



***Cambarus (Jugicambarus) adustus*, a new species of crayfish from northeastern Kentucky delimited from the *Cambarus (J.)* aff. *dubius* species complex**

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Abstract

A new species of burrowing crayfish, *Cambarus (Jugicambarus) adustus*, is described from Lewis County in northeastern Kentucky, USA. The new species is most similar morphologically to *C. dubius*. *Cambarus adustus* coloration differs from *C. dubius* by lacking red, orange and blue hues, and instead is brown over the entire body surface. Morphological differences between *C. dubius* and *C. adustus* exist in the form I male gonopod, with *C. adustus* possessing a caudal knob, while *C. dubius* does not. In addition, the lateral carapace of *C. adustus* is distinctly tuberculate, whereas in *C. dubius* the carapace lacks extensive tuberculation. *Cambarus (J.) adustus* appears to have an extremely small geographic range (~19.5 km²), and as such we suggest its consideration for both state and federal levels of protection.

Key words: Appalachian Mountains, Cambaridae, *Cambarus*, crayfish, Decapoda, new species

Introduction

Cambarus (Jugicambarus) dubius Faxon 1884, historically has been considered a widespread species complex native to the central and southern Appalachian Mountains (Deweese 1972; Jezerinac *et al.* 1995; Loughman *et al.* 2015; Taylor and Schuster 2004). In an effort to decipher the geographic distribution and range of variation within this complex, the authors of this paper formed a working group in 2011 with the goal of resolving the taxonomic status of this complex, especially as it relates to the currently known geographic variants (primarily color morphs). One of the first efforts undertaken by the group was a broad sampling and subsequent genetic analysis conducted by JWF. The preliminary results obtained suggested that some populations were not *C. dubius* *sensu stricto*, but instead had different taxonomic and evolutionary affinities. These initial results were quite encouraging, and helped to focus the efforts of the working group on obtaining a broader geographic sampling of *C. dubius* (and allies) from throughout its known geographic range.

GWS and Prof. Raymond F. Jezerinac were the first to collect specimens of *C. adustus*. Both communicated to RFT that they believed it to be a new species. The senior author (RFT) first collected the species in 2011, and also believed populations warranted elevation to the species level based on its morphological characteristics. *Cambarus adustus*, new species, has a highly restricted distribution, being confined to Quicks Run, Salt Lick Creek, and the upper most headwaters of Fry Branch (Lower Cabin Creek drainage) in Lewis County, Kentucky (Fig.1). The prior two streams were part of the headwaters of the preglacial Portsmouth River, a tributary to the Teays River basin (Stout *et al.* 1943). The river system existed prior to the onset of the Quaternary, at least 2.58 MYA (Gradstein *et al.* 2004).

Jezerinac *et al.* (1995) delimited the West Virginia population associated with typical *C. dubius* *sensu stricto*. Loughman *et al.* (2015) re-described and geographically delimited nominal *C. dubius* (Jezerinac's orange color morph) to southwestern Pennsylvania and northern West Virginia. With *C. dubius* thus restricted, the remaining

populations (color morphs) formerly part of that complex were designated *C. aff. dubius*, until such time that they could be further resolved (*aff.* = has affinities to). The current paper builds on the work of Loughman *et al.* (2015), and represents the second contribution by this working group to delimit one of the numerous undescribed species currently subsumed under the *Cambarus aff. dubius* complex.

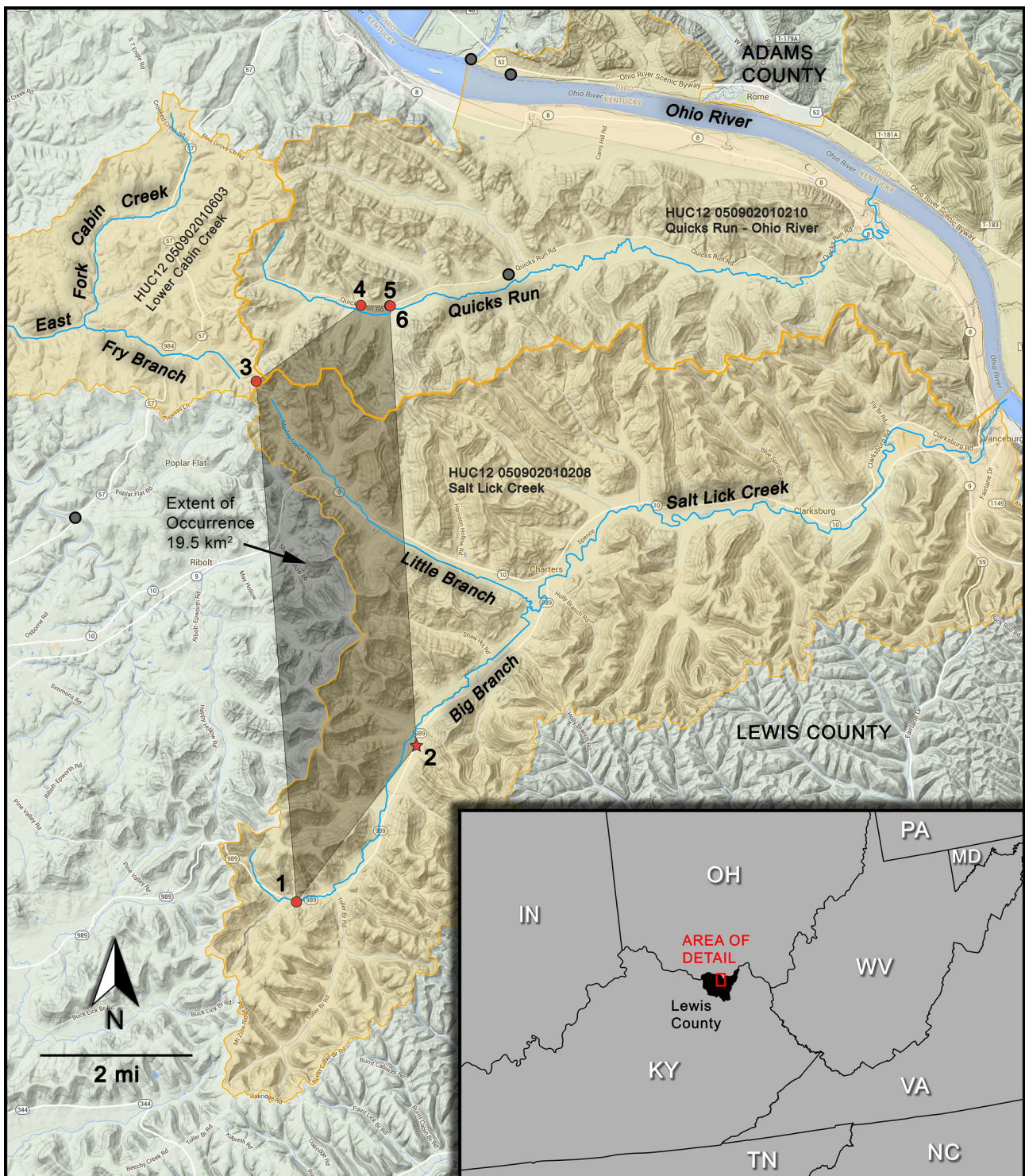


FIGURE 1. Map showing the known distribution of *Cambarus (Jugicambarus) adustus* in Lewis County, Kentucky. The red star indicates the location of the type locality and red circles indicate localities where the species was found. Numbers at sites indicate the locality as listed in the “Specimens Examined” section. Dark gray circles indicate sites where the species was not found. Gray polygon denotes the species known extent of occurrence. Light yellow areas indicate the three focal drainage basins discussed in the main text. Map generated using Google MyMaps.

