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Research Article

Shallow Waters, Deep Divergence: Epigean Amphipods Utilize Shallow Hyporheic Habitats in the Western Allegheny Plateau

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Two new species of freshwater amphipods, *Crangonyx ipnoecetes* n. sp. and *Crangonyx furnaricolus* n. sp., are described from intermittent bodies of water in Vinton Furnace State Forest (VFSF), Vinton County, Ohio, USA. The descriptions of these species are based on both morphological and molecular analyses, which identify them as only distantly related despite their syntopic occurrences. These species are estimated to have last shared a common ancestor during the Late Cretaceous or early Paleogene (73–50 Ma) and to have diverged from their closest congeners towards the end of this period. Given the region occupied by these species, it is likely that their distributions were influenced by both preglacial habitats, such as the Teays River, and postglacial structures, such as Lake Tight. Our results suggest that significant gaps in knowledge still exist for the amphipod fauna of the Western Allegheny Plateau (eastern USA), with implications for taxonomy and conservation of *Crangonyx* as well as the natural history of the plateau and surrounding region. Furthermore, the description of these species provides additional evidence for the utilization of shallow hyporheic habitats by epigean *Crangonyx* spp. These habitats may play significant roles not only for these species, but for other epigean taxa as well.

Keywords: *Crangonyx*; Lake Tight; Ohio; Teays River; Vinton County; Vinton Furnace State Forest

1. Introduction

When considering hypogean (subterranean) systems, habitats such as caves, wells, and aquifers that harbor organisms with obvious stygobitic/troglobitic features, such as a lack of coloration/eyes and greatly elongated appendages, are often considered. However, while common, organisms like these and the habitats they occupy comprise only a subset of such systems. So-called "shallow subterranean habitats" (SSHs), such as hyporheic zones, also fall under the definition of "hypogean" and remain relatively understudied [1]. In particular, the natural history and biodiversity of the organisms that inhabit SSH are poorly known, with many regions lacking proper inventories [1]. For areas that have been inventoried, a wide variety of taxa occupy such systems; some of these taxa are clearly hypogean/stygophilic, while others appear epigean but co-occur with hypogean taxa [1]. In addition, these systems are associated with high levels of diversity, often containing endemic taxa adapted to these unique environments. For example, the hypogean amphipod *Stygobromus anacostensis* Cannizzaro, Sawicki and Niemiller (in [2]) occurs in a single hypotelminorheic seepage spring in the Washington D. C. metropolitan area, while other genetically distinct *Stygobromus* spp. were recorded from similar springs throughout the region [2]. Such diversity suggests that these shallow springs may each harbor endemic *Stygobromus* spp., which further suggests that other taxa occupying the springs may also be unique. This trend may even extend to the "epigean" taxa which occupy these systems, for which even less is known when compared to "hypogean" inhabitants of SSH.

The genus *Crangonyx* [3] is one of the most species-rich amphipod genera in Nearctic freshwaters, with 49 described species known from the realm [4–6]. *Crangonyx* spp. are

broadly distributed throughout the eastern United States and Canada, where they are known to occupy a wide range of aquatic habitats, including rills, streams, rivers, lakes, caves, seeps, and similar habitats [4]. Within this broad range of ecosystems, they appear to be most common in groundwater-adjacent habitats such as seeps, springs, and cave streams. While hypogean and stygophilic *Crangonyx* are quite diverse, numerous members of the genus are also known from epigean systems. General knowledge of these epigean species is poor, especially in regard to diversity, distribution, and natural history. This is best exemplified in *Crangonyx floridanus* [7], which at one point was thought to occur throughout the eastern United States but has recently been revealed to be a complex of at least six species, with others still awaiting description [5, 6, 8]. Many of these species are local endemics, several of which have been observed to occur in temporary habitats or SSHs [5, 6]. Given that other species within the genus also show wide ranges and occupy similar habitats—*Crangonyx pseudogracilis* [9] and *Crangonyx richmondensis* [10] are examples—it is likely that additional species complexes exist within the genus.

The patterns of endemicity and diversity observed among epigean *Crangonyx* spp. may be due, in part, to their ecology and the habitats which they occupy. Like other amphipod crustaceans, *Crangonyx* spp. lack a dispersive larval stage and have few mechanisms for long-range dispersal. In addition, they appear to occupy cryptic freshwater ecosystems such as seeps, intermittent streams, and similar habitats, unlike other Nearctic amphipods such as *Gammarus* and *Hyalella* [4]. During changes in water level, *Crangonyx* spp. are thought to utilize the hyporheos or other SSH to escape desiccation and for colonizing new habitats [11, 12]. As a result, they are less likely to utilize overland dispersal. For example, clinging to birds has not been observed in Nearctic crangonyctids. The habitats occupied by *Crangonyx* spp. and their penchant to utilize groundwater, rather than surface water, for transportation act to limit dispersal and gene flow, which, in turn, promotes speciation and endemism. This trend is illustrated by both the high number of species within the genus and deep-set lineages common throughout the family, suggestive of a biogeographic history shaped by vicariance [5, 13].

While *Crangonyx* species have been recorded throughout the eastern and central United States and Canada, they appear to reach their highest level of diversity in Appalachia, with states such as Kentucky and Virginia possessing the highest species richness recorded for the genus, with 15 and 14 known species, respectively [4]. The Appalachian Mountains of the United States are a well-known hotspot of biodiversity, especially among freshwater taxa, with the region hosting a number of endemic invertebrates, including crayfishes and freshwater mussels [14, 15]. While freshwater amphipod diversity is comparatively high in this region, it is largely represented by hypogean taxa [4, 16]. The taxonomy of the epigean amphipods in this region is based on a single study [4]. Most species and populations within the region have not been examined using molecular tools, and as a result, amphipod diversity within Appalachia is likely underestimated.

Within the Appalachians, the Western Allegheny Plateau is an ecoregion of ~84,000 km^2 in northeastern Kentucky, southeastern Ohio, northwestern West Virginia, and southwestern Pennsylvania [17, 18]. The plateau is mostly characterized by oak and mixed mesophytic forests and is underlain by mostly sedimentary (often carboniferous) rock, in contrast to the limestone plains found to the west [17]. The region contains moderate topographic complexity and harbors considerable numbers of diverse aquatic taxa [19–21]. To date, approximately 17 native amphipod species in four genera from three families have been recorded from the Western Allegheny Plateau (Figure 1). It is likely that this number greatly underrepresents the region's true species richness; many records in the region are identified only to genus. This is especially apparent for *Crangonyx*, which possesses a substantial number of unidentified populations within the ecoregion (Figure 1A). As such, the Western Allegheny Plateau is an ideal region for examining potentially undescribed diversity. We describe two new epigean species of *Crangonyx* from small, intermittent bodies of water in Vinton County, Ohio, USA, based on morphological examination and molecular analyses of four loci commonly used in amphipod phylogenetic studies [5, 6, 22–24]. Our results provide insight into the biodiversity and historical biogeography of freshwater fauna from the Western Allegheny Plateau.

2. Materials and Methods

2.1. Collection of Specimens and Records. Specimens of *Crangonyx* were collected live from occupied rills and brooks using hand nets or small metal strainers. In total, specimens were taken from five localities, with ~5–10 individuals collected per site (Table 1). Specimens were preserved in 95% ethanol immediately upon collection and stored at −20°C until analyzed. From these localities, 1–5 individuals were sequenced per site (Table 1). In addition, 11 individuals of *Crangonyx* from Vinton Furnace were set aside for morphological analyses (Table 1 and materials examined sections).

2.2. Morphological Analyses. To aid in morphological analysis, specimens were cleared and stained prior to dissection. Individuals were digested in a mixture of 400 μL of Molecular Grade Water, 360 μL of 2x Digestion Buffer (Zymo Research, Irvine, CA, USA), and 40 μL of proteinase K; samples were incubated in this mixture at 37°C for 2 h and then at room temperature overnight. Once digested, specimens were transferred to a solution of Lignin Pink for 2–3 h to stain; after specimens were sufficiently stained, they were transferred to glycerin for dissection using a Nikon SMZ-800 stereomicroscope. Individual appendages were mounted on temporary glycerin slides to facilitate examination using a Nikon Alphaphot-2-YS2 compound microscope with attached drawing tube, used in combination with a Wacom Cintiq 27QHD drawing tablet. Both illustrations and plates were prepared using Adobe Illustrator CC. Body length measurements were taken by measuring the distance from the rostrum to the base of the telson, following the contour of the body using ImageJ software [25]. Nomenclature for setal patterns on the second and third segments of the mandibular palps follows Cole [26] and Stock [27], respectively. The term "defining angle" refers to the posterior margin of the palm and the distal-most point

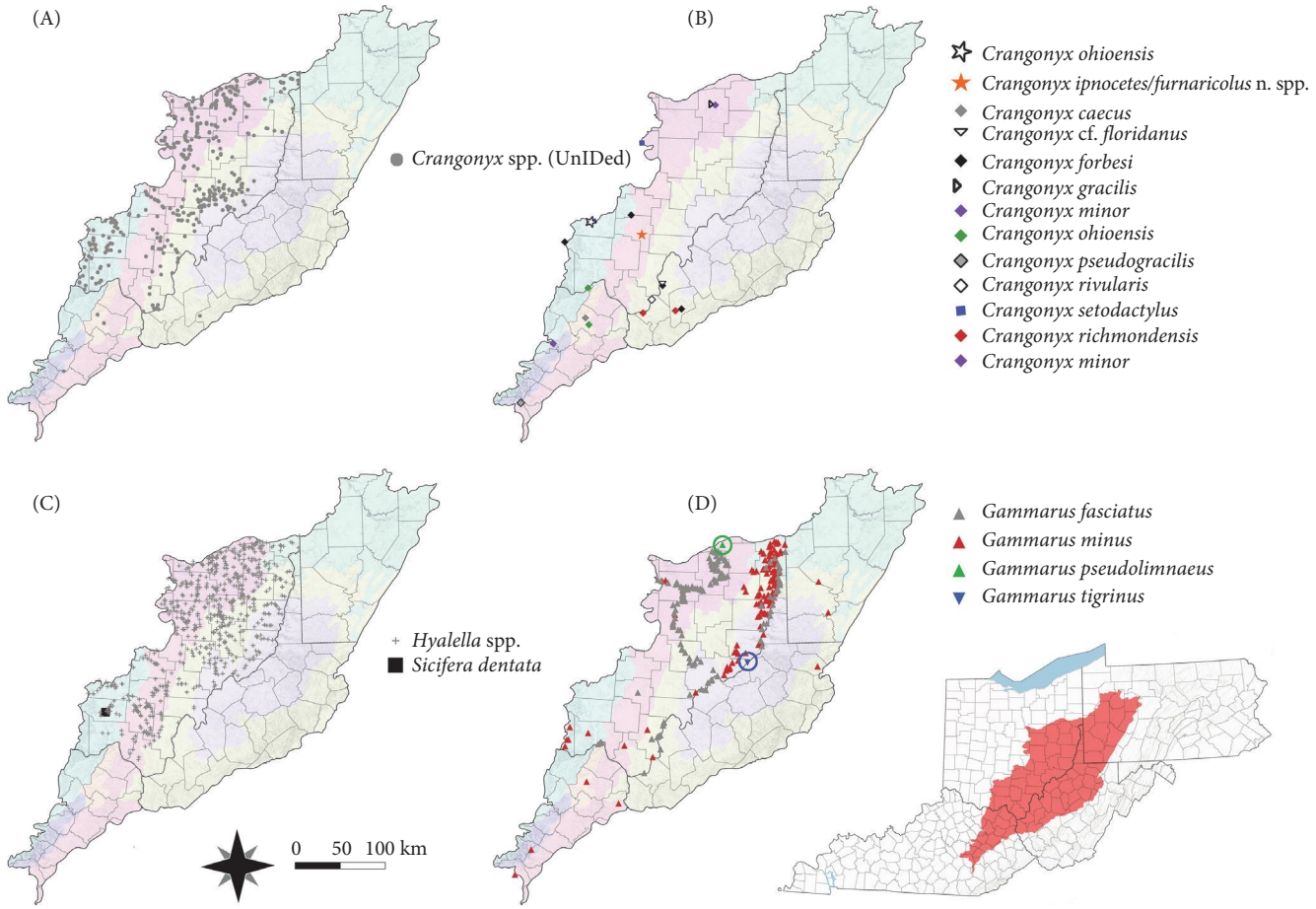

FIGURE 1: Amphipod diversity of the Western Allegheny Plateau: (A) records of *Crangonyx* spp., which have not been identified beyond genus; (B) identified *Crangonyx* spp., including species described herein; (C) *Sicifera dentata* (Crangonyctidae) and *Hyalella* spp. (Hyalellidae); and (D) *Gammarus* spp. (Gammaridae). Records of the introduced *Echinogammarus ischnus* are excluded. States, counties, and subregions of the Western Allegheny Plateau are highlighted for all maps. The locator map in the lower right indicates extent of the Western Allegheny Plateau. Distributional data presented here were collected from several sources, including personal collections, museum records, and gray literature, along with records from Zhang and Holsinger [4].

of the posterior margin of the propodus (the area where the tip of the dactylus closes on the propodus and rests when closed). The term "pereopod 7 gill" refers to the gill attached between the coxa and basis of pereopod 7 as described by Steele and Steele [28]. "Stout setae" on pereopod dactyli are intermediate in robustness relative to robust setae (traditionally referred to as spines) and thinner, more flexible setae [8]. "Clothespin setae" refer to notched, robust setae present on the basal segments of the pleopod inner rami [29].

