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Cirolanides wassenichae sp. nov., a freshwater, subterranean Cirolanidae (Isopoda, Cymothoida) with additional records of other species from Texas, United States

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Abstract

Cirolanides wassenichae **sp. nov.**, is described from the phreatic zone of the Edwards Aquifer, Texas, USA where it is sympatric with *Cirolanides texensis* Benedict, 1896. Its status as a new species is based on both morphological and molecular data. Number of antennula articles (3–5 vs 9–15), size (mean sizes of 9.5 and 8.8 mm *vs* 11.1 and 10.4 mm for males and females, respectively), morphology of pereopods 1–3 (haptorial to semi-haptorial in 1–3 *vs* only 1 haptorial), and shape of pleotelson (squared, slightly indented *vs* rounded) are key morphological characteristics that distinguish *C. wassenichae* **sp. nov.** from *C. texensis*. Phylogenies based on cytochrome oxidase 1 and large ribosomal subunit 28S show that divergent morphologies correspond to reciprocally monophyletic groups for both nuclear and mitochondrial datasets. The genus *Cirolanides* is in need of revision, as our description of *C. wassenichae* **sp. nov.** renders *C. texensis* paraphyletic.

Key words: Edwards Aquifer, Stygobiont, Aquifer Biodiversity, *Cirolanides texensis, Speocirolana hardeni,* cytochrome oxidase 1 (CO1), large ribosomal subunit 28S

Introduction

In 1896, the first stygobiont from Texas was described from an artesian well in San Marcos, Texas (Stejneger 1896). Additional species were described from the same site shortly after, including the cirolanid isopod, *Cirolanides texensis* Benedict, 1896. Texas is an extension of the peri-Caribbean–Mexican region, which harbors 43 endemic cirolanid species and includes several localities with sympatric species (Alvarez & Villalobos 2008; Iliffe & Botosaneanu 2006; Bruce *et al.* 2017). In Texas, *C. texensis* and *Speocirolana hardeni* Bowman, 1992 occur in the Edwards and Edwards–Trinity aquifers in the extensive limestones of the Balcones Escarpment and Edwards Plateau. They are a component of the state's larger stygofauna, which includes 80 species that have been documented from diverse groundwater habitats across the state (Camacho *et al.* 2017; Hutchins 2017; Hillis et al. 2001; Holsinger and Longley 1980; Külköylüoğlu *et al.* 2017a; Külköylüoğlu *et al.* 2017b; Külköylüoğlu *et al.* 2017c; Külköylüoğlu *et al.* 2017d; Külköylüoğlu *et al.* 2017e; Külköylüoğlu & Gibson 2018).

Cirolanides texensis has previously been reported from 66 localities, and the subspecies, *C. t. mexicensis* has been recorded from 3 widely separated locations in North Mexico (Botosaneanu & Iliffe 2002; Hutchins 2017; Hutchins *et al.* 2013; Krejca 2009) (Fig. 1). *S. hardeni* Bowman, 1992, has previously been reported from 12 localities (Bowman 1992; Hutchins 2017; Zara 2010; Zara 2014) in the Edwards Aquifer (Fig. 1). Relatively little is known about *S. hardeni*, but *C. texensis* has been the subject of several studies, summarized by Krejca (2009).

Following Benedict's original description of *C. texensis,* further description was provided by Ulrich (1902), Richardson (1905), and Van Name (1936). A formal redescription was provided by Bowman (1964), who briefly

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emended the diagnosis of the genus, discussed relationships among stygobiotic genera of Cirolanidae, and provided a key to Western Hemisphere genera. Iliffe & Botosaneanu (2006) provided an updated synopsis of Caribbean and Gulf of Mexico groundwater cirolanids, and since that time, additional species and genera of stygobiotic cirolanids continue to be described from the region (Alvarez & Villalobos 2008; Bruce *et al.* 2017).

The genus *Cirolanides* remained monospecific until *C. texensis mexicensis* Botosaneanu & Iliffe, 2002 was described based on a smaller body size and presence of an additional (4th) spiniform seta on the palm of pereopod 1 propodus. Here, we describe a new species of *Cirolanides* from the phreatic zone of the Edwards Aquifer, representing the 3rd stygobiotic cirolanid from Texas and the 44th species from the peri-Caribbean region. Still, the diagnosis of the genus *Cirolanides* is in need of revision. Specifically, the emended diagnosis provided by Bowman (1964) is primarily a brief comparison with the genus *Antrolana,* which was the focus of that work. That revision failed to adequately address, at the generic level, several mouthparts, pleonites, male *appendix masculina,* pleotelson, and sexual dimorphism. As discussed below, our current understanding of *Cirolanides* is incomplete and a revised diagnosis of the genus will require future examination of additional *Cirolanides* populations.