Material and methods

Specimen and tissue collection. All animals were collected from burrows by hand through burrow excavation from the six localities. 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), after which, specimens were preserved in 70% ethanol. Sampled legs were cut into 2–3 mm pieces and then placed directly into 1 mL of Cell Lysis Buffer (10 mM Tris, 100 mM EDTA, 2% SDS, pH 8.0) that also contained 10 μ L 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 a 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 μ L. Each PCR reaction contained the following components: 1X PCR buffer, 3 mM magnesium chloride, 1.25 mM each dNTP, 1 μ M 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 except that a universal primer sequence was added to the 5' end of the Forward and Reverse COI primers (T7 and T3, respectively). These non-degenerate, non-homologous 5' tails (in bold) were then used to sequence all resulting PCR products. Primer sequences used were: HybLCO 5'-**TAATACGACTCACTATAGGGGGTCAACAAATCATAAAGATATTGG**-3' and Hyb2HCO 5'-**ATTAACCCCTACTAAAGTAACTTCAGGGTGACCAAAAATCA**-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_{μ96} plates (Millipore, Cat#: LSKMPCR50) in preparation for DNA sequencing by a commercial sequencing service (Eurofins Genomics). Sequences obtained from the sequencing facility were initially corrected and aligned using the program Sequencher v5.01 (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 v3.04 (Maddison and Maddison 2015) to verify the presence of an open reading frame (i.e., no stop codons or indels), and to avoid incorporating mtDNA nuclear pseudogenes (=numts) in the analysis. The data were then imported and analyzed using PAUP* v4.0a147 (Swofford 2002) in order to output distance matrices. Phylogenetic analyses were conducted to examine higher level relationships of *Cambarus adustus* with other related species from the subgenus *Jugicambarus*. Different models of DNA sequence evolution were tested for their fit to the COI dataset. Twenty-four different models (three substitution schemes) of DNA sequence evolution were tested using jMODELTEST v2.1.10 (Darriba *et al.* 2012) with the best model selected by BIC for the Bayesian analyses. Partitioning the dataset by codon position was accomplished using MEGA v7.0.14 (Kumar *et al.* 2016). Both maximum likelihood (ML) and Bayesian inference (BI) optimality criteria were used to estimate phylogenies using raxmlGUI v1.3.1 (Silvestro and Michalak 2012, which includes RAXML v7.4.2 (Stamatakis 2006)), and MrBayes v3.2.6 (Ronquist and Huelsenbeck 2003), respectively. For RAXML, the GTR+G model was selected for use over the other alternative model (GTR+G+I), following the program 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 separate partitioning schemes. The first utilized the entire dataset, with a model selected by jMODELTEST, and was analyzed with standard methods (single default partition). For the second analysis, three separate partitions were created from the COI dataset based on codon position, with each subsequent partition run through jMODELTEST in order to obtain the best evolutionary model for each of the three data subsets. For this latter analysis, the parameters were unlinked so that independent values could be estimated for each partition. For both analyses, two simultaneous independent runs were conducted with one cold chain and three hot chains. The program was run for 5×10^6 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. Trees were then examined in FigTree v1.4.2.

Systematics

Cambarus (Jugicambarus) adustus, new species

Figures 2–4, Table 1

Cambarus aff. *dubius*: Loughman, Thoma, Fetzner and Stocker, 2015:534 [in part].

Diagnosis. Pigmented; eyes not reduced. Rostrum with margins slightly convergent, moderately thickened, without marginal spines or tubercles, lacking median carina, slightly excavated, and angled near end at slightly less than 90° to a well-defined acumen. Carapace vaulted, subovate in cross-sectional view, without cervical spines. Branchiostegal tubercles moderately developed. Suborbital angle obtuse to acute. Postorbital ridges developed, never ending in distinct spines or tubercles. Areola open, length 6.9 to 17.9 times width (\bar{x} = 12.6, n = 27), constituting, in adults, 28.1 to 42.5% (\bar{x} = 40.6%, n = 27) of entire carapace length, with room for 2 or 3 rows of punctuations in narrowest part. Antennal scale 1.4 to 2.8 times as long as wide (\bar{x} = 2.41, n = 27), usually broadest at mid-point. Mesial palm of chelae with two rows of tubercles. First row consisting of 5–8, second row of 2–7 well developed tubercles; first row cristiform. No tufts of elongate setae at base of opposable propodus. Opposable margin of propodus with four enlarged tubercles on basal half consisting, from base, of two smaller tubercles, an enlarged tubercle, a space, and a sharply pointed forth tubercle; denticles extending on average 69.0% (n = 27) of distal length. Opposable margin of dactyl from base with one enlarged tubercle, followed by two smaller tubercles, and then one enlarged tubercle, all on basal half; denticles extending on average 51.8% of distal length. Palm length to dactyl length ratio averaging 1.8. Strong dorsomedian longitudinal ridges on dactyl and propodus. Moderate to weak dorsolateral impression at base of propodus. Carpus with one large tubercle on mesial margin, just distal of midpoint, usually with two smaller tubercles proximal to base; row of three to four small tubercles dorsal to carpal tubercle. First pleopods of form I male adjoining at bases, shafts with convexity near mid length of cephalic surface; terminal elements consisting of a tapering, distally pointed central projection with a subapical notch, and equal in length to mesial process. Mesial process conically shaped at base and tapering to distal point(s). Both processes recurved at 90°. Mesial processes deflected slightly lateral. Caudal knob present. Hooks on ischium of third pereopods only, not opposed by tubercle on coxa. Female with asymmetrical annulus ventralis formed by two hardened caudal parts, one curved in a C shape the other a straight, rounded segment forming a flange that projects under the C-shaped portion creating a fossa. Cephalic half of annulus ventralis not sclerotized. Postannular sclerite a symmetrical bar shape, greatest height in center.

Holotypic male, Form I. (Fig. 2A, B, C, D, E, H, J, K & Fig. 3). Eyes pigmented, diameter 37.2% of width of rostrum at eyes. Body vaulted, subovate (Fig. 2J), width equal to depth (Fig. 2A). Abdomen narrower than cephalothorax; maximum width of carapace slightly greater than depth at caudodorsal margin of cervical groove. Areola narrow (1.1 mm) with two punctuations across narrowest part, length comprising 41.4% of total carapace length. Rostrum slightly curved downward distally with slightly convergent, thickened margins and a slightly less than 90° angle delimiting acumen, anterior tip upturned, not reaching to base of ultimate podomere of antennular peduncle; dorsal surface of rostrum slightly excavate with few punctuations, mostly on basal half. Subrostral ridge moderately developed. Postorbital ridge well developed, grooved dorsolaterally, and ending cephalically without tubercle, spine or corneous portion. Suborbital angle obtuse; branchiostegal spine small. Cervical spine absent. Hepatic and branchiostegal regions with small tubercles. Lateral carapace with granular tubercles, dorsally punctate. Abdomen slightly shorter than carapace, pleura short, rounded caudoventrally. Cephalic section of telson with two spines in caudolateral corners. Proximal podomere of uropod with weak distal spine on mesial lobe; mesial ramus of uropod with median rib ending distally as weak distomedian spine not overreaching margin of ramus.