2.3. DNA Extraction and PCR Preparation. Genomic DNA (gDNA) was extracted from the digested material prepared during the specimen clearing process. Extractions were performed using Tissue & Insect DNA MiniPrep kits (Zymo Research), utilizing a modified protocol [30]. Extracted DNA was stored at −20°C and quantified using a Qubit fluorometer.

Polymerase chain reactions (PCRs) using the primer pairs 18SF/18S700R, 18SF/18S1000F and 18S700F/18S1250R [22] amplified 600–1000 base pair (bp) segments of nuclear 18S rDNA; 28SF/28S1000R [23] or 28S_lev2/28S_des1 LEV [31] were used to amplify 600–900 bp of the nuclear 28S rDNA; 16StF [32] and 16SBR [33] were used to amplify 370 bp of the mitochondrial 16S rDNA; LCO1490/HCO2198 [34] or UCOIF/UCOIR [35] were used to amplify a 590 bp segment of the mitochondrial cytochrome c oxidase subunit I (COI). Total PCR volumes of 20 μL contained 30–70 ng of extracted gDNA. The PCR master mix contained 10 μL of GoTaq Master mix (Promega), 1 μL of each 10 μM primer, and 6 μL of molecular-grade water. The PCR was performed on a Bio-Rad T100 thermal cycler (Bio-Rad Laboratories). A negative control lacking only gDNA was included for all sets of PCRs to screen for contamination. The following thermal cycler protocols were followed: 18S: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 68.5°C for 30 s, and 72°C for 2 min, ending with a 10 min final extension at 72°C; 28S: 95°C for 5 min, followed by 38 cycles of 94°C for 45 s, 45°C for 45 s, and 72°C for 1 min, ending with a 5 min final extension at 72°C; 16S: 95°C for 5 min, followed by 36 cycles of 95°C for 45 s, 42°C for 40 s, and 72°C for 1 min, ending with a 5 min final extension at 72°C; and COI: 95°C for 5 min, followed by 38 cycles of 94°C for 45 s, 50–53°C for 45 s, and 72°C for 45 s, ending with a 5 min final extension at 72°C.

Table 1: GenBank accession numbers and collection information for individuals sequenced as a part of this study.

Catalog#	Species	Locality	Date	Coordinates	Collectors	18S acc.#	28S acc.#	16S acc.#	COI acc.#
AGC-59.3	*Crangonyx setodactylus*	Miami University Natural Areas, Butler County, OH	4/4/2020	39.53219, −84.7059	A.G. Cannizzaro	PV857753	—	—	PV857662
AGC-598.1	*Crangonyx* cf. *bousfieldi*	French Park, Hamilton County, OH	5/7/2022	39.19595, −84.42186	A. G. Cannizzaro, T.R. Sawicki	PV857744	—	—	PV857674
AGC-49.2	*Crangonyx minor*	Indian Creek Metropark, Butler County, OH	3/8/2020	39.440194, −84.7640	A.G. Cannizzaro	PV857750	—	—	PV857666
AGC-953.1	*Crangonyx furnaricolus* n. sp.	Vinton Furnace State Forest, Vinton County, OH	3/25/2023	39.18583, −82.39626	A.G. Cannizzaro et al.	PV857745	PV857754	—	PV857673
AGC-954.7	*Crangonyx furnaricolus* n. sp.	Vinton Furnace State Forest, Vinton County, OH	3/25/2023	39.18583, −82.39626	A.G. Cannizzaro et al.	PV857746	PV857755	—	PV857672
AGC-953.2	*Crangonyx ipnoecetes* n. sp.	Vinton Furnace State Forest, Vinton County, OH	3/25/2023	39.18583, −82.39626	A.G. Cannizzaro et al.	PV857747	PV857729	PV857737	PV857671
AGC-954.1	*Crangonyx ipnoecetes* n. sp.	Vinton Furnace State Forest, Vinton County, OH	3/25/2023	39.19851, −82.40891	A.G. Cannizzaro et al.	PV857748	PV857730	PV857739	PV857670
AGC-954.3	*Crangonyx ipnoecetes* n. sp.	Vinton Furnace State Forest, Vinton County, OH	3/25/2023	39.19851, −82.40891	A.G. Cannizzaro et al.	PV857749	PV857731	PV857740	PV857669
AGC-954.5	*Crangonyx ipnoecetes* n. sp.	Vinton Furnace State Forest, Vinton County, OH	3/25/2023	39.19851, −82.40891	A.G. Cannizzaro et al.	—	—	PV857738	PV857668
AGC-954.6	*Crangonyx ipnoecetes* n. sp.	Vinton Furnace State Forest, Vinton County, OH	3/25/2023	39.19851, −82.40891	A.G. Cannizzaro et al.	—	—	—	PV857667
AGC-1077.1	*Crangonyx ohioensis*	"Buzzard's Roost", Ross County, OH	4/21/2024	39.32693, −83.06535	A.G. Cannizzaro	PV857751	PV857732	PV857741	PV857665
AGC-1077.2	*Crangonyx ohioensis*	"Buzzard's Roost", Ross County, OH	4/21/2024	39.32693, −83.06535	A.G. Cannizzaro	PV857752	PV857733	PV857742	PV857664
AGC-1077.3	*Crangonyx ohioensis*	"Buzzard's Roost", Ross County, OH	4/21/2024	39.32693, −83.06535	A.G. Cannizzaro	—	PV857734	PV857743	PV857663

Note: Coordinates referenced to WGS 1984.

PCR products were prepared for sequencing using ExoCIP Rapid PCR Cleanup (New England Biolabs, Ipswich, MA, USA). Sanger sequencing of purified PCR products was carried out at Eurofins Genomics (Louisville, KY, USA) using industry-standard Sanger sequencing methodology. All sequences generated by this study were submitted to GenBank (Table 1).

2.4. Sequence Preparation and Alignment. Pairwise sequence alignment was conducted using MUSCLE [36] in GENEIOUS PRIME (www.geneious.com) and manually adjusted. For COI, amino acid translation was used to screen for pseudogenes (indicated by the presence of multiple stop codons); upon confirmation, multiple sequence alignment was conducted using MUSCLE in GENEIOUS PRIME. Multiple sequence alignments of the nuclear 18S/28S rDNA and mitochondrial 16S rDNA were performed separately utilizing the same parameters; gaps were retained in the final alignments. All markers were concatenated into a final alignment using SEQUENCE MATRIX v.1.8 [37]; this alignment comprised 5091 bp for 33 individuals.

2.5. Phylogenetic Analyses. Prior to analysis, substitution saturation was estimated for each marker using the test by Xia et al. [38] in DAMBE (Ver. 5.3.10, http://dambe.bio.uottowa.ca/DAMBE/dambe.aspx)[39]. Signification saturation levels were detected for the COI 3rd codon position ($I_{ss} > I_{ss.cSyn}$, $p = 0.0001$), and so this maker was not included in the analyses. Phylogenetic relationships were reconstructed using both maximum likelihood and Bayesian inference methods. Maximum likelihood analyses were conducted using IQTREE v.2.2 [40]. The IQTREE search was run using the MODELFINDER algorithm to select the best-fitting substitution models, which were analyzed under an edge-linked model. The following models were selected: 18S: Tamura 3-parameter with empirical base frequencies and a FreeRate model with three categories (TPM3 + F + R3); 28S and 16S: general time reversible model with equal empirical base frequencies, a proportion of invariant sites, and a gamma distribution with four categories (GTR + F + I + G4); COI (both 1st and 2nd codon positions, the 3rd codon position was excluded due to saturation detected in the DAMBE analysis): transition model with equal empirical base frequencies, a proportion of invariant sites, and a gamma distribution with four categories (TIM2 + F + I + G4). Statistical support was estimated using 1000 ultrafast bootstrap replicates [41] and the Shimodaira–Hasegawa approximate likelihood ratio test [42, 43]. Sequences from other crangonyctoid genera (*Crymostygius Sicifera*, *Stygobromus*) and the distantly related *C. islandicus* and *C. chlebnikovi* were downloaded from GenBank to serve as outgroups; in addition, sequences from 10 members of the *gracilis* group of *Crangonyx* were obtained from GenBank and comprised part of the ingroup. Accession numbers for individuals included in the phylogenetic analyses (including all outgroups) are provided in Table S1.

Bayesian inference was performed in BEAST 2.5.2 [44] with bModelTest [45] used to select the best-fit substitution model for each partition; evolutionary models were unlinked among genes. A relaxed clock with a log-normal distribution was applied to each partition, and the tree prior for the analysis was set to a birth–death model. For estimation of divergence times, calibrations based on fossil Amphipoda were utilized [13. The crown age of the Crangonyctoidea was modeled under an exponential distribution (mean: 60 Ma, offset: 35 Ma, highest posterior density (HPD): 38–215 Ma). Markov chain Monte Carlo (MCMC) simulations were run for 100 million generations and sampled every 1000 generations. Convergence and effective sample size (ESS) were checked using TRACER 1.6 [46]. The first 25% of resulting trees were discarded as burn-in based on parameter estimates determined in TRACER, and the resulting tree was summarized using TreeAnnotator 1.8.1 in BEAST.

3. Results

3.1. Molecular Phylogenetic Analyses. Our phylogenetic reconstructions of the genus *Crangonyx* using both maximum-likelihood and Bayesian inference place individuals collected from Vinton Furnace State Forest (VFSF) into two separate, well-supported clades distinct from other members of the genus (Figures 2, 3). The VFSF clades display distant affinity (Figures 2, 3). One species is sister to *Crangonyx ohioensis* [4], newly collected from Earl H. Barnhart "Buzzard's Roost" Nature Preserve in Ross County, Ohio, and the other is sister to *Crangonyx antennatus* Packard, 1881 in [47] and more distantly to the *C. floridanus* complex (Figures 2, 3). Based on our BEAST analysis, the most recent common ancestor of all three species we collected existed sometime during the Late Cretaceous or early Paleogene (~73−50 Ma; Figure 3). These species were revealed to have diverged from their closest congeners later in the Paleogene (~40−20 Ma; Figure 3).

3.2. Morphological Analyses. The unidentified *Crangonyx* collected from VFSF were observed to differ morphologically from each other and from described members of the genus in several taxonomically relevant characters, including gnathopod shape, pereopod armature, and structure/armature of the uropods. Based on these differences and the molecular distances derived from the phylogenetic analyses, we conclude that both populations are novel species and describe them below.

3.3. Systematics. Order AMPHIPODA Latrielle, 1816.
Suborder SENTICAUDATA Lowry & Myers, 2013.
Infraorder GAMMARIDA Latreille, 1802.
Superfamily CRANGONYCTOIDEA [48].
Family CRANGONYCTIDAE [48]; emended by [49].
Genus *Crangonyx* [3].
Crangonyx ipnoecetes n. sp. Cannizzaro & Berg.
ZOOBANK registration: urn:lsid:zoobank.org:act:75BB7DE4-4682-453E-A896-19745A1637E1.
(Figures 4–11)

Material examined: holotype, female 7.25 mm, in rills draining from small cave, VFSF, Vinton County, Ohio, USA (39.18583, −82.39626); collectors: Andrew G. Cannizzaro, Ashley Walters, and Sarah McKillop, 25 March 2023; OSUMC

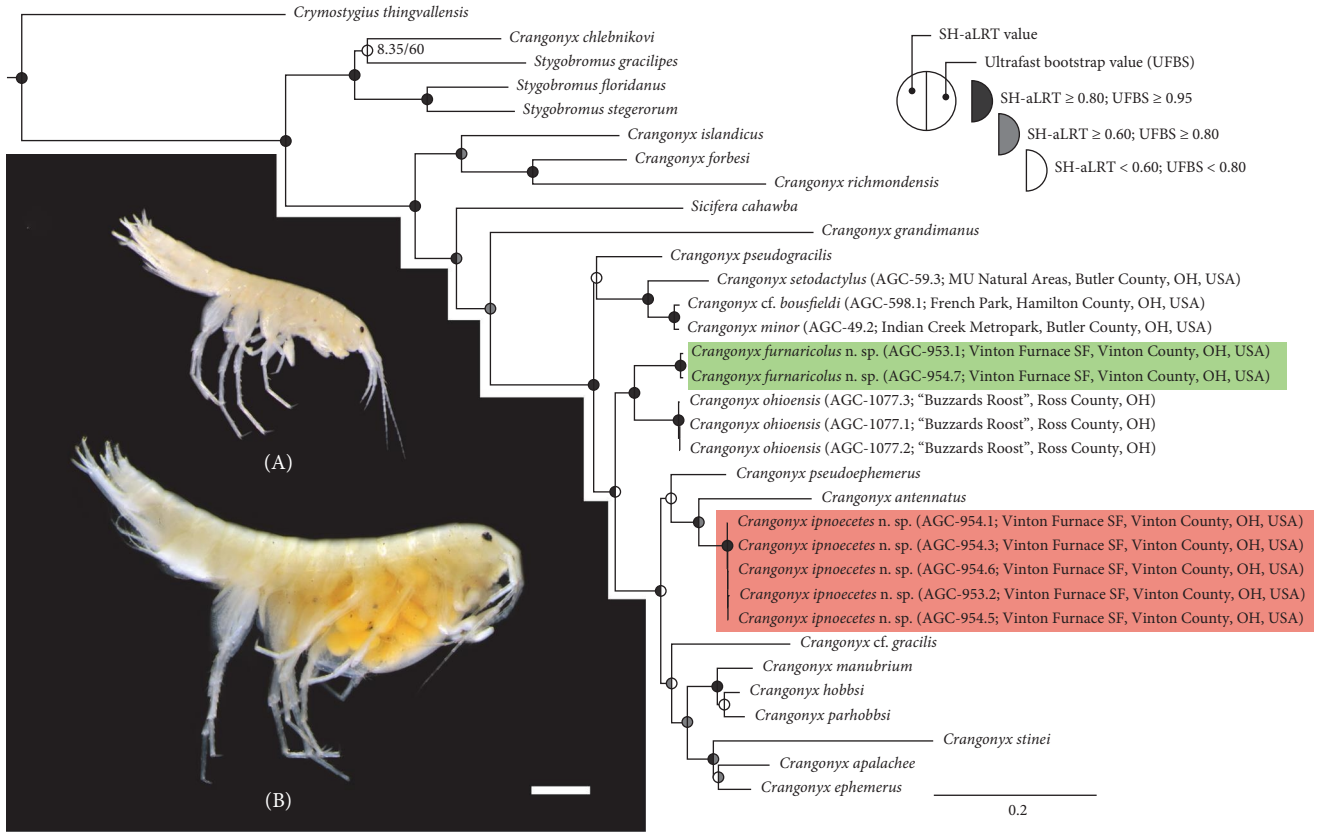

FIGURE 2: Maximum-likelihood phylogeny reconstructed from the concatenated dataset (18S rDNA, 28S rDNA, 16S rDNA, COI) using IQTREE. Node support using two methodologies is indicated by shaded circles placed on nodes. (A) *Crangonyx furnaricolus* n. sp., male, 4.23 mm, rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA, and (B) *Crangonyx ipnoecetes* n. sp. female, 6.75 mm, rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. Scale bar represents 1 mm. UBFS, ultrafast bootstrap, SHaLRT, Shimodaira–Hasegawa approximate likelihood ratio test.