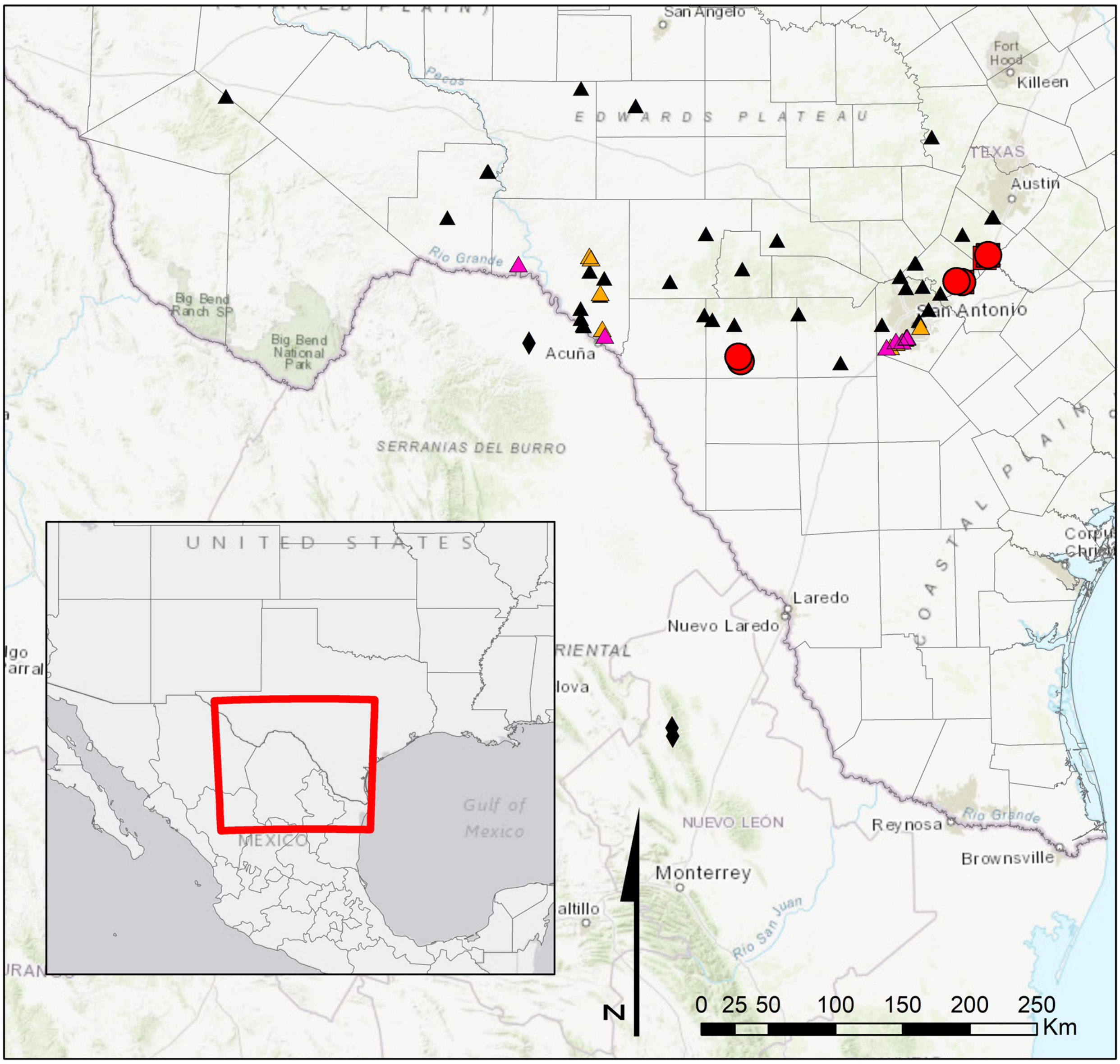

FIGURE 1. Range map for Cirolanides and Speocirolana hardeni in Texas and Mexico. *Cirolanides wassenichae* **sp. nov.** = large red circles; *C. wassenichae* **sp. nov.** and *C. texensis* = large red squares; *C. texensis* = small black triangles; *Speocirolana hardeni* = small purple triangles; *C. texensis* and *S. hardeni* = small orange triangles; *C. texensis mexicensis* = black diamonds.

TABLE 1. Samples used in phylogenetic analyses, with their associated locality data and GenBank accession numbers.