Cephalomedian lobe of epistome (Fig. 2D) pentagonal in shape with level margins, small setae; main body with shallow fovea; epistomal zygoma moderately arched. Ventral surface of antennal peduncle's proximal podomere without spine at base of distal third. Antennal peduncle without spines; antennal scale (Fig. 2H) 2.4 times as long as wide, broadest at midpoint, distal mesial border rounded, proximal border straight; distal spine moderate, not reaching distal extremity of antennular peduncle. Ischium of third maxilliped with abundant flexible setae; mesial margin with 20 teeth, numbers 1, 4, 6, 8 and 11 from distal end larger than adjacent teeth.

Length of right chela (Fig. 2K) 76% that of carapace; width 49% of chela length; palm length 36% of chela length; dactyl length 1.7 times palm length. Dorsal surface of palm covered with punctuations, mesial margin with

two rows of tubercles, first row seven, second row five; ventral surface mostly smooth, one low tubercle on articular rim opposite base of dactyl; lateral surface of chela slightly costate. Both fingers of chela with well-defined submedian dorsal ridges; opposable margin of fixed finger with row of four tubercles (third from base enlarged), gap between third and fourth tubercle, denticles extending distally from third tubercle over 71% of opposable margin of propodus. Opposable margin of dactyl with row of four tubercles along proximal half, first and fourth enlarged; single row of minute denticles extending distally from tubercle two over 61.9% of dactyl length; mesial surface of dactyl with three basal tubercles.

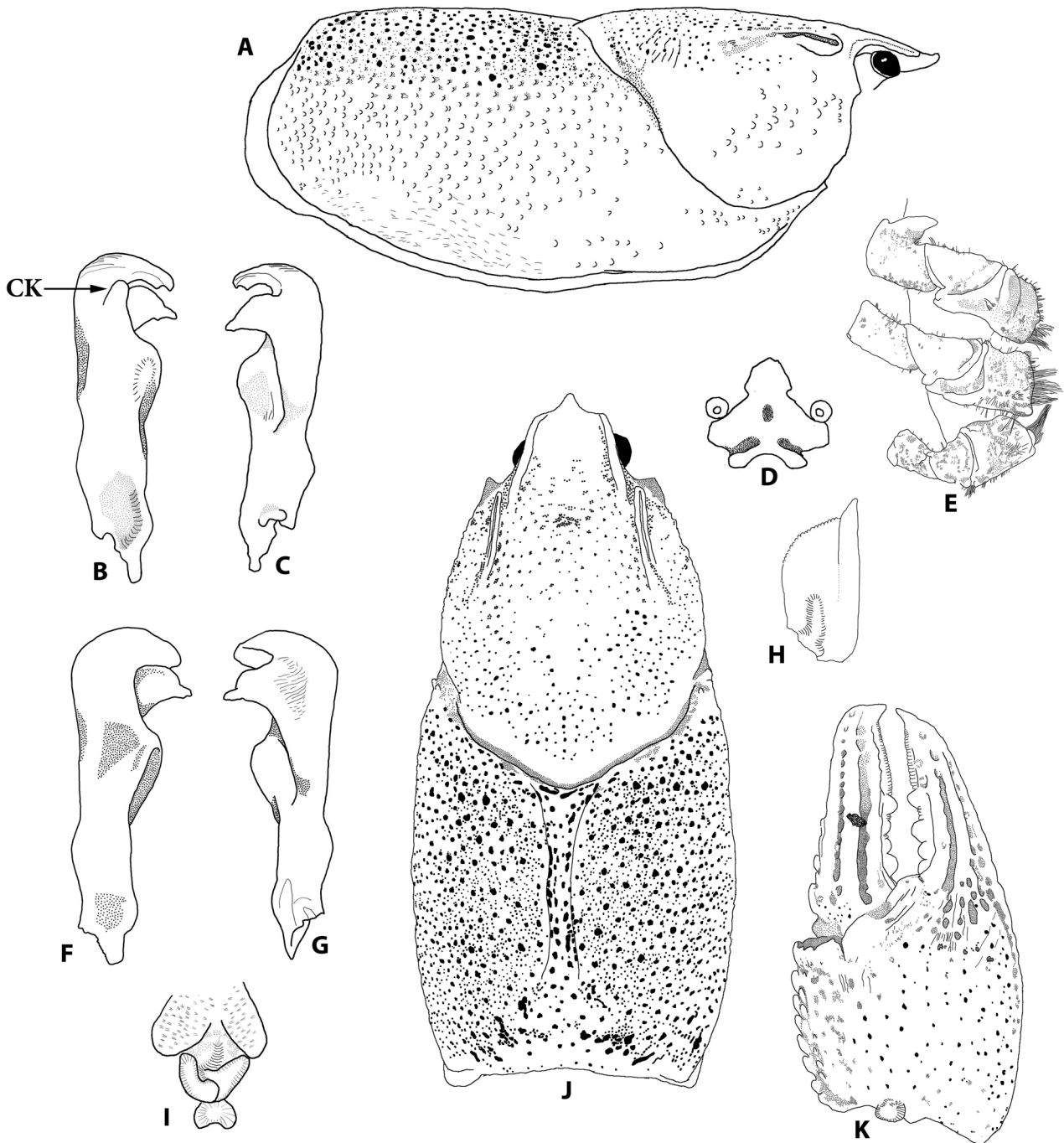


FIGURE 2. *Cambarus (Jugicambarus) adustus*, new species; all from holotype male, form I (USNM 1407169), except F and G from morphotype male, form II (USNM 1407171), and I from allotype female (USNM 1407170): A, lateral aspect of carapace; B and C, lateral and mesial aspect, respectively, of male I gonopod (first pleopod); D, epistome; E, ventral aspect of right third, fourth, and fifth pereopods; F and G, lateral and mesial aspect, respectively, of male II gonopod; H, dorsal aspect of antennal scale; I, ventral aspect of annulus ventralis; J, dorsal aspect of carapace; K, dorsal aspect of distal podomeres of right cheliped; CK, caudal knob.



FIGURE 3. *Cambarus (Jugicambarus) adustus*, new species, holotype male (form I). USNM 1407169



FIGURE 4. *Cambarus (Jugicambarus) adustus*, new species, from the type locality showing natural coloration.

Carpus of cheliped with distinct dorsal furrow; mesial surface with one large spiniform tubercle subtended by one small tubercle proximally, third small tubercle at proximal edge of carpus; ventral surface with small tubercle on distal articular rim, and small fifth tubercle positioned between largest mesial tubercle and articular rim tubercle. Merus with two pre-marginal, slightly spiniform, tubercles dorsally, ventromesial row of ten spiniform tubercles slightly increasing in size distally, ventrolateral row of nine. Ventral ridge of ischium without tubercles. Ischium of third pereopods (Fig. 2E) with strong hook extending proximally over basioischial articulation, not opposed by tubercles on basis. Coxa of fourth pereopods (Fig. 2E) with vertically disposed caudomesial boss; that of fifth pereopod lacking boss, its ventral membrane setiferous.

First pleopods adjoining at base, just reaching coxae of third pereopods when abdomen flexed; central projection (Fig. 2B, C) long, tapering with subapical notch, angled at 90°, shorter than mesial process; mesial process conical at base and tapered apically, directed caudolaterally, bent greater than 90°, terminating in single point, slightly defined caudal knob. Distal margin of proximal segment of lateral ramus of right uropod having fourteen small spines displayed distally, one larger, movable penultimate spine at lateral edge, median spine of mesial uropod ramus small, not overhanging distal margin.