11024 (AGC-954.5). Allotype male, 5.20 mm, from the same locality and collection date as the holotype, OSUMC 11025 (AGC-954.1). Paratype male, 4.20 mm from the same locality and collection date as the holotype, OSUMC 11026 (AGC-953.2). Paratype female, 6.52 mm, from same locality and collection date as the holotype, OSUMC 11027 (AGC-954.6). Female paratypes from the same locality and collection date as the holotype, OSUMC 11028. Male paratypes from the same locality and collection date as the holotype, OSUMC 11029.

Type locality: USA, Ohio, Vinton County, VFSF, rill draining from the small cave (39.18583, −82.39626; Figure 1).

Etymology: The specific epithet *ipnoecetes* is derived from the Ancient Greek *πνό* (furnace, oven, kiln, or lantern) and the suffix *oikētēs* (a dweller). The name is given in reference to both the species type locality (Vinton Furnace State Furnace) and the landscape it occupies (the Hanging Rock Iron Region), where many charcoal-fired blast furnaces once operated.

Diagnosis. Medium-sized, epigean species showing characteristics typical of the *gracilis* group; distinguished from all other species in the genus, except *C. bousfieldi* [4] and *C. stagnicolous* [4], by the presence of two setae along the posterior margin of the epimera, with one above the posterodistal corner. Further distinguished from these latter species by the following characteristics, maxilla 1 with three plumose setae on inner plate; palms of female gnathopods 1 and 2 with small robust setae; female uropod 1 with 6 outer robust setae on peduncle; female uropod 3 inner ramus lacking robust seta; female telson cleft 15% of length, with a depth-to-width ratio of ~0.30; male uropod 2 inner ramus with 4 robust setae; outer ramus narrowing distally, curved slightly with three robust outer setae, and one smaller inner seta, apex with 10 comb-like robust setae placed in a transverse row.

Description: Holotype female (Figures 4–9), 7.25 mm in length, with eye somewhat reduced, pigment yellowish in color when alive (Figure 4C).

3.3.1. Antennae. Antenna 1 (Figure 5A): 60% body length, ~1.25x longer than antenna 2; peduncle segment 1 with three dorsal robust setae; primary flagellum with 18 segments, aesthetascs on distal segments, aesthetascs shorter than respective segments; accessory flagellum 2-segmented, reaching to half the length of the second primary flagellar segment. Antenna 2 (Figure 5B): 30% body length, gland cone developed; peduncle ~1.7x length of flagellum, with three robust setae on segment 3; peduncular segment 4 subequal in length to segment 5; flagellum with seven segments, lacking calceoli.

3.3.2. Mouthparts. Mandibles: right mandible (Figure 5C) incisor 4-dentate, lacinia mobilis bifurcate, lobes with

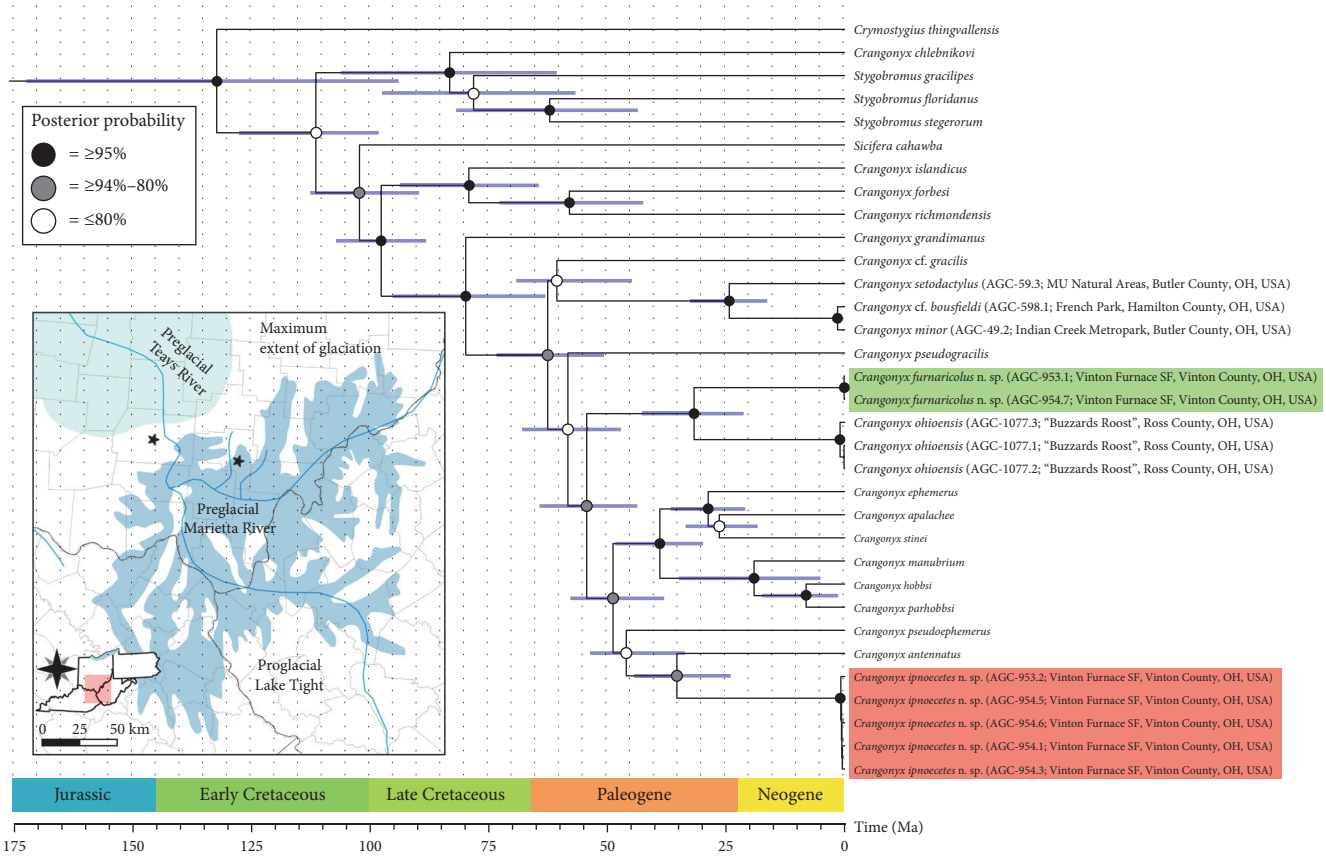

Figure 3: Time-calibrated multilocus phylogeny reconstructed using BEAST. Posterior probabilities are indicated by circles placed on nodes; node bars indicate the 95% HPD intervals of clade ages. Branches associated with species described as a part of this project are highlighted in red and covered with gradients. Inset: map of southeastern Ohio, northwestern West Virginia, and northeastern Kentucky detailing historically relevant features, including the general location of preglacial rivers such as the Teays, and the general location of proglacial Lake Tight. Stars indicate the localities of *Crangonyx ohioensis* (west) and *Crangonyx ipnoecetes/furnaricolus* n. spp. (east).

numerous fine dentations; accessory setal row with five plumose setae; molar process well-developed, cylindrical, triturative, with seta; palp 3-segmented, second segment subequal in length to third segment, with inner margin bearing 5 setae; third segment rounded distally, inner margin straight, lacking A-setae, 1 B-seta, 1 C-seta, 9 D-setae, and 4 E-setae; face of segment covered in numerous, fine pubescent setules. Left mandible (Figure 5D) incisor 6-dentate, lacinia mobilis 5-dentate, with five plumose accessory setae; molar similar in form to right mandible, with single plumose seta; palp as in right mandible. Upper lip (Figure 6A): rounded, apical margin of labrum with numerous setules. Lower lip (Figure 6B) inner lobes distinct, outer margin of inner and outer lobes covered in setules; face of lip covered in pubescent setules. Maxilla 1 (Figure 6C): inner plate shorter than outer plate, with three apical pappose setae, fine pubescent setules covering entire plate; outer plate with seven apical serrate setae, pubescent setules covering entire plate, decreasing laterally; palp two-segmented, distal segment covered in pubescent setules, apical margin of distal segment with three submarginal setae and four marginal robust setae. Maxilla 2 (Figure 6D): both inner and outer plates covered in pubescent setules; outer plate ~1.3x length of inner plate, with numerous apical setae; inner plate narrowing slightly distally, with numerous apical setae and three pappose facial setae. Maxilliped (Figure 9E): inner plate shorter than outer plate with two unarmed cuspidate setae and a pappose cuspidate seta along apical margin, surface of plate covered in pubescent setules; outer plate with numerous setae, surface of plate covered in pubescent setules; palp 4-segmented, second and third segments with numerous marginal, submarginal setae; distal surface of third segment with pubescent setules; dactylus with three inner marginal setae and an outer marginal seta.

3.3.3. Gnathopods. Gnathopod 1 (Figure 7A): coxal plate with six apical setae and sparse facial setae; basis narrowing proximally with long setae along anterior, medial, and posterior margins, and six shorter anterior and two posterior setae, posterior surface of segment with pubescent setules; ischium with four setae and pubescent setules along posterior margin; merus with pubescent setules covering posterior surface, seven pappose posterodistal setae, and a medial seta along anterior margin; carpus ~50% length of propodus, with two setae along anterior margin, four setae inserted at anterodistal corner, posterior margin with numerous pappose setae; propodus ~1.4x longer than broad, with three

Figure 4: Habitus photographs of *Crangonyx* spp. collected as a part of this study. (A) *Crangonyx furnaricolus* n. sp. (OSUMC 11,029), holotype female, 7.10 mm, rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (B) *Crangonyx furnaricolus* n. sp. (OSUMC 11,030), allotype male, 6.21 mm, same locality. (C) *Crangonyx ipnocetes* n. sp. holotype female, 7.25 mm (OSUMC 11,024), same locality. (D) *Crangonyx ipnocetes* n. sp. allotype male, 5.20 mm (OSUMC 11,025), same locality. (E) *Crangonyx ohioensis* female, 6.98 mm (AGC-1077.1), rill near parking area, Earl H. Barnhart "Buzzard's Roost" Nature Preserve, Ross County, Ohio, USA. (F) *Crangonyx ohioensis* male, 5.50 mm (AGC-1077.2), same locality as E. Scale bar represents 1 mm.

marginal anterior setae, six setae inserted at anterodistal corner, four inferior medial setae, and eight pappose posterior setae; palm weakly convex with four setae along outer margin; seven outer and six inner bifid robust setae; inner margin of defining angle with four robust setae, outer margin with two robust setae, large robust split-tip seta; dactylus with outer seta, and four inner setae. Gnathopod 2 (Figure 7B): coxal plate with six apical setae and sparse facial setae; basis narrowing proximally with long setae along anterior and posterior margins, and two shorter anterior and two posterior setae; ischium with three short setae and pubescent setules along posterior margin; merus with pubescent setules covering posterior surface, and three posterodistal setae; carpus ~66% length of propodus, with a seta along anterior margin, three setae inserted at anterodistal corner, posterior margin with numerous pappose setae; propodus ~1.6x longer than broad, with a marginal anterior seta, seven setae inserted at anterodistal corner, four superior medial setae, two inferior medial setae, and four clusters of pappose posterior setae; palm weakly convex with six setae along inner

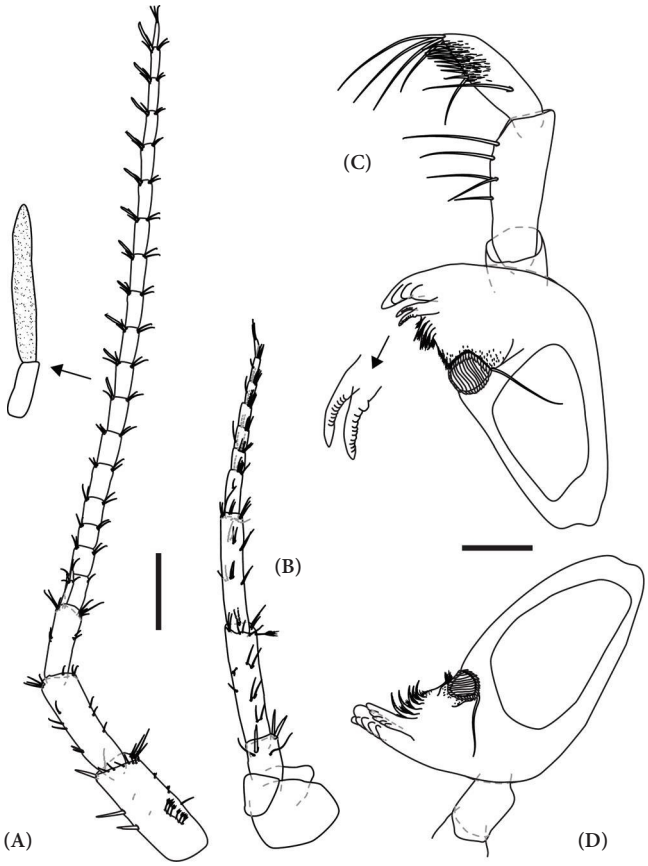

Figure 5: *Crangonyx ipnoecetes* n. sp. holotype female, 7.25 mm (OSUMC 11,024), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Antenna 1 (aesthetasc enlarged), (B) antenna 2, (C) right mandible (lacinia mobilis enlarged), and (D) left mandible (palp omitted). Scale bars: 0.25 mm (A, B) and 0.1 mm (C, D).