Species	Sex	Length (mm)	Locality	County	Specimen ID	Co1	28S
C. texensis	*male*	12.0	San Marcos Artesian Well	Hays	SMAW001	MK333115	MK333151
C. texensis	*male*	11.8	San Marcos Artesian Well	Hays	SMAW002	MK333114	MK333150
C. texensis	*female*	13.6	San Marcos Artesian Well	Hays	SMAW003	MK333113	MK333149
C. texensis	*male*	11.3	San Marcos Artesian Well	Hays	SMAW004	MK333112	MK333148
C. texensis	*male*	12.5	San Marcos Artesian Well	Hays	SMAW007	MK333111	-
C. texensis	*female*	7.3	San Marcos Artesian Well	Hays	SMAW008	MK333110	MK333147
C. texensis	*male*	12.7	San Marcos Artesian Well	Hays	SMAW009	MK333109	-
C. texensis	*female*	7.1	San Marcos Artesian Well	Hays	SMAW010	MK333108	MK333146
C. texensis	*female*	14.7	San Marcos Artesian Well	Hays	SMAW011	MK333107	MK333145
C. texensis	*female*	11.3	San Marcos Artesian Well	Hays	SMAW012	MK333106	MK333144
C. texensis	*female*	6.7	San Marcos Artesian Well	Hays	SMAW013	MK333105	MK333143
C. texensis	*female*	12.9	San Marcos Artesian Well	Hays	SMAW014	MK333104	MK333142
C. texensis	*female*	11.8	San Marcos Artesian Well	Hays	SMAW015	MK333103	MK333141
C. texensis	*female*	9.1	San Marcos Artesian Well	Hays	SMAW016	MK333102	MK333140
C. texensis	*female*	13.6	San Marcos Artesian Well	Hays	SMAW017	MK333101	MK333139
C. texensis	*female*	13.4	San Marcos Artesian Well	Hays	SMAW018	-	MK333138
C. texensis	*male*	9.3	San Marcos Artesian Well	Hays	SMAW019	MK333100	-
C. texensis	*female*	11.0	Panther Canyon Well	Comal	PC021	MK333099	MK333137
C. texensis	*female*	16.4	Comal Springs	Comal	CS025	MK333096	MK333135
C. texensis	*female*	11.1	Panther Canyon Well	Comal	PC027	MK333095	-
C. texensis	*female*	15.9	Panther Canyon Well	Comal	PC028	MK333094	MK333134
C. texensis	*female*	15.0	Panther Canyon Well	Comal	PC030	MK333093	MK333133
C. wassenichae **sp. nov.**	*female*	12.3	Panther Canyon Well	Comal	PC024	MK333097	-
C. wassenichae **sp. nov.**	*juvenile*	3.4	Comal Springs	Comal	CS031	MK333092	MK333132
C. wassenichae **sp. nov.**	*juvenile*	3.8	Comal Springs #7	Comal	CS032	MK333091	MK333131
C. wassenichae **sp. nov.**	*female*	7.2	Mission Valley Bowling Alley Well	Comal	MV033	MK333090	MK333130
C. wassenichae **sp. nov.**	*juvenile*	3.4	Comal Springs	Comal	CS034	MK333089	MK333129
C. wassenichae **sp. nov.**	*juvenile*	3.5	Comal Springs #3	Comal	CS035	MK333088	MK333128
C. wassenichae **sp. nov.**	*juvenile*	3.5	Comal Springs #7	Comal	CS036	MK333087	MK333127
C. wassenichae **sp. nov.**	*male*	10.7	Comal Springs #7	Comal	CS037	MK333086	MK333126
C. wassenichae **sp. nov.**	*male*	9.1	San Marcos Artesian Well	Hays	SMAW038	MK333085	-
C. wassenichae **sp. nov.**	*juvenile*	3.2	San Marcos Artesian Well	Hays	SMAW039	MK333084	-
C. wassenichae **sp. nov.**	*juvenile*	3.2	San Marcos Artesian Well	Hays	SMAW040	MK333083	MK333125
C. wassenichae **sp. nov.**	*juvenile*	3.3	San Marcos Artesian Well	Hays	SMAW041	-	MK333124
C. wassenichae **sp. nov.**	*male*	8.4	San Marcos Artesian Well	Hays	SMAW042	MK333082	MK333123
C. wassenichae **sp. nov.**	*juvenile*	3.5	San Marcos Artesian Well	Hays	SMAW043	-	MK333122
C. wassenichae **sp. nov.**	*juvenile*	4.0	San Marcos Artesian Well	Hays	SMAW044	-	MK333121
C. wassenichae **sp. nov.**	*female*	5.6	San Marcos Artesian Well	Hays	SMAW045	-	MK333120
C. wassenichae **sp. nov.**	*male*	9.4	San Marcos Artesian Well	Hays	SMAW046	-	MK333119

...Continued on next page

TABLE 1. (Continued)

Species	Sex	Length (mm)	Locality	County	Specimen ID	Co1	28S
C. wassenichae **sp. nov.**	*juvenile*	3.5	San Marcos Artesian Well	Hays	SMAW047	MK333081	MK333118
C. wassenichae **sp. nov.**	*juvenile*	3.7	San Marcos Artesian Well	Hays	SMAW048	MK333080	MK333117
C. wassenichae **sp. nov.**	*female*	10.3	San Marcos Artesian Well	Hays	SMAW049	MK333079	MK333116
C. wassenichae **sp. nov.**	*juvenile*	3.1	San Marcos Artesian Well	Hays	SMAW050	MK333078	-
C. wassenichae **sp. nov.**	*female*	5.3	San Marcos Artesian Well	Hays	SMAW051	MK333077	-
C. wassenichae **sp. nov.**	*female*	7.4	San Marcos Artesian Well	Hays	SMAW052	MK333076	-
C. wassenichae **sp. nov.**	*juvenile*	3.9	San Marcos Artesian Well	Hays	SMAW053	MK333075	-
C. wassenichae **sp. nov.**	*juvenile*	3.5	Comal Springs #3	Comal	CS023	MK333098	MK333136

Material and methods

Specimen collection. The new species described herein was first noticed in 2014 during periodic drift-netting on the outflow of the San Marcos Artesian Well (SMAW) in the Edwards Aquifer, on the Texas State University campus, San Marcos, TX, USA. Between February 13, 2013 and August 21, 2015, aquifer invertebrates were periodically collected in a 100 µm net. Isopods were sorted from bulk samples. All specimens were preserved in 95% ethanol and archived in the Texas State University Edwards Aquifer Research and Data Center (EARDC) collection.

Additionally, curated specimens previously identified as *C. texensis* in collections at the U.S. Fish and Wildlife Service San Marcos Aquatic Resources Center, EARDC, and University of Texas at Austin, Department of Integrative Biology, Biodiversity Collections (formerly Texas Memorial Museum) were re-examined. During examination of museum specimens, previously unpublished records for *C. texensis* and *S. hardeni* were also documented. All coordinates provided in WGS84.