Allotypic female. Other than secondary sexual characters, differing from holotype in following respects: right chela 69% carapace length; dactyl of pereopod I 1.75 times length of palm; palm of chela with two rows of seven tubercles in first row and five in second; eye diameter 36% of rostrum width at eyes; denticles extending distally over 64% of inner margin of propodus, denticles extending distally over 58.4% of dactyl length. Annulus ventralis (Fig. 2I) sclerotized on caudal margin, moderately embedded in V-shaped sternum, asymmetrical in shape, formed by two hardened caudal parts, right curved in a weak C shape, left a straighter, rounded segment with a flange projecting under C-shaped portion and forming a fossa. Cephalic half of annulus ventralis not sclerotized. Postannular sclerite a symmetrical oval shape with a distal indentation, greatest height in center.

Morphotypic male, Form II. Differing from holotype in following respects: palm of chela with two rows of tubercles (first row = 7, second = 4); areola length 42.3% of carapace length; areola 11.7 times as long as wide; antennal scale 2.64 times as long as wide; right chelae 60% of carapace length; palm length 35% of chela length; eye diameter 48.7% of rostral width at eyes; merus with row of five ventrolateral spines; central projection of first pleopod (Fig. 2F, G) non corneous and blunt, equal in length to mesial process. Hook on ischium of third pereopod smaller, not overhanging basioischial articulation. Distal margin of proximal segment of lateral ramus of right uropod having 16 spines displayed distally.

Type locality. Holotype, morphotype, and allotype collected from roadside ditch serving as a tributary to Big Branch of Salt Lick Creek adjacent KY Rt. 898 just N of Hatcher Branch, 4.67 km SW of Charters, 3.60 km NE of Glen Springs, (38.53930, -83.46565, WGS84), Lewis County, Kentucky. The holotype and allotype were both collected on 16 October 2014; morphotype was collected on 12 June 2013.

Disposition of types. The holotype, allotype, and morphotype are in the collection of the National Museum of Natural History (USNM 1407169, 1407170, and 1407171 respectively), Suitland, Maryland along with paratypes (USNM 1409733). Additional paratypes are housed in the Cleveland Museum of Natural History (CMNH 1179) and Carnegie Museum of Natural History (CM Acc. 38597).

Range and specimens examined. This species has only been found in Lewis County, Kentucky in the Quicks Run, Salt Lick Creek and the eastern edge of Cabin Creek basins. Adjacent areas in Kentucky and Ohio have been searched but no other populations have been found. Taylor and Schuster (2004) did not report *C. dubius* from Lewis County, KY. Their closest record was a single site reported from Rowan County, KY in the Licking River basin.

KENTUCKY: *Lewis County*: **1)** CMNH 1179—ditch adjacent KY Rt. 989, tributary to Big Branch of Salt Lick Creek of Ohio River, 0.95 km W of Glen Springs, 18.52 km SW of Vanceburg, (38.50859, -83.49575, WGS84), Roger F. Thoma (RFT) and Jim Grow (JG), 2 MI, 1 MII, 12 F, 3 M_{juv}, 3 F_{juv}, 31 August 2013; **2)** USNM 1407169, 1407170, 1407171 [Holotype, Allotype, Morphotype], CM Acc. 38597 [Paratypes]—roadside ditch tributary to Big Branch of Salt Lick Creek adjacent KY Rt. 898, 4.67 km SW of Charters, 3.60 km NE of Glen Springs, (38.53930, -83.46565, WGS84), RFT and G. Whitney Stocker (GWS), 1 MI, 1 MII, 1 F, 1 M_{juv}, 1 F_{juv}, 12 June 2013; **3)** CMNH 1180—roadside ditch in Manley Hollow of Fry Branch of East Fork Cabin Creek adjacent Manley Hollow Road, 16.27 km W of Vanceburg, 12.09 km SE of Manchester, (38.61050, -83.50592, WGS84), RFT & GWS, 1 MI, 1 F, 12 June 2013; **4)** CMNH 1182—ditch adjacent Quicks Run Road, 3.67 km W of Queens, 6.97 km NE of Tollesboro, (38.625357, -83.479688, WGS84), RFT, 1 MI, 1 F, 1 M_{juv}, 2 F_{juv}, 12 May 2011; **5)** CMNH 1183—roadside ditch tributary to Quicks Creek adjacent Quicks Creek Road at Muses Church and cemetery, 6.99

km SSE of Concord, 13.76 km SE of Manchester, (38.62527, -83.47250, WGS84), RFT & GWS, 2 MI, 3 MII, 12 June 2013; 6) CMNH 1181—ditch adjacent Quicks Run Road and graveyard/church, 3.06 km W of Queens, 7.25 km NE of Tollesboro, (38.625246, -83.472327, WGS84), RFT, 2 MII, 2 M_{juv}, 1 F_{juv}, 1 body part, 12 May 2011; 7) USNM 1409733—Salt Lick Creek adjacent Salt Lick Road at #3073 Salt Lick Road, 3.6 km NE of Glen Springs, 14.7 km SW of Vanceburg, (38.539320, -83.465670, WGS84), RFT, JG, Max Luehrs (ML), Mitch Farley (MF) 7 MI, 1 MII, 4 F, 16-Oct-15; 8) CMNH 1184—roadside ditch adjacent Big Branch of Salt Lick Creek and KY Rt 989, 1.12 km WSW of Glen Springs, 18.59 km SW of Vanceburg, (38.508790, -83.496560, WGS84), RFT, JG, ML, MF, 16 October 2015. No material collected by Jezerinac and Stocked was available for examination.

Conservation status. *Cambarus (J.) adustus*, new species, is narrowly distributed, with a current extent of occurrence of 19.5 km² in Lewis County, Kentucky. It is best classified as Endangered following Taylor *et al.* (2007), Endangered (B2ab(iii), ver. 3-1) using IUCN criteria (IUCN 2001), G1 using NatureServe. It should be listed as S1 by the state of Kentucky, and Endangered by the USFWS.

Color notes. All specimens observed have been a concolorous brown (Fig. 4). Tubercles on the opposable margins of the chelae fingers are cream-yellow. The tips of chelae fingers can be orange.

Variation. No geographic variation has been identified in the highly limited range of this species. Within population variation consist primarily of ontogenetic changes and sexual dimorphism.

Size. The three largest individuals were female, with the largest female having a carapace length of 45.9 mm. Mature form I males average TCL was 37.0 mm (34.60–39.8 mm), form II males average TCL was 32.3 mm (27.2–35.4 mm), and females TCL averaged 37.0 mm (30.0–45.2 mm) carapace length.

TABLE 1. Morphological measurements (mm) of the primary type specimens of *Cambarus (J.) adustus*, new species.

Character	Holotype	Allotype	Morphotype
Carapace			
Height	18.20	19.30	12.57
Width	18.68	20.63	12.80
Length	37.70	40.99	27.24
Areola			
Length	15.60	17.34	11.52
Width	1.05	1.63	0.98
Rostrum			
Width at base	5.80	6.15	3.90
Width at eyes	5.08	5.44	3.37
Length	5.50	7.05	5.12
Eye			
Diameter	1.89	1.96	1.64
Chela (right)			
Length lateral margin	28.47	28.14	16.45
Length mesial palm	4.90	4.00	4.50
Width of palm	5.90	5.10	5.50
Length of dactyl	7.90	6.80	7.50
Abdomen			
Length	20.70	20.10	18.90
Width	8.30	8.30	7.90
Gonopod			
Length	4.50	N/A	4.60
Antennal scale			
Length	3.30	3.40	3.70
Width	1.40	1.40	1.50

Life history. Collections have been made in May, June, August, September, and October. First form males, second form males, and females have been observed in all months sampled. No ovigerous females or young-of-year have been observed.