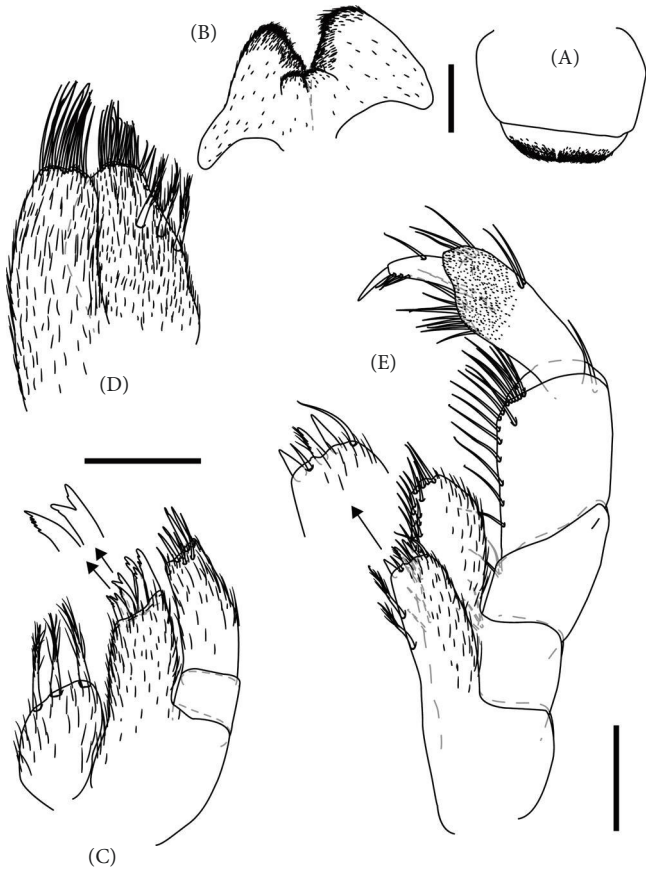

Figure 6: *Crangonyx ipnoecetes* n. sp. holotype female, 7.25 mm (OSUMC 11,024), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Upper Lip, (B) lower Lip, (C) maxilla 1 (outer plate apical serrate setae enlarged), (D) maxilla 2, and (E) maxilliped (apical margin of inner plate enlarged). Scale bars: 0.1 mm.

margin; six outer and five inner bifid robust setae; inner margin of defining angle with four robust setae, outer margin with three robust setae, large robust split-tip seta; dactylus with outer seta, and four inner setae.

3.3.4. Pereopods. Pereopod 3 (Figure 8A): coxal plate with 7 apical setae and sparse facial setae, plate not excavated posteroproximally; basis with numerous long setae inserted along anterior and posterior margins; merus ~1.8x longer than carpus; carpus ~90% length of propodus; dactylus ~50% length of propodus, with plumose seta proximally on anterior margin, stout seta placed distally along posterior margin and proximal to nail. Pereopod 4 (Figure 8B): subequal to pereopod 3 in length, coxal plate with nine apical setae and several facial setae, plate with distinct posteroproximal excavation; basis with numerous long setae along anterior, medial, and posterior margins; merus ~1.4x longer than carpus; carpus ~90% length of propodus; dactylus ~35% length of propodus, setation as in pereopod 3. Pereopod 5 (Figure 8C): coxal plate large, bilobate with distinct anterior and posterior lobes, each with a seta on respective distal corners; basis posterior margin weakly convex with six blunt serrations and a weakly convex distal corner, posterior surface of segment with sparse facial setae, proximal anterior margin with three long setae, anterior margin with five split-tipped robust setae, four setae along anterodistal margin; merus ~80% length of carpus; carpus subequal in length to propodus; dactylus ~35% length of propodus, setation as in other pereopods. Pereopod 6 (Figure 8D): coxal plate weakly bilobate, with produced posterior lobe bearing two setae along posterodistal margin; basis posterior margin straight with seven blunt serrations and a weakly convex distal corner, posterior surface of segment with sparse facial setae, proximal anterior margin with three long setae, anterior margin with six split-tipped robust setae, three setae along anterodistal margin; merus ~85% length of carpus; carpus subequal in length to propodus; dactylus ~40% length of propodus, setation as in other pereopods. Pereopod 7 (Figure 8E): coxal plate small, subtriangular, with two posterior setae; basis posterior margin convex with nine shallow serrations and with weakly rounded distal corner, posterior face of segment with sparse facial setae, proximal anterior margin with four long setae, anterior margin with five split-tipped robust setae, two robust setae along anterodistal margin; merus ~85% length of carpus; carpus subequal in length

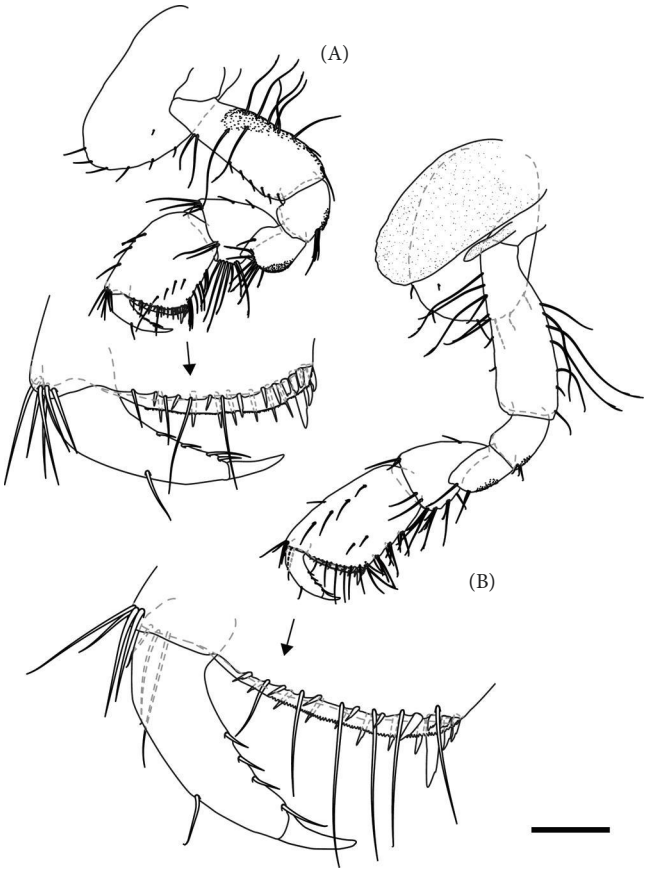

Figure 7: *Crangonyx ipnoecetes* n. sp. holotype female, 7.25 mm (OSUMC 11,024), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Gnathopod 1 (palmar margin and dactylus enlarged) and (B) gnathopod 2 (palmar margin and dactylus enlarged). Scale bar: 0.25 mm.

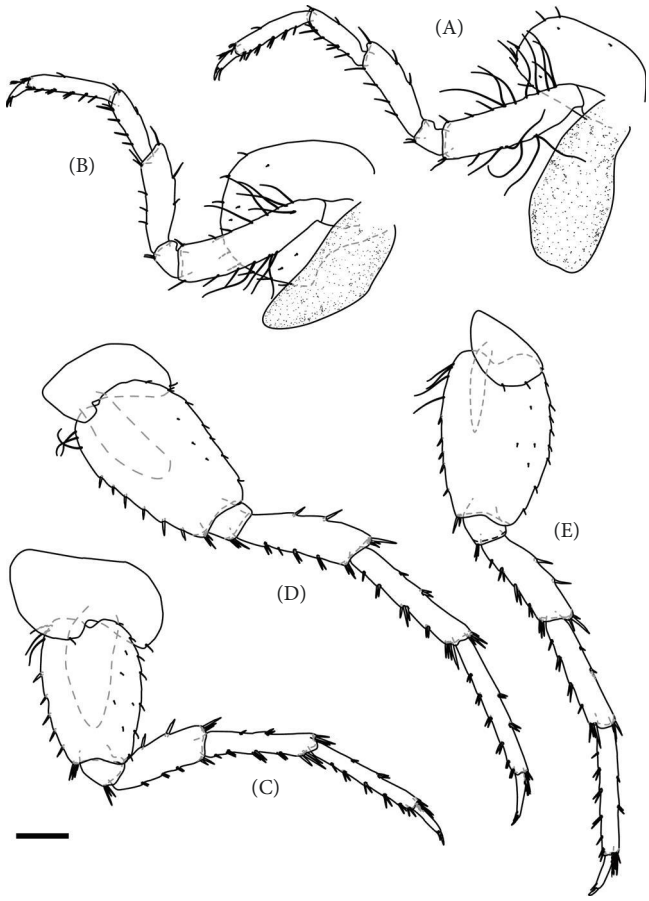

Figure 8: *Crangonyx ipnoecetes* n. sp. holotype female, 7.25 mm (OSUMC 11,024), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Pereopod 3, (B) pereopod 4, (C) pereopod 5, (D) pereopod 6, (E) and pereopod 7. Scale bar: 0.25 mm.

to propodus; dactylus ~35% length of propodus, setation as in other pereopods.

3.3.5. Gills. (Figures 7B, 8A–E) Coxal gills are present on somites 2–6, and somite 7 with a small pereopod 7 gill. Slender sternal gills present on somites 6 and 7 (damaged in holotype). Brood plates present on somites 2–5, decreasing in size posteriorly (not developed in holotype).

3.3.6. Pleon. Epimera (Figure 9A): first epimeron ventral margin unarmed, strongly oblique, posterodistal corner with weak tooth-like extension, posterior margin with two setae; second epimeron ventral margin with two robust setae, posterodistal corner blunt, produced, posterior margin with two setae; third epimeron ventral margin with two robust setae, posterodistal corner blunt, weakly produced, posterior margin with two setae. Pleopod 1 (Figure 9B): peduncle ~55% length of inner ramus, lacking setae, with two coupling hooks; outer and inner rami with 9 and 12 segments, respectively, basal segment of inner ramus with plumose clothespin setae. Pleopod 2 (not figured): peduncle similar to pleopod 1, outer and inner rami with 8 and 9 segments, respectively. Pleopod 3 (not figured): peduncle similar to pleopods 1 + 2, outer and inner rami with 8 and 9 segments.