Genetics. DNA was extracted from partial specimens using the DNeasy Blood and Tissue Kit following manufacturer's instructions. DNA was amplified for fragments of the mitochondrial gene cytochrome oxidase 1 (*Co1*) and nuclear gene large ribosomal subunit 28S (*28S*). Specimen data and GenBank accession numbers are provided in Table (1). *Co1* was amplified using the general metazoan primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.* 1994). *28S* was amplified using newly designed primers for *Cirolanides*: Ciro_28S_F (5'-GATCAGAGCTCGCTCGAATAAA-3') and Ciro_28S_R (5'-GGAGGTAGAGGAAGAAGAA-3'). PCR reactions were typically 13 µL in total volume, containing 1 µL of ~100 ng/µL of DNA template, 2.5 µL 5x Green GoTaq Reaction Buffer (Pro-Mega), 8 µL RNAse free H?O, 0.75 µL of 25 mM MgCl?, 0.25 µL of 10 mM dNTPs, 0.125 µL GoTaq G2 DNA polymerase, and 0.25 µL of 10 µM forward and reverse primers. PCR products were verified using gel electrophoresis on a 1.0% agarose gel stained with ethidium bromide. Unincorporated nucleotides were removed from PCR products using 1 µL of 20% ExoSAP-IT (USB Corp) per 10 µL of PCR product. Cycle sequencing reactions were typically ~10 µL in volume, containing 3µL of purified product, 2.5 µL of RNAse free H?O, 2 µL of 5M Betanine, 1.5 µL of 1 µM forward or reverse primer, 0.5 µL of BigDye 5X Sequencing Buffer, and 0.4 µL of BigDye v3.1 (Applied Biosystems, Inc.). Products were filtered through a plate containing Sephadex G-50 (Invitrogen Corp) then sequenced on an ABI 3130xl (Applied Biosystems, Inc.). Forward and reverse chromatographs were visualized, assembled into contigs, and edited in Sequencher v5.2 (Gene Codes Corp).

We collected 43 *Co1* (~694 bp) and 36 *28S* (~439 bp) sequences. *Speocirolana hardeni* and *C. t. mexicensis Co1* sequences were included from GenBank for a final dataset of 47 *Co1* sequences. *Speocirolana* was chosen as an outgroup due to its presumed close phylogenetic relationship with *Cirolanides*, due to its previous status as a subgenus within *Cirolanides* (Bowman 1964). No available closely related species suitable as an outgroup could be found for *28S*. Sequences were aligned in MUSCLE using MEGA7.0 (Kumar *et al.* 2016) based on default parameters. Best-fit models of nucleotide substitution were estimated for *Co1* in PartitionFinder2.0 (Lanfear *et al.* 2016) which uses the "Greedy" algorithm (Lanfear *et al.* 2012) and PhyML 3.0 (Guindon *et al.* 2010) to estimate substitution models. Nucleotide substitution models were set by codon position to TrNef (1^{st} Codon), GTR (2^{nd}

Codon), and HKY (3rd Codon). PAUP* v4.0a152 (Swofford 2002) was used to calculate uncorrected *p* genetic distance among samples.

Bayesian phylogenetic analyses were performed in BEAST v2.4.6 (Bouckaert *et al.* 2014) using a strict clock, and coalescent-fixed population size model. Runs consisted of four chains (three heated, one cold) run for 100 million generations and sampled every ten thousand with a random starting tree and the first 10% discarded as burn-in. *28S* and *Co1* were treated separately for phylogenetic analyses. Results were examined in Tracer v1.6 (Rambaut *et al.* 2014) to evaluate stationarity of likelihood.

Taxonomy

Order ISOPODA Latreille, 1817

Family CIROLANIDAE Dana, 1852

Genus *Cirolanides* Benedict, 1896

Type species. *Cirolanides texensis* Benedict 1896; by monotypy.

Included species. Previously monotypic with two described subspecies: *Cirolanides texensis texensis* Botosaneanu and Iliffe 2002, *Cirolanides texensis mexicensis* Botosaneanu and Iliffe 2002.

Remarks. Bowman (1964) provided a diagnosis of the genus *Cirolanides.* That diagnosis is in need of revision which will require additional morphologic and molecular examination of putative *C. texensis* populations.

Cirolanides texensis Benedict 1896

Cirolanides texensis Benedict 1896: 616–617.
Cirolanides texensis.—Ulrich (1902): 88–90, plate 15; Richardson (1905): 120–123, figs 121–122; Van Name (1936): 427–428, figs 427; Bowman (1964): 229, 232–235, plates 55–57.

Material examined. Texas: Bexar Co., Longhorn Cement—Bexar Well #F-106 (29.53472°N, 98.4°W), coll. M. Brzozowski, September 2, 1981 (EARDC Cat# BLC 810902-1); Comal Co., Comal Springs, Spring #7 (29.71533°N, 98.1349°W), coll. R. Gibson, Dec. 28, 2016 (EARDC Cat# CS7 161228-1); Hays Co., Sessom Creek Springs (29.8904°N, 97.9369°W), coll. A. Everett, June 6, 2017 (EARDC Cat# SS 170606-1); Spring Lake Outflow Well (29.89292°N, 97.9314°W), coll. J. Crow and V Cantu, May 4, 2014 (EARDC Cat# SL 140504-1); Uvalde Co., George Ligocky Well (29.19362°N, 99.8106°W), coll. R.C. Wiedenfeld, February 18, 1980 (EARDC Cat# GL 800218-1); Val Verde Co., Pitaya Pit (Lake Amistad National Recreation Area—exact location unavailable), coll. P. Sprouse, Sept. 23, 2015 (EARDC Cat# PP 150923-1); Medina County, Robert Fassler Farm well (29.18468°N, 99.0806°W), coll. R.C. Wiedenfeld, February 25, 1980 (EARDC Cat# RFF 800225-1). All specimens curated at Texas State University Edwards Aquifer Research and Data Center except for Pitaya Pit (University of Texas Austin, Biodiversity Center Collections).