Crayfish associates. *Cambarus thomai* and *Cambarus ortmanni* are found burrowing in adjacent areas. In streams of the area there is a member of the *Orconectes* subgenus *Procericambarus* present. It is not clear if these populations represent an undescribed species or are just a geographic variant of *Orconectes raymondi* Thoma and Stocker 2009.

Relationships. *Cambarus adustus* is assigned to the subgenus *Jugicambarus* based on the presence of a cristiform row of tubercles on the mesial margin of the chelae. It appears to be most closely related to *C. dubius*, *C. pauleyi*, and other members of the *C. aff. dubius* complex. It is hypothesized that the species had become established in the preglacial Portsmouth River prior to the onset of the Quaternary. *Cambarus adustus* displays a caudal knob similar to, but smaller than, *Cambarus bouchardi* Hobbs 1970. This character state is not thought to reflect a close relationship between the two species.

Comparisons. Within the genus *Cambarus*, *C. (J.) adustus* new species, falls within the group of species with a vaulted carapace. Almost all members of this morphological group display dramatic color patterns involving bright reds and blues in life. In contrast, *Cambarus adustus* is all brown. When specimens are keyed using morphological characters, the couplets lead to *C. dubius* (Thoma 2016). Both species have a truncated rostrum, narrow areola, cristiform palmer tubercles, slightly vertically rotated chelae, and a vaulted carapace. It is easily distinguished from *C. dubius* in several ways. The slight caudal knob on the MI gonopod is not present in *C. dubius* or any closely related taxa/forms (see Loughman *et al.* 2015), a lack of red and blue coloration in *C. adustus* is a distinguishing characteristic in live animals, and the lateral carapace of *C. adustus* is more strongly granular with many small tubercles, while *C. dubius* tuberculation is much reduced.

A superficial similarity exist between *C. adustus* and *C. ortmanni*. Both are primary burrowers and both are brown. *Cambarus ortmanni* lacks a cristiform row of tubercles on the chelae palm, has only one row of palmer tubercles, has a very narrow areola with only one row of punctations, and a much reduced suborbital angle being almost obsolete, as opposed to a more obtuse suborbital angle in *C. adustus*. Two other brown colored primary burrowers (*Cambarus nodosus* Bouchard and Hobbs 1976 and *Cambarus causeyi* Riemer 1966) are found in the subgenus *Jugicambarus*. *Cambarus causeyi* differs in the abundant setae found on the chelae. *Cambarus nodosus* differs in its dramatically enlarged central projection and mesial process of the MI gonopod and a lack of small tubercles on the lateral surface of the posterior thorax.

Within the subgenus *Jugicambarus*, the group of stream dwelling species related to *C. distans* (*Cambarus bouchardi*, *Cambarus crinipes* Bouchard 1973, *Cambarus obeyensis* Hobbs and Shoup 1947, *Cambarus tuckasegee* Cooper and Schofield 2002, *Cambarus unestami* Hobbs and Hall 1969, *Cambarus parvoculus* Hobbs and Shoup 1947, and *Cambarus jezerinaci* Thoma 2000) all differ in the shape of their carapace, which is dorso-ventrally compressed, as opposed to laterally compressed (vaulted).

Etymology. The species epithet (*adustus* = L. brown) is chosen to recognize the overall coloration of this species. The suggested vernacular name is Dusky Mudbug.

Results

A total of 22 new unique COI sequences was generated for this study, including 11 from members of the subgenus *Jugicambarus* and 11 that represent taxa for an extended outgroup used to help root the phylogeny. Seven additional *Jugicambarus* sequences were gathered from Genbank and are from previously published studies. Sequenced specimens included four *C. adustus* from three of the six known localities, one *C. dubius* from the type locality at Terra Alta, one *C. pauleyi* from its type locality in Moncove Lake State Park, and two *C. aff. dubius*, including one from WV (black body, orange claw morph) and the other from NC (taken from Genbank). An additional 13 taxa from *Jugicambarus*, the presumed closest relatives to the new species, were also included, along with several burrowing species from other *Cambarus* subgenera. Outgroups included a total of nine representative species sampled from the genera *Procambarus* (n=3), *Orconectes* (3), *Distocambarus* (1), *Hobbseus* (1), and *Fallicambarus* (1) (Table 2). Unique haplotype sequences generated as part of this study were deposited in GENBANK under accession numbers (KX417092-KX417114), and existing sequences from GENBANK used in the analysis are listed in Table 2.

TABLE 2. Summary of locality data for genetic samples examined in this study. N is the number of sequences examined from each location. The H1 and H2 designations in the Specimen Code field for *C. adustus* indicates different mtDNA haplotypes. The Genbank accession numbers listed for *C. adustus* indicate those for haplotypes H1 (KX417092) and H2 (KX417093), respectively.