3.3.7. Urosome. Somites are free, bare dorsally. Uropod 1 (Figure 9C): peduncle subequal in length to rami, with six outer robust setae and an inner robust seta; outer ramus ~80% length of inner ramus, with three robust setae on inner margin, two robust setae on outer margin, and five robust setae on apex; inner ramus with two robust setae on both inner and outer margins, four robust setae on apex, and a small seta placed proximally on ventral margin. Uropod 2 (Figure 9D): peduncle subequal in length to rami, with three outer robust setae and two paired inner robust setae; outer ramus ~90% length of inner ramus, with two robust setae on inner margin, three robust setae on outer margin, and four robust setae on apex; inner ramus with two robust setae on inner margin, three robust setae on outer margin, and five robust setae on apex. Uropod 3 (Figure 9E): peduncle ~66% length of outer ramus, with two distal robust setae, and a proximal seta; inner ramus reduced, scale-like, lacking setae; outer ramus ~3x longer than broad, ~5x longer than inner ramus, with three groups of robust, split-tipped setae on outer margin and a split-tipped seta on inner margin, apex with a slender seta paired with a short robust seta. Telson (Figure 9F): ~1.2x broader than long, cleft ~15% of length,

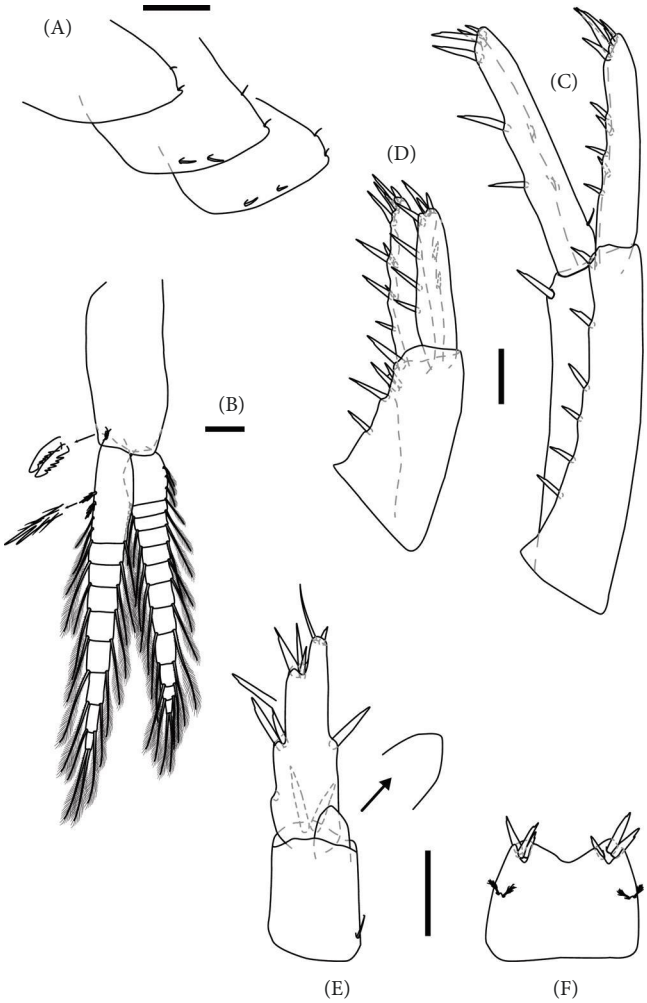

Figure 9: *Crangonyx ipnoecetes* n. sp. holotype female, 7.25 mm (OSUMC 11,024), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Epimera, (B) pleopod 1 (coupling spines and clothespin seta enlarged), (C) uropod 1, (D) uropod 2, (E) uropod 3, and (F) telson. Scale bars: 0.25 mm (A) and 0.1 mm (B–F).

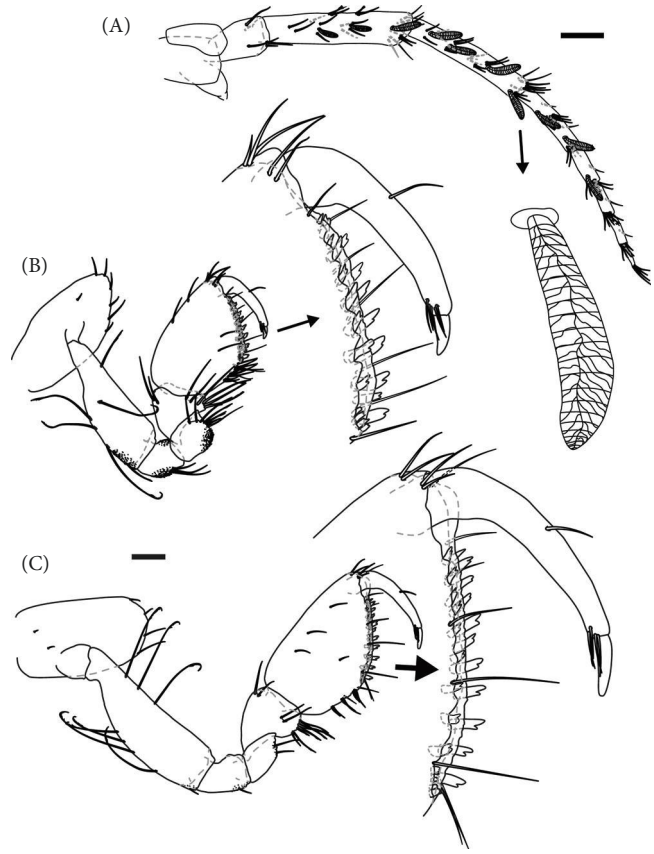

Figure 10: *Crangonyx ipnoecetes* n. sp. allotype male, 5.20 mm (OSUMC 11,025), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Antenna 2 (single calceolus enlarged), (B) gnathopod 1 (palmar margin and dactylus enlarged), and (C) gnathopod 2 (palmar margin and dactylus enlarged). Scale bars: 0.1 mm.

with a depth-to-width ratio of ~0.30, apical lobes armed with three robust split-tipped setae; two plumose setae arise dorsolaterally from the outer margin of both lobes.

Male: (Figures 4D, 10, 11): OSUMC 11025 (AGC-954.1), 5.20 mm in length. Differing from females in smaller body size, shorter antennae, presence of calceoli on the peduncle and flagellum on antenna 2, more robust gnathopods with enlarged propodi and more robust setae on the palmar margins, pereopod 7 posterodistal serrations, uropod 1 setation, uropod 2 setation and rami structure, uropod 3 setation, and telson shape and setation. Structures not described below as in females.

3.3.8. Antennae. Antenna 1 (not illustrated, but see Figure 4D): 50% body length, ~1.7 times longer than antenna 2; primary flagellum with 14 segments, aesthetascs on distal segments, aesthetascs shorter than respective segments; accessory flagellum 2-segmented, reaching to half the length of the second primary flagellar segment. Antenna 2 (Figure 10A): 30% body length, gland cone developed; peduncle ~1.5 times length of flagellum; peduncular segment 4 subequal in length to segment 5; flagellum with six segments; calceoli present on segments 4 + 5 of peduncle and proximal flagellar segments.

3.3.9. Gnathopods. Gnathopod 1 (Figure 10B): coxal plate narrowing distally, with six apical setae and sparse facial setae; basis narrowing proximally with long setae along anterior, medial, and posterior margin, posteroproximal surface of segment with pubescent setules; ischium with three setae and pubescent setules along both posterior and anterior margins; merus with pubescent setules covering posterior surface and seven pappose posterodistal setae; carpus ~45% length of propodus, with two setae along anterior margin, posterior margin with numerous pappose setae; propodus ~1.4x longer than broad, with two marginal anterior setae, five setae inserted at anterodistal corner, two inferior medial setae, and three clusters of posterior setae; palm weakly convex with four setae along outer margin and two setae along inner margin; eight outer and seven inner bifid robust setae; inner margin of defining angle with four robust setae, outer margin with a single bifid robust seta; dactylus with outer seta, and

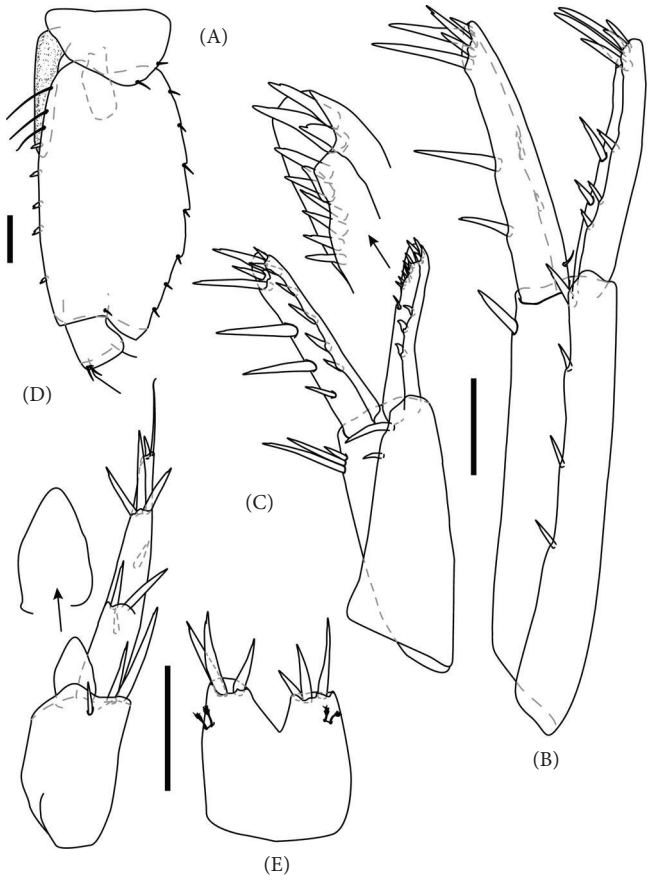

Figure 11: *Crangonyx ipnoecetes* n. sp. allotype male, 5.20 mm (OSUMC 11,025), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Pereopod 7 coxal plate, basis, and ischium, (B) uropod 1, (C) uropod 2 (tip of outer ramus enlarged), (D) uropod 3 (inner ramus enlarged), and (E) telson. Scale bars: 0.25 mm (A) and 0.1 mm (B–D).

five inner setae located near nail. Gnathopod 2 (Figure 10C): coxal plate with four apical setae and sparse facial setae; basis narrowing proximally with long setae along anterior and posterior margins, and a single short posterior seta; ischium with a posterior seta and pubescent setules along posterior margin; merus with sparse pubescent setules posterodistally, and two posterodistal setae; carpus ~40% of propodus with two anterior setae, two medial setae, and numerous pappose posterior setae; propodus ~2.0x longer than broad, with five setae inserted at anterodistal corner, three superior medial setae, one inferior medial seta, and four clusters of posterior setae; palm straight with three setae along inner margin and two setae along outer margin; 10 outer and nine inner bifid robust setae; inner margin of defining angle with six short robust setae; outer margin with a single larger bifid robust seta; dactylus with outer seta, and four inner setae.

3.3.10. Pereopods. Pereopod 7 (Figure 11A): coxal lobes indistinct, with two posterior setae; basis posterior margin convex, with seven serrations (increasing in depth distally), distal corner distinctly produced, anterior margin with five robust split-tipped setae and 3 setae inserted along anterodistal corner.

3.3.11. Urosome. Uropod 1 (Figure 11B): peduncle 1.5x length of rami, with four outer robust setae and an inner robust seta; outer ramus ~90% length of inner ramus, with two robust setae on inner margin, three robust setae on outer margin, and five robust setae on apex; inner ramus with two robust setae on inner margin, two robust setae on outer margin, five robust setae on apex, and a small seta placed proximally on ventral margin. Uropod 2 (Figure 11C): peduncle 1.3x length of rami, with three clustered inner robust setae, and two outer robust setae; inner ramus ~1.2x length of outer ramus, with four robust setae on outer margin, three robust setae on inner margin, and five robust setae on apex; outer ramus narrowing distally, curved slightly with three robust outer setae, and one smaller inner seta, apex with 10 comb-like robust setae placed in a transverse row. Uropod 3 (Figure 11E): peduncle ~60% length of outer ramus, with three distal robust setae; inner ramus reduced, scale-like, lacking setae; outer ramus ~3.5x longer than broad, ~6x longer than inner ramus, with two groups of robust, split-tipped setae on both inner and outer margins, apex with a slender seta paired with a short robust seta. Telson (Figure 11E): as broad as long, cleft ~30% of length, with a depth-to-width ratio of ~0.80, apical lobes armed with three robust split-tipped setae, two plumose setae arise dorsolaterally from the outer margin of both lobes.

3.4. Variation. Individuals examined were shown to vary notably in several morphological characteristics (Table 2).

3.5. Distribution and Ecology. Based on collections within and outside the immediate area of VFSF in Vinton County, Ohio, *C. ipnoecetes* n. sp. is currently known only from its type locality, although future sampling may discover additional populations. The type locality is located within ~50 km^2 of primarily oak forest in southwestern Vinton County, located on the Ironton Plateau, a region of the Western Allegheny Plateau characterized by coarse-grained, coal-bearing rock sequences and remnants of the Teays Valley [50, 51]. As a result, VFSF and the surrounding region are dissected by ridges and ravines; due in part to this topography, the region hosts a suite of unique flora and fauna and has been referred to as "one of the most biologically diverse ecosystems in the United States" [50].

Within VFSF, individuals of *C. ipnoecetes* n. sp. were collected in several rills located within ~4 km of each other. These rills were all very shallow and were clearly intermittent in nature, likely flowing due to rains that occurred the day prior to collection. In addition to these habitats, individuals were also observed in what appeared to be temporary, shallow pools of water present on the forest floor with no obvious surface connections to other bodies of water, suggesting the species likely utilizes shallow groundwater environments to disperse. We observed ovigerous females when collecting; it is likely that the species enters its reproductive peak during spring, as hypothesized for other *Crangonyx*. In addition, individuals of *C. furnaricolus* n. sp. were observed to occur syntopically with *C. ipnoecetes* n. sp. Similar patterns have been observed for other species pairs within the genus. In Florida, *Crangonyx ephemerus* Cannizzaro and Sawicki [52], and *Crangonyx*

Table 2: Noteworthy morphological variation observed among materials examined for each species of *Crangonyx* in this study.

Characters	Variability (*Crangonyx ipnoecetes* n. sp.)	Variability (*Crangonyx furnaricolus* n. sp.)
♀ Antenna 1 flagellar segments	17–19	19–20
Antenna 1 acc. flagellum to primary flagellum length	Shorter or longer	Shorter or longer
♀ Antenna 2 flagellar segments	6–8	6–8
♀ Antenna 1 length to body length	55%–62%	50%–55%
Maxilla 1 inner plate pappose setae	3–4	3–4
♀ Gnathopod 1 palmar margin robust setae (in./out.)	5–7/6–7	5–6/6–8
♀ Gnathopod 2 palmar margin robust setae (in./out.)	5–6/5–6	3–6/3–8
♀ Pereopod 7 basis posterior setae	9–11	13–18
♀ Uropod 1 peduncle outer robust setae	6–7	8–15
♂ Antenna 1 flagellar segments	12–14	14–17
♂ Antenna 2 flagellar segments	6–7	6–7
♂ Gnathopod 1 palmar margin robust setae (in./out.)	6–7/6–7	6–8/7–10
♂ Gnathopod 2 palmar margin robust setae (in./out.)	6–7/6–8	8–11/9–12
♂ Uropod 1 peduncle outer robust setae	3–4	11–12

pseudoephemerus Cannizzaro and Sawicki [52] were collected syntopically in ephemeral pools, and were revealed to be members of distinct lineages. Furthermore, individuals of *Crangonyx setodactylus* [9], *C. minor* [9], and *C. rivularis* [9] have also been observed to occur syntopically. In Butler/Hamilton counties in Ohio, individuals of these species have been collected from several temporary habitats analogous to those where *C. ipnoecetes* n. sp. and *C. furnaricolus* n. sp. were collected (AGC, personal observation). These observations suggest that *Crangonyx* spp. can often co-occur. The details on how they partition niches in such limited environments remain unclear.