Remarks. New records of *C. texensis* consist of new collections and older (1980–1981) archived samples at EARDC that were not previously published.

Cirolanides wassenichae sp. nov.
Figs 2–6

Material examined. *Holotype:* ♀ (11.3 mm), EARDC Cat#: AW 130305-1, USNM 1480594 San Marcos Artesian Well (SMAW), Hays County, Texas, U.S.A.: 29.88963°N, 99.9364°W, ~177mASL, 25 March 2013, coll. B. Hutchins, B. Schwartz, and Z. Schwartz.

Paratypes: ♂ (10.2 mm), EARDC Cat#: AW 111026-1, USNM 1480595 San Marcos Artesian Well (SMAW), Hays County, Texas, U.S.A, 29.88963°N, 99.9364°W, ~177 m ASL, 26 October 2011, coll. unknown—identified in

2018 from EARDC archived samples. USNM 1480596 and 1480597, Panther Canyon Well, Comal County, Texas, U.S.A.: 29.71361°N, 98.13830°W, ~204 m ASL, 30 September 2014, and 23 September 2015, coll. Randy J. Gibson.

Additional material. (Note: most juveniles were sacrificed for molecular analysis. For most mature specimens, one set of pereopods was used for molecular analysis. EARDC catalog numbers are provided for archived specimens) TEXAS, USA**:** COMAL CO., Comal Springs, Spring # 3 (29.71352°N, 98.1371°W), coll. R. Gibson, 27 Oct., 2016, 2 juv.; Comal Springs, Spring #7 (29.71533°N, 98.1349°W), coll. R. Gibson, 28 Oct., 2016, 1 ♂ (EARDC Cat #: CS7 161028-1); Panther Canyon Well (29.71361°N, 98.1383°W), coll. R. Gibson, 24 Jul., 2015, 1 ♂, 1 ♀ (EARDC Cat #: PC 150725-1, PC 150725-1); Mission Valley Bowling Well (29.72056°N, 98.1794°W), coll. R. Gibson, 7 Jun., 2013, 1 ♀ (EARDC Cat #: MV 130607-1); HAYS CO., San Marcos Artesian Well (29.88963°N, 97.9364°W) (paratypes, coll. B. F. Schwartz, B. T. Hutchins, A. P. Swink, V. Castillo III), 8 May, 1976, 1 ♀; 14 Feb., 2013, 1 ♂; 18 Feb., 2013, 1juv.; 26 Mar., 2013, 1juv.; 28 Mar., 2013, 1juv.; 15 Nov., 2013, 1 ♂, 1 juv.; 19 Nov., 2013 1 juv.; 22 Nov., 2013, 1juv.; 29 Nov., 2013, 1 juv.; 2 Dec., 2013, 1 juv.; 5 Dec., 2013, 1 juv.; 12 Dec., 2013, 1 juv.; 19 Feb., 2014, 1 ♂, 1 juv.; 14 Jul., 2014, 1 ♀; 16 Jun., 2015, 2 ♀; 3 Jul., 2015, 1 ♀; 17 Aug., 2015, 1 ♀; 6 Sep., 2017, 1 ♂, (EARDC Cat. #s: AW 130214-1, AW 131115-2, AW 140219-1, AW 150616-1, AW 150616-2, AW 150703-1, AW 150817-1, AW 170906-1, AW 760508-1); San Marcos Springs, Deep Hole Spring (29.89237°N, 97.9323°W), coll. G. Longley, 15 Nov., 1976 (EARDC Cat #: DH 761115-1), 1 juv.; Ezell's Cave, coll. J. Krejca, 1 Jun., 2006, 1 ♂; Spring Lake Outflow Well (29.89292°N, 97.93143°W), coll. A. Everett, 24 Jul., 2017, 1 juv (EARDC Cat #: SLO 170724-1); UVALDE CO., Bill Stockton well (29.2002°N, 99.844°W) (identified by R. C. Wiedenfeld as *C. texensis*), coll. R. C. Wiedenfeld, 1 May, 1980, 1 damaged (EARDC Cat #: BSW 800501-1); R. K. Dunbar Farm Well (29.23139°N, 99.8614°W) (identified by R. C. Wiedenfeld as *C. texensis*), coll. R. C. Wiedenfeld, 10 Jun., 1980, 1 ♂ (EARDC Cat #: RKD 800610-1) . All specimens curated at Texas State University Edwards Aquifer Research and Data Center (EARDC) except for Ezell's Cave (University of Texas Austin, Biodiversity Center Collections) and type material, discussed above.