Species	N	State	County	Location	Lat	Long	Collector(s)	Specimen Code	Genbank
<i>Cambarus (J.) adustus</i>	2	KY	Lewis	Quick Creek	38.62527	-83.47250	RFT	JF13762 (H1), JF13764 (H2)	KX417092
<i>Cambarus (J.) adustus</i>	1	KY	Lewis	Fry Branch	38.61050	-83.50592	RFT	JF13767 (H2)	KX417093
<i>Cambarus (J.) adustus</i>	1	KY	Lewis	Big Branch	38.53930	-83.46565	RFT	JF13770 (H2)	—
<i>Cambarus (J.) dubius</i>	1	WV	Preston	Terra Alta	39.45491	-79.52711	ZJL	JF14706	KX417094
<i>Cambarus (J.) aff. dubius</i>	1	WV	Greenbrier	Roadside ditch	37.90995	-80.70275	ZJL	JF15037	KX417095
<i>Cambarus (J.) pauleyi</i>	1	WV	Monroe	Moncove Lake State Park	37.62033	-80.35337	ZJL	JF15064	KX417096
<i>Cambarus (J.) aff. dubius</i>	1	NC	Haywood	Cold Spring Creek	35.73784	-83.02345	K. Crandall	KC1390	JX514462
<i>Cambarus (J.) asperimanus</i>	1	NC	Avery	Watauga River	36.12304	-81.82690	RFT	JF11041	KX417097
<i>Cambarus (J.) batchi</i>	1	KY	Madison	Central KY Wildlife Area	37.62035	-84.20811	G. Schuster	JF16004	KX417098
<i>Cambarus (J.) carolinus</i>	1	SC	Greenville	Barton Creek	35.11435	-82.36269	JWF, RFT	JF7943	KX417099
<i>Cambarus (J.) clivosus</i>	1	TN	Cannon	Trib. EF Stones River	35.84724	-85.95328	RFT	JF5984	KX417100
<i>Cambarus (J.) cryptodytes</i>	1	FL	Jackson	Salamander Pond Cave	--	--	K. Crandall	KC784	JX514483
<i>Cambarus (J.) distans</i>	1	KY	McCreary	Trib. Cumberland River	36.83672	-84.34557	JWF, RFT	JF7218	KX417102
<i>Cambarus (J.) genivri</i>	1	TN	Dickson	Williams Branch	36.17964	-87.27985	JWF	JF2508	KX417101
<i>Cambarus (J.) magerae</i>	1	VA	Wise	South Fork Powell River	36.83606	-82.70552	RFT, R. Puckett	JF8698	KM099317
<i>Cambarus (J.) monongalensis</i>	1	PA	Greene	Crabapple Creek	39.91631	-80.43712	JWF, GWS	JF2727	JX514463
<i>Cambarus (J.) nodosus</i>	1	TN	Polk	Ocoee River	35.04041	-84.44896	RFT, L. Hersh	JF11444	KX417103
<i>Cambarus (J.) aculabrum</i>	1	AR	Benton	Bear Hollow Cave	--	--	K. Crandall	KC574	JX514482
<i>Cambarus (J.) setosus</i>	1	MO	Dade	Carrico Cave	--	--	K. Crandall	KC593	JX514464
<i>Cambarus (J.) tartarus</i>	1	OK	Delaware	Stansbury-January Cave	--	--	K. Crandall	KC578	JX514465
<i>Cambarus (C.) bartonii cavatus</i>	1	KY	Bell	Trib. Clear Creek	36.74288	-83.70293	JWF, RFT	JF5011	KM099336
<i>Cambarus (L.) diogenes</i>	1	IL	Ford	Roadside ditch	40.53021	-88.25401	JWF	JF10165	KX417104
<i>Cambarus (D.) deweeseae</i>	1	KY	Russell	Roadside ditch	37.05342	-84.96669	C. Mason	JF9854	KX417105
<i>Hobbsseus valliculus</i>	1	MS	Choctaw	Trib. Tibby Creek	33.22883	-89.15555	JWF, C. Taylor	JF4006	KX417111
<i>Distocambarus devexus</i>	1	GA	Oglethorpe	Goosepond Creek	33.97072	-82.86642	C. Skelton	JF2802	KX417106
<i>Fallicambarus (C.) fodiens</i>	1	OH	Jackson	Little Salt Creek	39.02170	-82.61440	RFT	JF15099B	KX417107
<i>Orconectes (C.) eupunctus</i>	1	MO	Oregon	Eleven Point River	36.55338	-91.18764	MO Dept. Con.	JF10971	KX417108
<i>Orconectes (F.) limosus</i>	1	MD	Prince Georges	Paint Branch	39.03235	-76.95331	JWF, RFT	JF8295	KX417109
<i>Orconectes (P.) punctimanus</i>	1	MO	Oregon	Eleven Point River	36.64648	-91.20105	MO Dept. Con.	JF12635	KX417110
<i>Procambarus (O.) zotangulus</i>	1	LA	East Baton Rouge	LSU Ponds	30.37066	-91.18794	R. Romaine	JF10162	KX417112
<i>Procambarus (O.) viaeviridis</i>	1	AL	Tuscaloosa	Roadside ditch	33.06675	-87.64611	M. Kendrick	JF15129	KX417113
<i>Procambarus (S.) clarkii</i>	1	AL	Tuscaloosa	Roadside ditch	33.06730	-87.64520	M. Kendrick	JF15151	KX417114

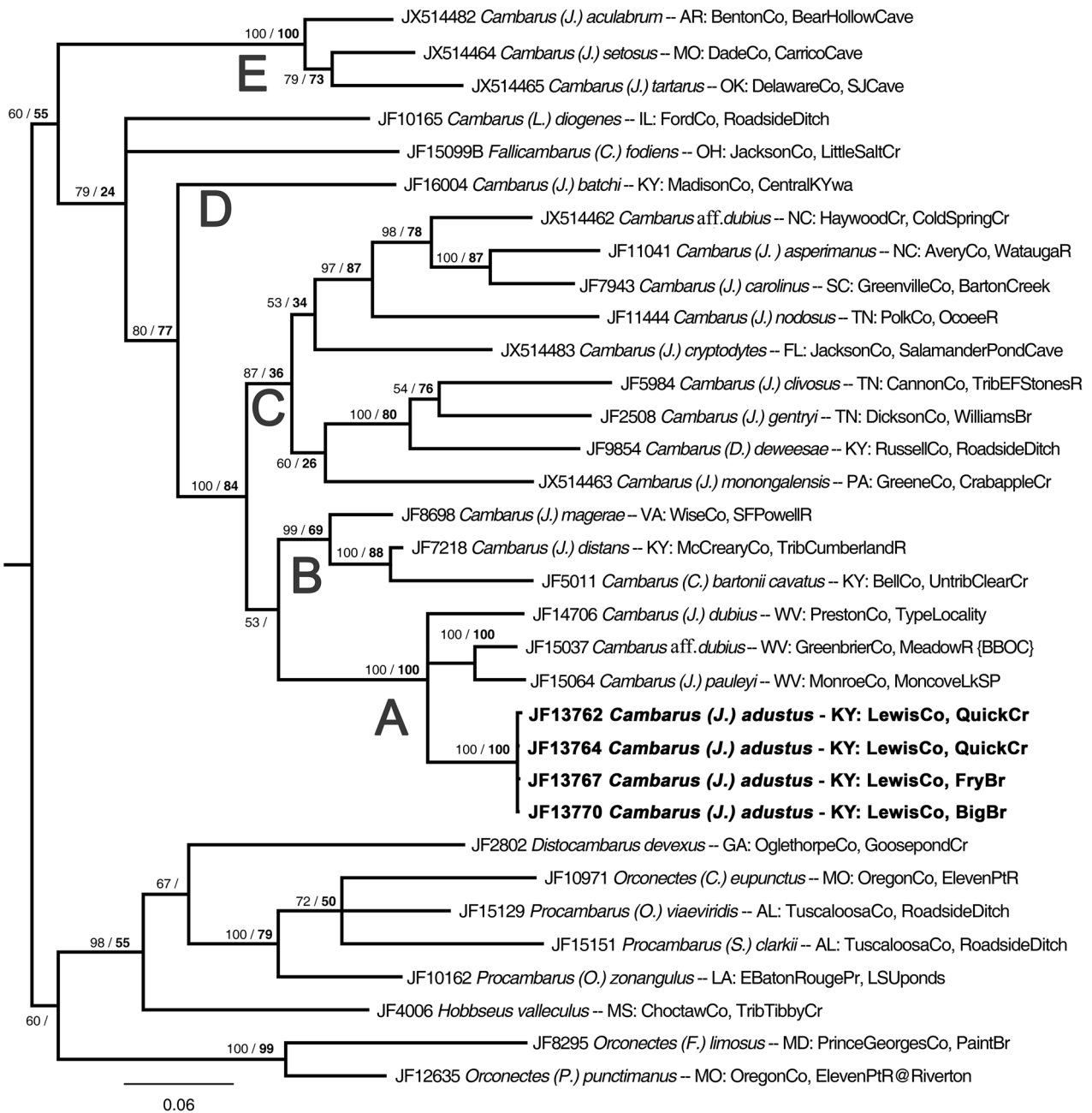


FIGURE 5. Bayesian phylogenetic tree depicting relationships among *Cambarus (J.) adustus* and other closely related species from the subgenus *Jugicambarus*, along with selected outgroup taxa. This tree was generated in MrBayes by treating all data as a single partition using the HKY+I+G model of sequence evolution. Numbers at nodes indicate both posterior probabilities (regular font) from MrBayes and bootstrap values (in bold), with the latter generated by RAxML using the GTR+G model and 1000 pseudoreplicate datasets. Letters indicate supported clades that are discussed in the main text. Three nodes without bootstrap values indicate nodes that were not found in the ML tree.