Crangonyx furnaricolus n. sp. Cannizzaro & Berg.

ZOOBANK registration: urn:lsid:zoobank.org:act:9DA986AC-AE73-45E9-ABF3-6E7C8F34F957.

(Figures 4, 12–17).

Material examined: holotype, female 7.10 mm, in rills draining from small cave, Vinton Furnace State Furnace, Vinton County, Ohio, USA (39.18583, −82.39626); collectors: Andrew G. Cannizzaro, Ashley Walters, and Sarah McKillop, 25 March 2023, OSUMC 11029 (AGC-953.1). Allotype male, 6.21 mm, from the same locality and collection date as the holotype, OSUMC 11030 (AGC-953.3). Paratype female, 6.75 mm, from the same locality and collection date as the holotype, OSUMC 11031 (AGC-954.7).

Type locality: USA, Ohio, Vinton County, VFSF, rill draining from the small cave (39.18583, −82.39626; Figure 1).

Etymology: The specific epithet *furnaricolus* is given from the Latin *fornax* (a furnace, oven, kiln) and the suffix, *cola* (to inhabit). This etymology is paired with *C. ipnoecetes* n. sp. (in Ancient Greek instead of Latin), to also highlight that these two species co-occur in the same locality.

Diagnosis. Medium-sized, epigean species showing characteristics typical of the *gracilis* group. Distinguished from other members of the *gracilis* group through the combination of the following characteristics: eyes present; mandibular palp with 1 C-seta; dorsoposterior margins of epimera with weak teeth-like extensions; female uropod 1 peduncle with 10 outer robust setae and three inner robust setae; female uropod 3 peduncle short, subequal in length to outer ramus, inner ramus unarmed; male uropod 1 with 4 inner setae on peduncle; male uropod 2 peduncle armed with setae on outer margin, outer ramus slightly recurved with three slender robust setae lining outer margin, apex with four slender robust setae arranged in a row; male telson with long robust setae on apices.

Description: Holotype female (Figures 4, 12–16), 7.10 mm in length, with eye somewhat reduced, pigment yellowish in color when alive (Figure 4A).

3.5.1. Antennae. Antenna 1 (Figure 12A): 52% body length, ~1.3x longer than antenna 2; peduncle lacking robust setae; primary flagellum with 19 segments, aesthetascs on distal segments, aesthetascs shorter than respective segments; accessory flagellum 2-segmented, shorter in length than primary flagellar segment. Antenna 2 (Figure 12B): 28% body length, gland cone weak; peduncle ~1.6x length of flagellum, with three robust setae on segment 3; peduncle segment 4 subequal in length to segment 5; flagellum with eight segments, lacking calceoli.

3.5.2. Mouthparts. Mandibles: right mandible (Figure 12C) incisor 4-dentate, lacinia mobilis bifurcate, lobes with numerous fine dentations; accessory setae row with five plumose setae; molar process well-developed, cylindrical, triturative, with seta; palp 3-segmented, second segment ~1.3x length of third segment, with inner margin bearing 6 setae, and sparse fine pubescent setules; third segment

strongly rounded distally, inner margin convex, lacking A-setae, 1 B-seta, 1 C-seta, 10 D-setae, and 3 E-setae; face of segment covered in numerous, fine pubescent setules. Left mandible (Figure 12D): incisor 4-dentate, lacinia mobilis 4-dentate, with four serrate and plumose accessory setae; molar similar form to right mandible, but lacking seta. Upper lip (Figure 13A): rounded, apical margin of labrum with numerous setules. Lower lip (Figure 13B): inner lobes distinct, outer margins of inner and outer lobes covered in setules; face of lip covered in pubescent setules. Maxilla 1 (Figure 13C): inner plate shorter than outer plate, with four apical pappose setae and fine pubescent setules covering entire plate; outer plate with seven apical serrate setae and pubescent setules covering entire plate, decreasing laterally; palp two-segmented, distal segment covered in pubescent setules, apical margin of distal segment with three submarginal setae and five marginal robust setae. Maxilla 2 (Figure 13D): both inner and outer plates covered in pubescent setules; outer plate ~1.3x length of inner plate, with numerous apical setae; inner plate narrowing slightly distally with numerous apical setae and four pappose facial setae. Maxilliped (Figure 13E): inner plate shorter than outer plate with two unarmed cuspidate setae and a pappose cuspidate seta along apical margin, surface of plate covered in pubescent setules; outer plate with numerous submarginal setae, surface of plate covered in pubescent setules, distal portion of inner margin with semicuspidate setae; palp 4-segmented, second and third segments with numerous marginal, submarginal setae; distal surface of third segment with pubescent setules; dactylus with three inner marginal setae and an outer marginal seta.

3.5.3. Gnathopods. Gnathopod 1 (Figure 14A): coxal plate with six long apical setae and sparse facial setae; basis with long setae along anterior, medial, and posterior margins, and three shorter distal setae; ischium with three setae and pubescent setules along posterior margin; merus with pubescent setules covering posterior surface, numerous pappose posterodistal setae, and a medial seta along anterior margin; carpus ~65% length of propodus, with four setae inserted at anterodistal corner, four medial setae, posterior margin with numerous pappose setae; propodus ~1.2x longer than broad, with three marginal anterior setae, five setae inserted at anterodistal corner, two inferior medial setae, and three groups of pappose posterior setae; palm convex with six setae along outer margin; six outer and five inner bifid robust setae; inner margin of defining angle with four bifid robust setae, outer margin with two bifid robust setae and a large robust split-tip seta; dactylus with outer seta and five distal setae inserted near base of nail. Gnathopod 2 (Figure 14B): coxal plate with seven long apical setae and sparse facial setae; basis narrowing proximally with long setae along anterior and posterior margins, and three shorter distal setae; ischium with two setae and pubescent setules along posterior margin; merus with pubescent setules covering posterior surface and three posterodistal setae; carpus ~60% length of propodus, with two setae along anterior margin, two setae inserted at anterodistal corner, and two medial setae, posterior margin with three groups of pappose setae; propodus ~2x longer than broad, with a marginal anterior seta, four setae inserted at anterodistal corner, four superior medial setae, two inferior medial setae, and three clusters of pappose posterior setae; palm convex with seven setae along outer margin and three setae along inner margin; three outer and three inner bifid robust setae; inner margin of defining angle with five serrate robust setae, outer margin with two serrate robust setae and a larger robust split-tip seta; dactylus with outer seta and five distal setae.

FIGURE 12: *Crangonyx furnaricolus* n. sp. holotype female, 7.10 mm (OSUMC 11,029), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Antenna 1 (aesthetasc enlarged), (B) antenna 2, (C) right mandible (lacinia mobilis enlarged), and (D) left mandible (palp omitted). Scale bars: 0.25 mm (A, B) and 0.1 mm (C, D).

3.5.4. Pereopods. Pereopod 3 (Figure 15A): coxal plate with eight apical setae increasing in length posteriorly and several facial setae, plate not excavated posteroproximally; basis with numerous long setae inserted along anterior and posterior margins; merus ~1.2x longer than carpus; carpus subequal in length to propodus; dactylus ~50% length of propodus, with plumose setae proximally on anterior margin, while stout seta placed distally along posterior margin, proximal to nail. Pereopod 4 (Figure 15B): subequal to pereopod 3 in length, coxal plate with 12 apical setae and several facial setae, plate with distinct posteroproximal excavation; basis with numerous long setae along anterior and posterior

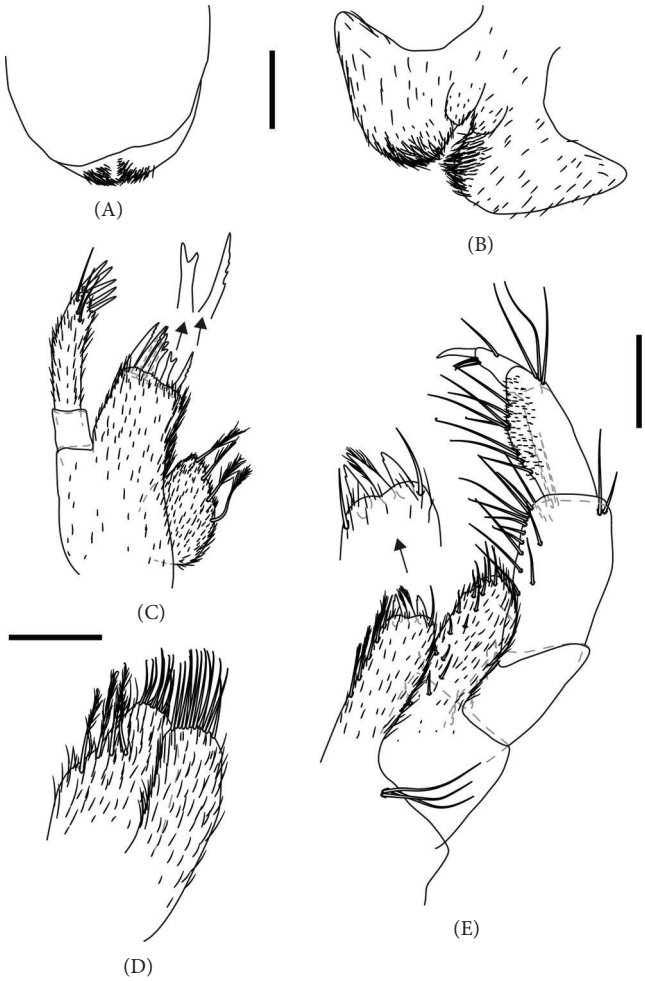

Figure 13: *Crangonyx furnaricolus* n. sp. holotype female, 7.10 mm (OSUMC 11,029), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Upper lip, (B) lower lip, (C) maxilla 1 (outer plate apical serrate setae enlarged), (D) maxilla 2, and (E) maxilliped (apical margin of inner plate enlarged). Scale bars: 0.1 mm.

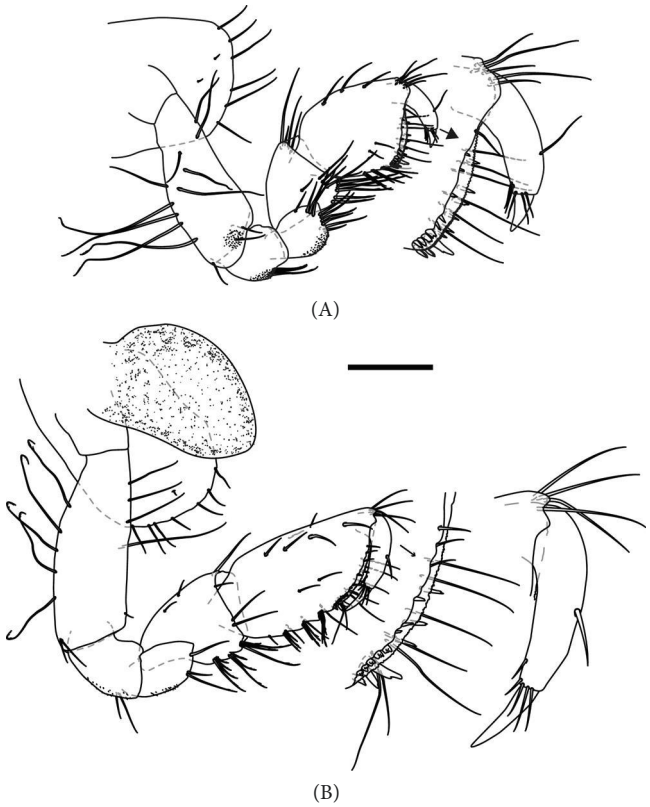

Figure 14: *Crangonyx furnaricolus* n. sp. holotype female, 7.10 mm (OSUMC 11,029), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Gnathopod 1 (palmar margin and dactylus enlarged) and (B) gnathopod 2 (palmar margin and dactylus enlarged). Scale bar: 0.25 mm.

margins; merus ~1.3x longer than carpus; carpus ~75% length of propodus; dactylus ~40% length of propodus, setation as in pereopod 3. Pereopod 5 (Figure 15C): coxal plate large, bilobate with distinct anterior and posterior lobes, each with seta on respective distal corners; basis posterior margin weakly convex with nine blunt serrations and a straight distal corner, posterior surface of segment with sparse facial setae, anterior margin with five split-tipped robust setae, two robust setae, and two long setae along anterodistal margin; merus ~80% length of carpus; carpus subequal in length to propodus; dactylus ~50% length of propodus, setation as in other pereopods. Pereopod 6 (Figure 15D): coxal plate weakly bilobate, with produced posterior lobe bearing a seta along posterior distal margin; basis posterior margin weakly convex with 11 blunt serrations and a convex distal corner, posterior surface of segment with sparse facial setae, proximal anterior margin with a long seta, anterior margin with five split-tipped robust setae, a robust seta and two long setae along anterodistal margin; merus ~75% length of carpus; carpus subequal in length to propodus; dactylus ~40% length of propodus, setation as in other pereopods. Pereopod 7 (Figure 15E): coxal plate subtriangular, with three posterior setae; basis posterior margin convex with 13 shallow serrations and with rounded distal corner, anterior margin with five split-tipped robust setae, one long seta along anterodistal margin; merus ~80% length of carpus; carpus subequal in length to propodus; dactylus ~45% length of propodus, setation as in other pereopods.