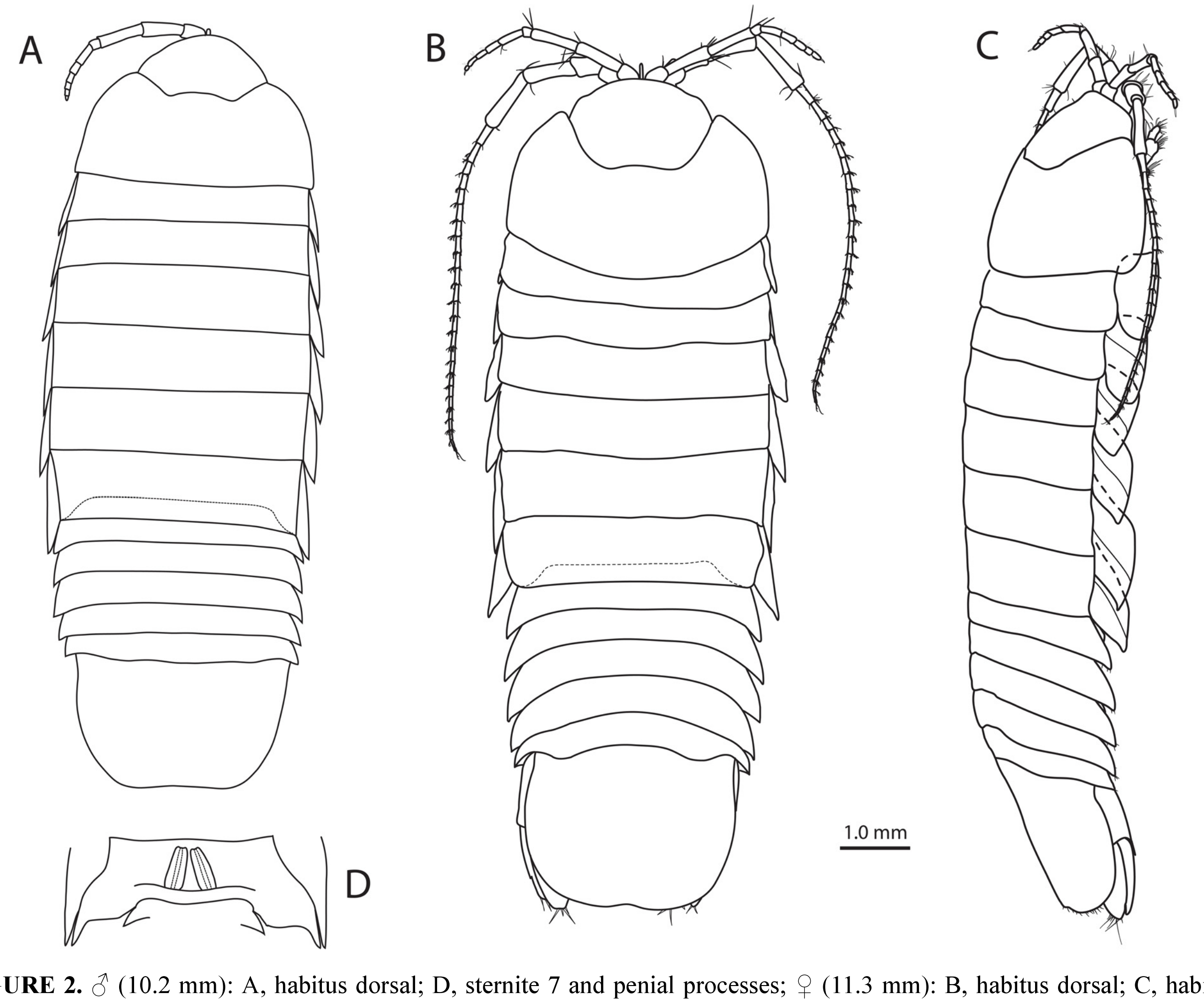

FIGURE 2. ♂ (10.2 mm): A, habitus dorsal; D, sternite 7 and penial processes; ♀ (11.3 mm): B, habitus dorsal; C, habitus lateral