Genetic divergences (uncorrected p-distances) among the three sampled populations of *C. adustus* were low (0.2%), with only two COI haplotypes being detected which differed by a single mutation. Keeping in mind the limited taxon and geographic sampling of this study, *C. adustus* appears most closely related to *C. dubius*, *C. pauleyi*, and other *C. aff. dubius* populations from West Virginia, differing from them on average by 6.8% sequence divergence (range: 6.1–7.4%) (Table 3). *Cambarus adustus* differed from other members of the subgenus *Jugicambarus* by an average divergence of 11.5% (range: 9.4–13.8%), and from the outgroups by 13.8% (range: 12.4–16.3%).

TABLE 3. Average genetic distances among species using sequences from the COI gene, uncorrected p-distances below diagonal, absolute distances (= number of mutations) above diagonal.

#	Specimen	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	JF13762 <i>Cambarus (J.) adustus</i>	****	1	1	1	39	43	45	65	65	74	71	69	61	71	59
2	JF13764 <i>Cambarus (J.) adustus</i>	0.002	****	0	0	38	42	44	64	64	73	71	70	60	70	58
3	JF13767 <i>Cambarus (J.) adustus</i>	0.002	0	****	0	28	31	33	47	47	53	49	52	44	53	44
4	JF13770 <i>Cambarus (J.) adustus</i>	0.002	0	0	****	38	42	44	64	65	73	71	70	60	70	58
5	JF14706 <i>Cambarus (J.) dubius</i>	0.064	0.062	0.061	0.062	****	46	42	77	78	83	88	72	67	76	62
6	JF15037 <i>Cambarus (J.) aff. dubius</i>	0.070	0.069	0.067	0.069	0.070	****	27	72	80	84	80	77	73	77	61
7	JF15064 <i>Cambarus (J.) pauleyi</i>	0.074	0.072	0.071	0.072	0.064	0.041	****	76	80	88	84	78	75	68	64
8	JX514462 <i>Cambarus (J.) aff. dubius</i>	0.106	0.104	0.101	0.104	0.119	0.111	0.117	****	52	82	47	76	64	70	55
9	JF11041 <i>Cambarus (J.) asperimanus</i>	0.106	0.106	0.102	0.106	0.119	0.122	0.122	0.080	****	88	44	74	63	76	68
10	JF16004 <i>Cambarus (J.) batchi</i>	0.121	0.119	0.116	0.119	0.126	0.128	0.134	0.127	0.134	****	94	76	86	79	64
11	JF7943 <i>Cambarus (J.) carolinus</i>	0.116	0.116	0.106	0.116	0.134	0.122	0.128	0.073	0.067	0.143	****	83	70	78	67
12	JF5984 <i>Cambarus (J.) clivosus</i>	0.113	0.114	0.114	0.114	0.109	0.117	0.119	0.117	0.112	0.116	0.126	****	60	60	65
13	JX514483 <i>Cambarus (J.) cryptodytes</i>	0.101	0.099	0.096	0.099	0.103	0.113	0.116	0.100	0.097	0.132	0.108	0.092	****	73	61
14	JF2508 <i>Cambarus (J.) genryi</i>	0.116	0.114	0.114	0.114	0.116	0.117	0.103	0.108	0.116	0.120	0.119	0.091	0.112	****	72
15	JF8698 <i>Cambarus (J.) magerae</i>	0.096	0.094	0.094	0.094	0.094	0.093	0.097	0.085	0.103	0.097	0.102	0.099	0.094	0.109	****
16	JX514463 <i>Cambarus (J.) monogalensis</i>	0.114	0.112	0.114	0.112	0.128	0.111	0.123	0.106	0.103	0.120	0.117	0.099	0.108	0.109	0.097
17	JF11444 <i>Cambarus (J.) nodosus</i>	0.120	0.121	0.118	0.121	0.132	0.131	0.141	0.104	0.103	0.134	0.109	0.134	0.117	0.137	0.108
18	JX514482 <i>Cambarus (J.) aculabrum</i>	0.134	0.132	0.126	0.132	0.142	0.142	0.136	0.129	0.136	0.153	0.135	0.150	0.114	0.134	0.128
19	JX514464 <i>Cambarus (J.) setosus</i>	0.131	0.129	0.123	0.129	0.131	0.134	0.129	0.122	0.129	0.140	0.135	0.124	0.112	0.134	0.128
20	JX514465 <i>Cambarus (J.) tartarus</i>	0.135	0.134	0.138	0.134	0.146	0.141	0.141	0.144	0.149	0.155	0.146	0.149	0.137	0.144	0.137
21	JF5011 <i>Cambarus (C.) bartonii cavatus</i>	0.105	0.103	0.108	0.103	0.107	0.115	0.113	0.105	0.113	0.115	0.113	0.098	0.110	0.098	0.072
22	JF10165 <i>Cambarus (L.) diogenes</i>	0.125	0.123	0.134	0.123	0.122	0.128	0.128	0.128	0.119	0.123	0.112	0.135	0.120	0.117	0.122
23	JF9854 <i>Cambarus (D.) deweesae</i>	0.108	0.106	0.107	0.106	0.117	0.128	0.123	0.120	0.114	0.129	0.117	0.099	0.095	0.091	0.109
24	JF2802 <i>Distocambarus devexus</i>	0.139	0.137	0.135	0.137	0.140	0.146	0.141	0.132	0.132	0.146	0.132	0.129	0.125	0.132	0.116
25	JF15099B <i>Fallicambarus (C.) fodiens</i>	0.133	0.132	0.126	0.132	0.123	0.125	0.129	0.122	0.129	0.117	0.126	0.126	0.123	0.135	0.106
26	JF10971 <i>Orconectes (C.) eupunctus</i>	0.133	0.132	0.128	0.132	0.129	0.138	0.137	0.124	0.137	0.129	0.140	0.134	0.138	0.126	0.120
27	JF8295 <i>Orconectes (F.) limosus</i>	0.155	0.155	0.163	0.155	0.152	0.153	0.153	0.134	0.153	0.129	0.149	0.141	0.148	0.126	0.125
28	JF12635 <i>Orconectes (P.) punctimanus</i>	0.156	0.155	0.161	0.155	0.150	0.147	0.143	0.141	0.157	0.134	0.152	0.138	0.137	0.123	0.135
29	JF4006 <i>Hobbsseus valleculeus</i>	0.128	0.127	0.124	0.127	0.140	0.137	0.147	0.116	0.135	0.128	0.131	0.123	0.124	0.129	0.112
30	JF10162 <i>Procambarus (O.) zonangulus</i>	0.137	0.136	0.137	0.136	0.129	0.135	0.138	0.130	0.129	0.132	0.134	0.128	0.113	0.120	0.116
31	JF15129 <i>Procambarus (O.) viaeviridis</i>	0.129	0.127	0.128	0.127	0.125	0.129	0.135	0.128	0.131	0.138	0.141	0.129	0.130	0.125	0.108
32	JF15151 <i>Procambarus (S.) clarkii</i>	0.134	0.136	0.136	0.136	0.134	0.138	0.146	0.136	0.141	0.143	0.143	0.134	0.124	0.143	0.116

.....continued on the next page

TABLE 3. (Continued)

