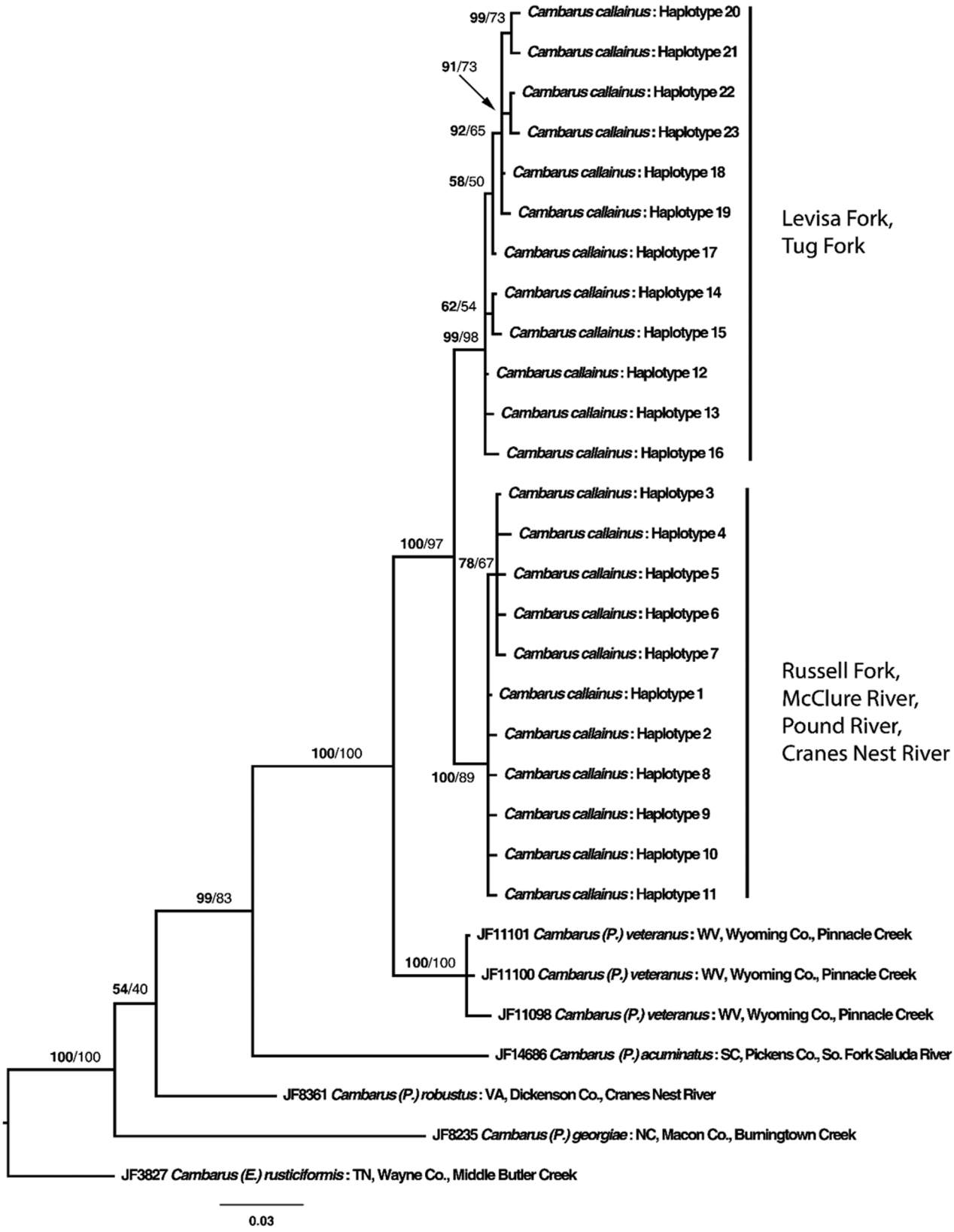

FIGURE 5. Bayesian phylogenetic tree depicting relationships among *Cambarus callainus* COI haplotypes and selected outgroup taxa. Number at nodes of the tree indicate the Bayesian posterior probabilities (in bold) followed by bootstrap values from the RAxML maximum likelihood search.

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