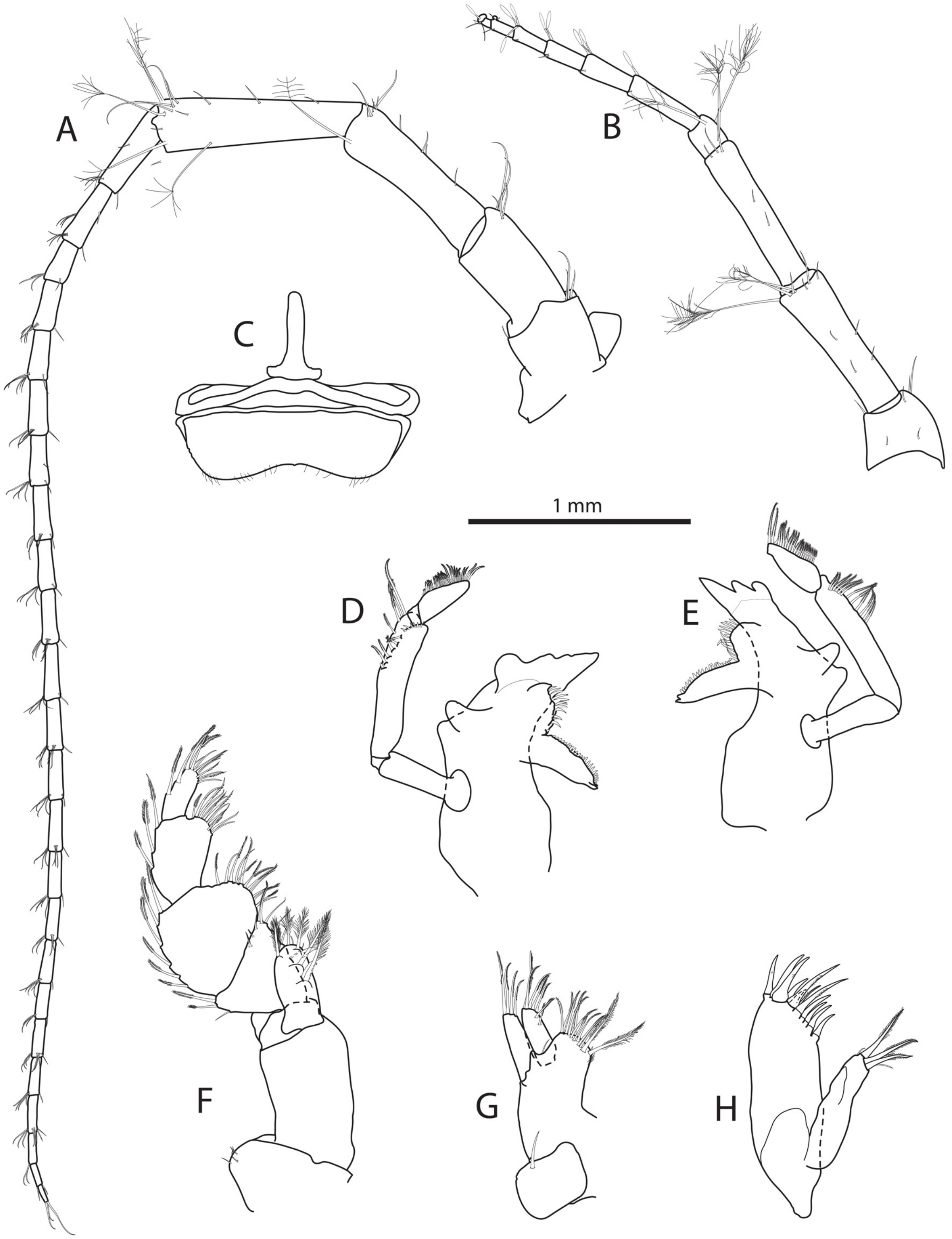

FIGURE 3. ♀ (11.3 mm): A, antenna; B, antennula; C, clypeus; D, left mandible; E, right mandible; F, left maxillula 1; G, left maxilla 2; H, left maxilliped.

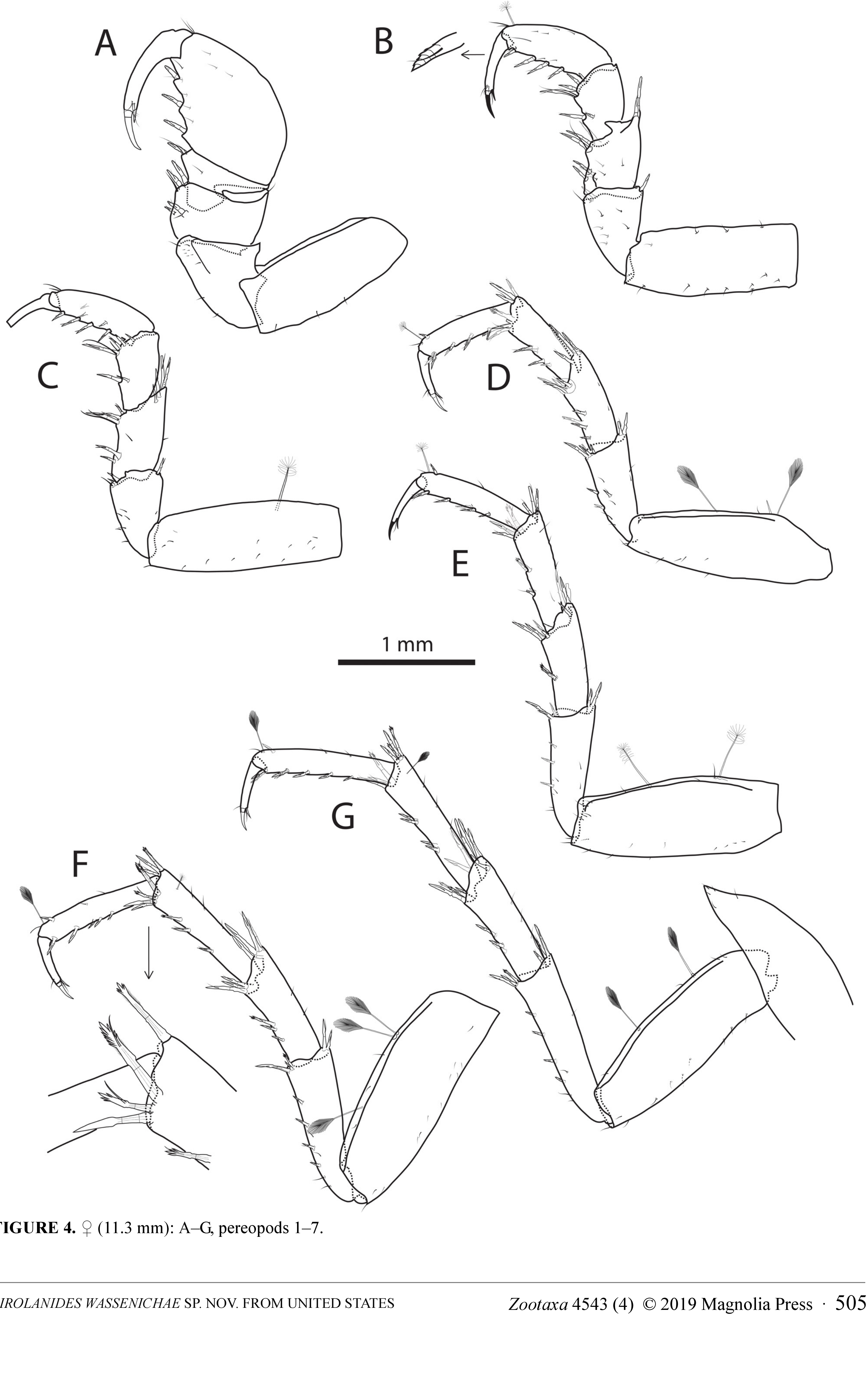

FIGURE 4. ♀ (11.3 mm): A–G, pereopods 1–7.