Specimen	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1 JF13762 <i>Cambarus (J.) adustus</i>	70	73	81	80	83	64	77	66	85	82	82	95	96	79	84	79	82
2 JF13764 <i>Cambarus (J.) adustus</i>	69	74	80	79	82	63	76	65	84	81	81	95	95	78	83	78	83
3 JF13767 <i>Cambarus (J.) adustus</i>	52	54	58	56	63	50	61	49	62	58	59	74	74	57	62	58	62
4 JF13770 <i>Cambarus (J.) adustus</i>	69	74	80	79	82	63	76	65	84	81	81	95	95	78	83	78	83
5 JF14706 <i>Cambarus (J.) dubius</i>	84	87	93	85	90	70	80	77	92	81	85	100	99	92	85	82	88
6 JF15037 <i>Cambarus (J.)</i> aff. <i>dubius</i>	73	86	93	87	87	75	84	84	96	82	91	101	97	90	89	85	91
7 JF15064 <i>Cambarus (J.) paulleyi</i>	81	93	89	84	87	74	84	81	93	85	90	101	94	97	91	89	96
8 JX514462 <i>Cambarus (J.)</i> aff. <i>dubius</i>	69	67	83	79	89	68	77	78	85	79	80	87	91	75	84	83	88
9 JF11041 <i>Cambarus (J.) asperimanus</i>	68	68	89	84	92	74	81	75	87	85	90	101	103	89	85	86	93
10 JF16004 <i>Cambarus (J.) batchi</i>	79	88	100	91	96	75	74	85	96	77	85	85	88	84	87	91	94
11 JF7943 <i>Cambarus (J.) carolinus</i>	77	72	88	88	90	74	89	77	87	83	92	98	100	86	88	93	94
12 JF5984 <i>Cambarus (J.) clivosus</i>	65	88	98	81	92	64	79	65	85	83	88	93	91	81	84	85	88
13 JX514483 <i>Cambarus (J.) cryptodytes</i>	70	76	74	72	84	71	76	62	81	80	89	96	89	80	73	84	80
14 JF2508 <i>Cambarus (J.) gentryi</i>	72	90	88	87	89	64	80	60	87	89	83	83	81	85	79	82	94
15 JF8698 <i>Cambarus (J.) magerae</i>	64	71	84	83	85	47	66	72	76	70	79	82	89	74	76	71	76
16 JX514463 <i>Cambarus (J.) monongalensis</i>	***	75	91	81	95	64	74	69	83	91	91	94	96	80	91	83	89
17 JF11444 <i>Cambarus (J.) nodosus</i>	0.114	***	91	95	95	85	83	85	91	92	95	94	100	91	94	89	95
18 JX514482 <i>Cambarus (J.) acutabrum</i>	0.139	0.139	***	44	48	91	82	92	79	88	85	90	91	81	76	87	89
19 JX514464 <i>Cambarus (J.) setosus</i>	0.124	0.146	0.068	***	43	91	78	90	90	80	86	87	89	79	76	83	81
20 JX514465 <i>Cambarus (J.) tartarus</i>	0.153	0.154	0.078	0.070	***	90	72	95	89	84	87	83	81	84	84	90	85
21 JF5011 <i>Cambarus (C.) bartonii cavatus</i>	0.098	0.130	0.140	0.140	0.146	***	79	74	90	86	92	86	94	89	85	88	88
22 JF10165 <i>Cambarus (L.) diogenes</i>	0.112	0.126	0.125	0.120	0.116	0.121	***	86	75	71	65	71	73	80	77	62	78
23 JF9854 <i>Cambarus (D.) deweeseae</i>	0.105	0.129	0.141	0.138	0.154	0.113	0.131	***	87	93	91	97	97	96	88	89	95
24 JF2802 <i>Distocambarus devexus</i>	0.126	0.138	0.121	0.138	0.144	0.138	0.114	0.132	***	89	74	84	89	67	67	65	72
25 JF15099B <i>Fallicambarus (C.) fodiens</i>	0.138	0.140	0.135	0.123	0.136	0.132	0.108	0.141	0.135	***	72	89	70	75	85	80	77
26 JF10971 <i>Orconectes (C.) eupunctus</i>	0.138	0.144	0.130	0.132	0.140	0.141	0.099	0.138	0.112	0.109	***	86	73	75	60	48	58
27 JF8295 <i>Orconectes (F.) limosus</i>	0.143	0.143	0.138	0.134	0.134	0.132	0.108	0.147	0.128	0.135	0.131	***	59	81	72	77	75
28 JF12635 <i>Orconectes (P.) punctimanus</i>	0.146	0.152	0.139	0.137	0.131	0.144	0.111	0.147	0.135	0.106	0.111	0.090	***	78	75	76	76
29 JF4006 <i>Hobbseus vallecullus</i>	0.122	0.138	0.124	0.122	0.136	0.136	0.122	0.146	0.102	0.114	0.114	0.123	0.119	***	71	69	72
30 JF10162 <i>Procambarus (O.) zonangulus</i>	0.138	0.143	0.116	0.117	0.136	0.130	0.117	0.134	0.102	0.129	0.091	0.109	0.114	0.108	***	53	52
31 JF15129 <i>Procambarus (O.) viaeviridis</i>	0.126	0.135	0.133	0.128	0.146	0.135	0.094	0.135	0.099	0.122	0.073	0.117	0.116	0.105	0.081	***	54
32 JF15151 <i>Procambarus (S.) clarkii</i>	0.135	0.144	0.136	0.125	0.137	0.135	0.119	0.144	0.109	0.117	0.088	0.114	0.116	0.109	0.079	0.082	***

The best model selected for the standard Bayesian analysis by jMODELTEST, using the BIC criterion, was the HKY+I+G model with the following settings: base=(0.2643 0.0894 0.1814) nst=2 tratio=5.4723 rates=gamma shape=0.7950 ncat=4 pinvar=0.5620. For the “by-conda” analysis, jMODELTEST selected the SYM+G [base=equal nst=6 rmat=(1.9673 4.6558 1.4334 1.1630 32.9412) rates=gamma shape=0.2060 ncat=4 pinvar=0], HKY+I [base=(0.1367 0.2378 0.1692) nst=2 tratio=2.9879 rates=equal pinvar=0.8210], and the GTR+G [base=(0.3246 0.0391 0.1483) nst=6 rmat=(0.0100 11.0056 0.4680 2.7547 22.8151) rates=gamma shape=1.7510 ncat=4 pinvar=0] models for the first, second and third codon datasets, respectively.

Both the Bayesian and Maximum-Likelihood (ML) phylogenetic trees placed *C. adustus* within a clade that also contains *C. dubius*, *C. pauleyi* and *C. aff. dubius* with very high nodal support (i.e., pp=100, bootstrap=100) (Fig. 5, A). The trees reconstructed with different optimality criteria each recovered the same groups of species (all trees not shown), but they differed slightly in their level of resolution at some of the internal nodes. In total, five supported groups of *Jugicambarus* species were recovered in the trees (see Fig. 5, A–E). Group E contained the cave species from AR, MO, and OK, and tended to be more distantly removed from the other species, so the discussion below does not consider this group further. The standard Bayesian analysis grouped the remaining clades as (((A+B)+C)+D), while the RAxML analysis (not shown) grouped them as ((A+C)+B)+D). The by-codon analysis depicted the relationships as a polytomy, ((A+B+C)+D). Given the low support in all of the trees for these nodes, the latter unresolved result is probably the best depiction of relationships among these various groups of species. A broader taxonomic and geographic sampling of *Jugicambarus*, and/or *Cambarus* in general, may help to improve support for the various groups recovered in the analysis, or increasing the size of the dataset by including additional genes may help to resolve their relationships.

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