Gills (Figures 14B, 15A–E) coxal gills present on somites 2–6, somite 7 with small pereopod 7 gill. Slender sternal gills present on somites 6 and 7 (damaged in holotype).

3.5.5. Pleon. Epimera (Figure 16A): first epimeron ventral margin unarmed, strongly oblique, posterodistal corner with weak tooth-like extension, posterior margin with a seta placed above posterodistal corner; second epimeron ventral margin with three robust setae, posterodistal corner with weak tooth-like extension, posterior margin produced with a seta placed above posterodistal corner; third epimeron ventral margin with three robust setae, posterodistal corner blunt, weakly produced, posterior margin with a seta placed above posterodistal corner. Pleopod 1 (Figure 17B): peduncle ~35% length of inner ramus, lacking setae, with two coupling hooks; outer and inner rami with 10 and 14 segments respectively, basal segment of inner ramus with plumose clothespin

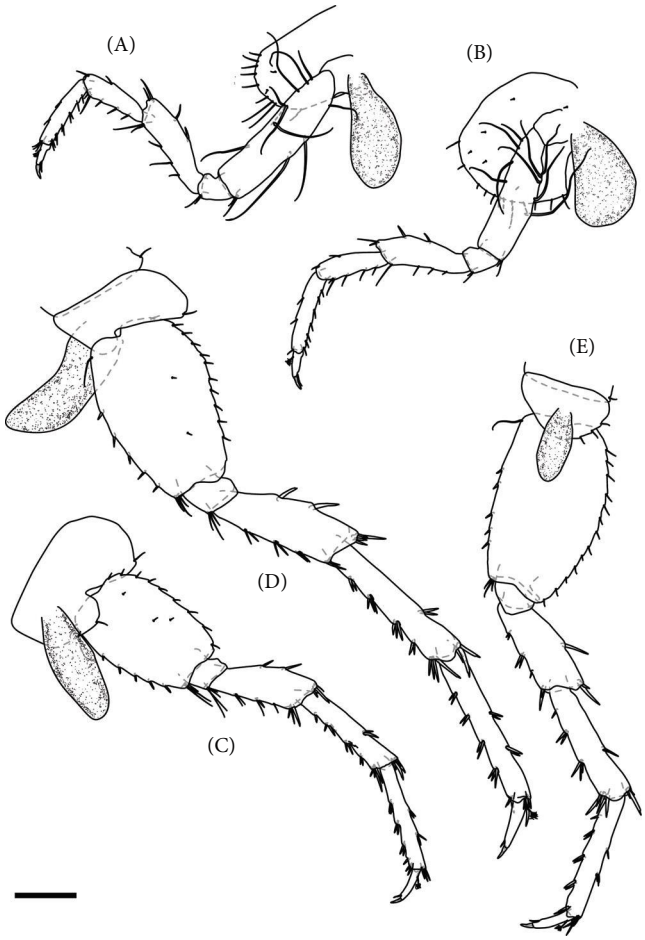

Figure 15: *Crangonyx furnaricolus* n. sp. holotype female, 7.10 mm (OSUMC 11,029), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Pereopod 3, (B) pereopod 4, (C) pereopod 5, (D) pereopod 6, and (E) pereopod 7. Scale bar: 0.25 mm.

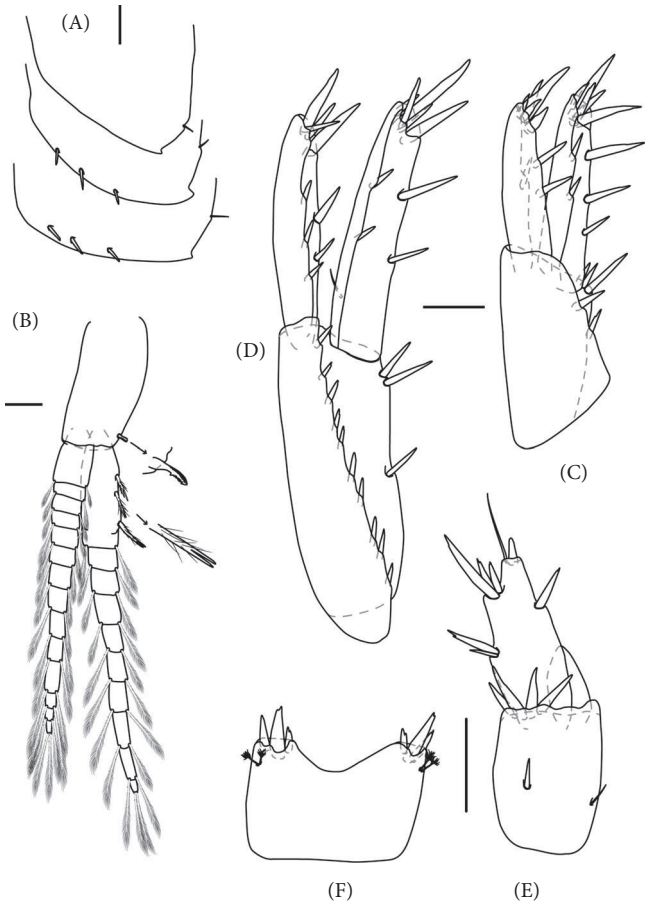

Figure 16: *Crangonyx furnaricolus* n. sp. holotype female, 7.10 mm (OSUMC 11,029), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Epimera, (B) pleopod 1 (coupling spines and clothespin seta enlarged), (C) uropod 1, (D) uropod 2, (E) uropod 3, and (F) telson. Scale bars: 0.25 mm (A) and 0.1 mm (B–F).

setae. Pleopod 2 (not figured): peduncle similar to pleopod 1, outer and inner rami with 10 and 11 segments, respectively. Pleopod 3 (not figured): peduncle similar to pleopods 1 + 2, outer and inner rami with 8 and 9 segments, respectively.

3.5.6. Urosome. Somites are free, bare dorsally. Uropod 1 (Figure 16C): peduncle ~1.2x length of rami with 10 outer robust setae and three inner robust setae, distal-most paired; outer ramus ~75% length of inner ramus, with two robust setae on inner margin, three robust setae on outer margin, and four robust setae on apex; inner ramus with two inner robust setae, two outer robust setae, four robust setae on apex, and a small seta placed proximally on ventral margin. Uropod 2 (Figure 16D): peduncle ~90% length of rami, with two outer robust setae and three paired inner robust setae; outer ramus ~90% length of inner ramus, with two robust setae on inner margin, a robust seta on outer margin, and five robust setae on apex; inner ramus with three robust setae on inner margin, two robust setae on outer margin, and five robust setae on apex. Uropod 3 (Figure 16F): peduncle subequal in length to outer ramus, with four distal robust setae, an inner proximal seta, and an outer proximal robust seta; inner ramus reduced, scale-like, lacking setae; outer ramus ~1.5x longer than broad, ~3x longer than inner ramus, with two groups of robust, split-tipped setae on outer margin, and a group of robust split-tipped setae on inner margin, apex with a slender seta paired with a short robust seta. Telson (Figure 16E): ~1.3x broader than long, cleft ~25% of length, with a depth-to-width ratio of ~0.3, apical lobes armed with three robust split-tipped setae, 2 plumose setae arise dorsolaterally from outer margins of both lobes.

Male: (Figures 4F, 17, 18): OSUMC 11030 (AGC-953.3), 6.21 mm in length. Differing from females in smaller body size; shorter antennae, presence of calceoli on the peduncle, and flagellum on antenna 2; more robust gnathopods with enlarged propodi and more robust setae on the palmar margins; pereopod 7 posterodistal serrations; uropod 1 setation; uropod 2 setation and rami structure; uropod 3 setation; and telson shape and setation. Structures not described below as in female.

3.5.7. Antennae. Antenna 1 (not illustrated, but see Figure 4B): 47% body length, ~1.6x longer than antenna 2; primary

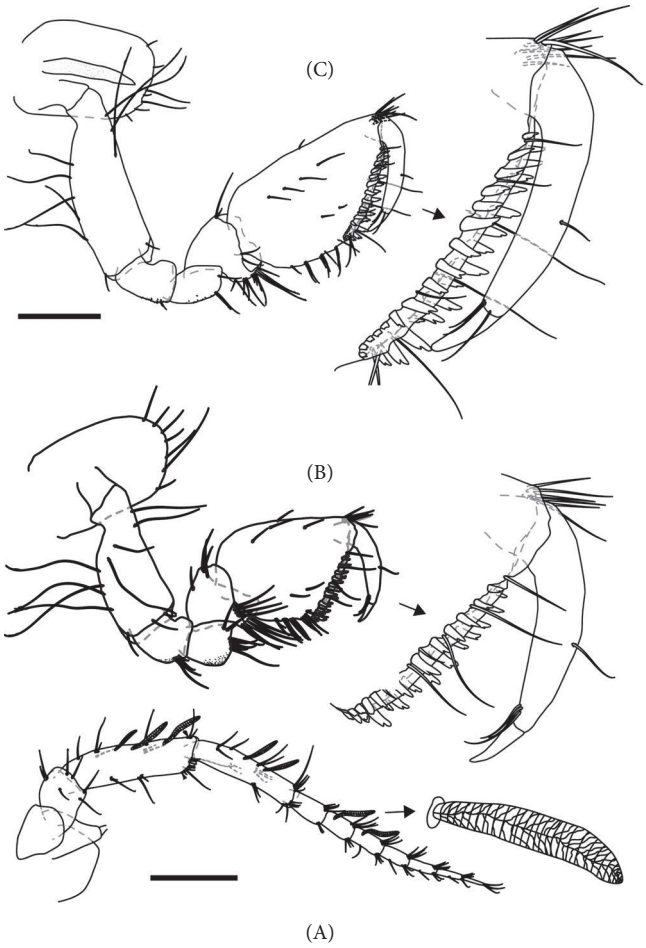

FIGURE 17: *Crangonyx furnaricolus* n. sp. allotype male, 6.21 mm (OSUMC 11,030), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Antenna 2 (single calceolus enlarged), (B) gnathopod 1 (palmar margin and dactylus enlarged), and (C) gnathopod 2 (palmar margin and dactylus enlarged). Scale bars: 0.1 mm.

flagellum with 17 segments, aesthetascs on distal segments, aesthetascs shorter than respective segments; accessory flagellum 2-segmented, reaching past the length of the first primary flagellar segment. Antenna 2 (Figure 17A): 62% body length, gland cone developed; peduncle ~1.6x length of flagellum; peduncular segment 5 ~80% length of segment 4; flagellum with seven segments; calceoli present on segments 4 + 5 of peduncle and proximal flagellar segments.

3.5.8. Gnathopods. Gnathopod 1 (Figure 17B): coxal plate narrowing distally, with seven long apical setae; basis narrowing proximally with long setae along anterior, medial, and posterior margins; ischium with four setae and pubescent setules along the posterior margin; merus with pubescent setules covering posterior surface, and five pappose posterodistal setae; carpus ~50% length of propodus, with a seta along anterior margin, three setae inserted at anterodistal corner, posterior margin with numerous pappose setae; propodus ~1.2x longer than broad, with four marginal anterior setae, five setae inserted at anterodistal corner, two inferior medial setae, and four clusters of posterior setae; palm straight with six setae along inner margin; 10 outer and eight inner bifid robust setae; inner margin of defining angle with four bifid robust setae, outer margin with three bifid robust setae, large robust split-tip seta; dactylus with outer seta, and three inner setae located near nail. Gnathopod 2 (Figure 17C): coxal plate with eight apical setae; basis narrowing proximally with long setae along anterior and posterior margins, and two short posterior setae; ischium with two posterior setae and pubescent setules along posterior margin; merus with sparse pubescent setules posterodistally, and three posterodistal setae; carpus ~60% of propodus with two anterior setae, two medial setae, and four groups of pappose posterior setae; propodus ~1.6x longer than broad, with an anterior seta, seven setae inserted at anterodistal corner, four superior medial setae, two inferior medial setae, and three clusters of posterior setae; palm weakly convex with three setae along inner margin and three setae along outer margin; 12 outer and 11 inner bifid robust setae; inner margin of defining angle with five short bifid robust setae, outer margin with three larger bifid robust setae; dactylus with outer seta, and three inner setae located near nail.

3.5.9. Pereopods. Pereopod 7 (Figure 18A): coxal lobes indistinct, with three posterior setae; basis posterior margin convex, with 10 serrations (increasing slightly in depth distally), distal corner weakly produced, anterior margin with four robust split-tipped setae, three robust setae inserted along anterodistal corner, and a long anteroproximal seta.

3.5.10. Urosome. Uropod 1 (Figure 18B): peduncle 1.7x length of rami, with 12 outer robust setae and four inner robust setae with the distal-most paired; outer ramus subequal in length to inner ramus, with two robust setae on inner margin, four robust setae on outer margin, and five robust setae on apex; inner ramus with two robust setae on inner margin, three robust setae on outer margin, five robust setae on apex, and a small seta placed proximally on ventral margin. Uropod 2 (Figure 18C): peduncle 1.3x length of rami, four inner robust setae with the distal 3 clustered, and three outer setae; inner ramus ~1.2x length of outer ramus, with two robust setae on outer margin, two robust setae on inner margin, and four robust setae on apex; outer ramus narrowing distally, slightly recurved with three slender robust setae lining outer margin, apex with four slender robust setae arranged in a row. Uropod 3 (Figure 18D): peduncle ~75% length of outer ramus, with four distal robust setae, an outer robust seta, and an inner seta; inner ramus reduced, scale-like, lacking setae; outer ramus ~3x longer than broad, ~4x longer than inner ramus, with two groups of robust, split-tipped setae on outer margin and a single group on the inner margin, apex with a slender seta paired with a short robust seta. Telson (Figure 18E): as broad as long, cleft ~30% of length, with a depth-to-width ratio of ~0.20, apical lobes armed with 2–3 long robust split-tipped setae and two simple setae, two plumose setae arise dorsolaterally from outer margin of both lobes.

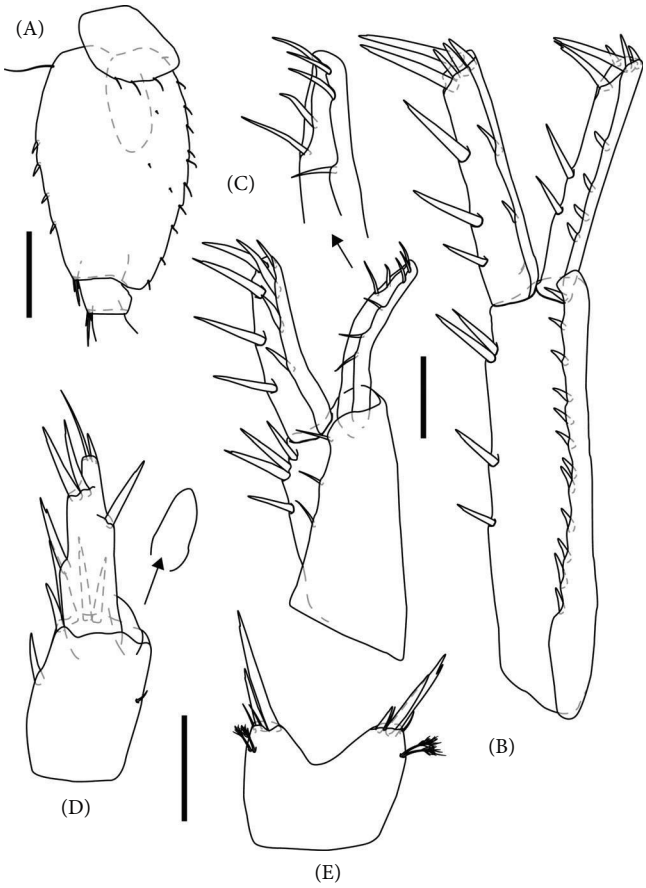

FIGURE 18: *Crangonyx furnaricolus* n. sp. allotype male, 6.21 mm (OSUMC 11,030), rill draining small cave, Vinton Furnace State Forest, Vinton County, Ohio, USA. (A) Pereopod 7 coxal plate, basis, and ischium, (B) uropod 1, (C) uropod 2 (tip of outer ramus enlarged); (D) uropod 3 (inner ramus enlarged); and (E) telson. Scale bars: 0.25 mm (A) and 0.1 mm (B–D).

3.6. Variation. Individuals examined were shown to vary notably in several morphological characteristics (Table 2).

3.7. Remarks. Crangonyx furnaricolus n. sp. appears similar to *C. ohioensis*, *C. castellanum* [4], and *C. acicularis* [4] in several points of morphology, but can be separated using a combination of characteristics. *Crangonyx ohioensis* and *C. furnaricolus* n. sp. share many points of morphology, especially in the gnathopods and pereopods, but males of these species differ considerably in uropod 2, where *C. ohioensis* possesses larger hook-like setae on the outer ramus, while in *C. furnaricolus* n. sp. these setae are much slenderer. *Crangonyx acicularis* and *C. castellanum* show somewhat similar morphology on the male uropod 2 and the telson, but these species differ considerably in the structure and armature of the gnathopods, epimera, and third uropods.

3.8. Distribution and Ecology. Crangonyx furnaricolus n. sp. is currently known only from its type locality, VFSF in Vinton County, Ohio, where it occurs syntopically with *C. ipnoecetes* n. sp. Individuals of *C. furnaricolus* n. sp. appeared to be somewhat rare, with only two individuals collected.

4. Discussion

Our results add insight into the poorly known amphipod fauna of the Western Allegheny Plateau. New collections from within Ross and Vinton counties have resulted in the identification of two new *Crangonyx* species (*C. ipnoecetes* n. sp. and *Crangonyx furnaricolus* n. sp.), and a range extension for another (*Crangonyx ohioensis*) (Figures 1–3). These results are corroborated by both morphological and molecular lines of evidence, with all three species forming well-supported monophyletic groups under all analytical methods utilized (Figures 2, 3). Not only do these species form monophyletic groups, but the two newly described species appear to belong to separate lineages. *Crangonyx ipnoecetes* n. sp. and *C. furnaricolus* n. sp. show more phylogenetic affinity to several other *Crangonyx* spp. than to each other. *Crangonyx ipnoecetes* n. sp. was recovered as sister to *C. antennatus*, and more distantly to members of the *C. floridanus* complex (Figures 2, 3); *Crangonyx furnaricolus* n. sp. was recovered as sister to *C. ohioensis* (Figures 2, 3). Notably, both of these species had relatively large genetic distances from their closest congeners, unlike other sequenced species in the genus, especially those within the *C. floridanus* complex (Figures 2, 3). This suggests that there could be additional species, yet to be sequenced, which may fill in gaps in the phylogeny.

Results of the BEAST analysis suggest fairly deep divergences for the species we examined. Based on these results, *C. ipnoecetes* n. sp. and *C. furnaricolus* n. sp. last shared a common ancestor sometime during the Late Cretaceous or early Paleogene (73–50 Ma; Figure 3). In addition, these species appear to have split from their closest congeners later into the Paleogene, ~20–45 Ma (Figure 3). These splits are consistent with those observed for other *Crangonyx* spp., with numerous lineages displaying similar divergence times ([5, 6]; Figure 3). With increasing analysis of the genus, it is becoming clear that the Paleogene was an important period in the evolutionary history of *Crangonyx*. The Appalachians and Western Allegheny Plateau are ancient systems, with origins stretching as far back as the Paleozoic [53]. However even into the Paleogene, geologic forces were still shaping the region. During the Mesozoic, it is likely that a large portion of the region was near-completely eroded, with uplift in the Cenozoic leading to the modern topography [53, 54]. The exact timing of these events is still unclear, with studies suggesting anywhere from the late Cenozoic to the Mesozoic–Cenozoic boundary [54–56]. Despite uncertainties on exact timing, it is clear that uplift during the Cenozoic would have not only begun to settle the region's topography but also influenced its hydrology. Such events occurring during the Cenozoic may explain the splitting of lineages among Appalachian *Crangonyx* spp. during the period that we observed (Figure 3).

Following Appalachian uplift in the Cenozoic, a large portion of the region containing the Western Allegheny Plateau was dominated hydrologically by the Teays River, a now-disrupted Cenozoic river which flowed from North Carolina northwards to Ohio and west into Illinois [57]. The Teays was a "precursor" to the Mississippi and Ohio

rivers; when it was active, drainage patterns in the region influenced by the river were drastically different from those present today [57, 58]. Vestiges of these drainages can still be observed in the phylogeographic patterns of taxa like the genus *Hypentelium* [59]. Given the period of time in which both the Teays existed and lineages within *Crangonyx* appear to have split, it is possible that the drainages interrupted by the river played a role in such splits. It is notable that *Crangonyx furnaricolus* n. sp. occurs west of the river's proposed path, and both *C. ohioensis*/*ipnoecetes* n. sp. are found east of this path (Figure 3). However, the recovery of *C. furnaricolus* n. sp. and *C. ohioensis* as sister taxa is inconsistent with this hypothesis. What is clear is that further sampling and phylogeographic analyses of *Crangonyx* spp., especially of Appalachian taxa, are needed to better reconstruct the evolutionary history of this region and of the genus.

Although it appears that most lineages within *Crangonyx* originated prior to the Pleistocene and were likely influenced by forces during the Paleogene, the impacts of glaciation on their modern distributions cannot be understated. The region occupied by the *Crangonyx* spp. we examined was heavily affected by glacial forces, with events in the Pleistocene causing major hydrologic shifts. As glaciers began to advance southward, they dammed the Teays River and started to fill valleys within the southwestern portion of the Western Allegheny Plateau leading to the formation of proglacial Lake Tight ([60, 61]; Figure 3), which is thought to have covered an area of ~26,000 km^2 in the region where Ohio, Kentucky, and West Virginia meet ([60, 61]; Figure 3). While subject to significant fluctuation, the lake reached depths of over 275 m and was responsible for considerable changes to the lowlands in the area underlying it [60, 61]. Areas above 275 m in elevation became islands and likely acted as refugia for organisms in the region. Following the lake's ~ 20,000+ year existence, water levels eventually rose to a point where they breached drainage divides, leading to the draining of Lake Tight, creating new drainages, and forming the modern Ohio River system [62]. While many plants and terrestrial animals would have been able to disperse rather quickly out of these islands following the lake's overflowing, amphipods and other dispersal-limited aquatic organisms would likely have been slower to recolonize new habitats. As a result, changes to Lake Tight may help explain strange distribution patterns on the Western Allegheny Plateau, such as those we observed in *Crangonyx* spp. However, distributional information at a species level is still lacking, and additional data is needed in order to make stronger inferences.

In addition to their distributions, the habitats occupied by *Crangonyx furnaricolus* n. sp. and *C. ipnoecetes* n. sp. are also of note. Both species were discovered in very shallow temporary habitats within the forest; many of these, including small rainwater-filled depressions, had no obvious surface connections to other bodies of water. This suggests that both species may utilize subterranean connections to disperse and occupy new habitats. Similar hypotheses have been proposed for other epigean members of the genus like *C. pseudogracilis* [12]. SSHs like these are poorly inventoried, especially for what are traditionally considered epigean taxa, which are often considered "visitors" to these habitats [1]. However, it is likely that despite their lack of obvious stygophilic characteristics, species such as *C. furnaricolus* n. sp. and *C. ipnoecetes* n. sp., are reliant on such habitats for both dispersal and as refugia during cold or dry months. Surveys of amphipods in Butler and Hamilton counties in Soutwestern Ohio seem to support this; *Crangonyx* spp. are often only observed/collected during the spring and are absent from both temporary and permanent bodies of water the rest of the year (AGC personal observations). Thus, SSHs may be more important to "epigean" taxa than previously considered. Investigations of other taxa occupying these habitats—isopods, gastropods, and insects, for example—may provide insights into the relationships between groundwater and surface water habitats, along with the organisms occupying them.

Our descriptions of *Crangonyx furnaricolus* n. sp. and *C. ipnoecetes* n. sp. highlight a significant knowledge gap for the amphipod fauna of the Appalachians. While only two localities were sampled, both revealed the presence of undescribed species; extrapolating this trend throughout the region suggests that there is a large amount of additional diversity awaiting analysis and description. The Appalachian Mountains and associated areas comprise one of the most biodiverse regions in the United States, especially when considering aquatic taxa, including endemic fishes, freshwater mussels, and crayfishes [14, 63, 64]. Many of these unique Appalachian taxa are of conservation concern due to their restricted ranges and habitat changes within the region [63, 65, 66]. Very little attention has been given to the conservation biology of amphipods and other "little things." Given their ecology and habits, it is likely that they are subject to the same anthropogenic threats as better-studied aquatic taxa within this region. The Linnaean shortfall plaguing such groups suggests a severe underestimation of not only diversity, but also conservation status, as populations are often either identified only to genus or are incorrectly assumed to be conspecific with wide-ranging taxa, which in turn are likely complexes of cryptic species. This suggests that populations of large numbers of amphipods and other small aquatic taxa could be of immediate conservation concern once they are described or could become extinct and/or locally extirpated before being formally recognized, a fate that has befallen a large number of similar taxa [67].

Gaining a better understanding of freshwater amphipod diversity has implications beyond just conservation and taxonomy. Amphipods often possess unique natural histories, with different lineages not only occupying varying habitats, but also varying considerably in age, with taxa having diversified from the Cretaceous to the Neogene [68, 69]. As a whole, freshwater amphipods do not represent a monophyletic group, and numerous lineages have colonized freshwater habitats. They likely utilized an impressive range of strategies to achieve contemporary and historical distributions, including both vicariance and dispersal [13, 69–71]. These factors suggest that amphipods are ideal, but often underutilized, models for addressing a range of evolutionary,

ecological, and biogeographic hypotheses. Investigations of such hypotheses utilizing amphipods as models have been highly successful in Palearctic systems [72–74], but have not been widely explored in the Nearctic, likely due in part to the significant Linnean shortfall. The addressing of these shortfalls, as we demonstrate here, has great potential to uncover more about the unique natural history of the regions in which these species occur.

Data Availability Statement

The data underlying this article are available in the GenBank Nucleotide Database at www.ncbi.nlm.nih.gov/genbank/ and can be accessed via the accession numbers provided in Table 1. Datasets generated during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest

Author Contributions

Andrew G. Cannizzaro conceived the ideas for the manuscript, performed all analyses, and led the writing. David J. Berg provided supervision, funding and reviewed/edited the manuscript.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. (*Supporting Information*)
Table S1. GenBank or BOLD (*) accession numbers of sequences utilized in this study.

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