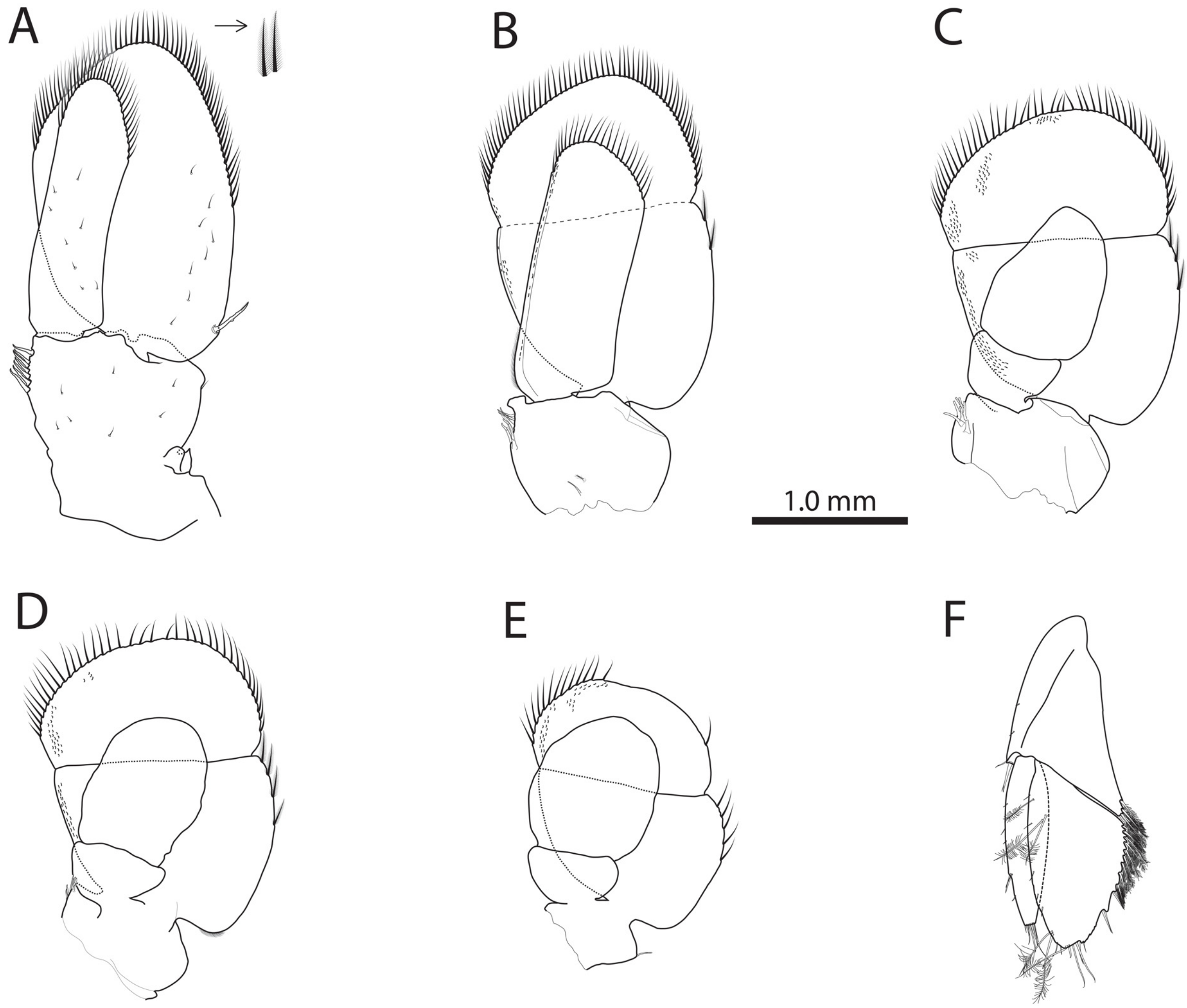

FIGURE 5. ♀ (11.3 mm): A–E, pleopods 1–5; F, uropod.

Diagnosis: Antenna reaching pereonite 5, flagellum composed of 20 articles; frontal lamina narrow, extending to distal margin of antennula peduncle article 1; pereopods 1-2 haptorial, pereopod 3 intermediate between haptorial and ambulatory; posterior margin of pleotelson quadrate, lateral margins parallel, posterior margin medially concave; distomedial angle of uropodal protopod more acutely produced than in *C. texensis*, extending to middle of pleotelson, exopod and endopod subequal in length, extending to posterior margin of pleotelson.

Description of holotype female: Body 11.3 mm long, without pigment, 2.7 times as long as greatest width, widest at pereonite 4, lateral margins parallel (Figs 2–5); *pereonite 1* and *coxae 2* with posteroventral angle rounded, *coxae 3–7* with acute posteroventral angle and single, entire oblique carina, (Fig. 2); *pereonite 7* with anterior internal concave 'suture' (Fig. 2); *pleonites 1–5* with posteroventral angles acute, not concealed by pereonite 7 which extends 2/3rds length of first pleonite (Fig. 2); *pleotelson* quadrate, 0.8 times as wide as long, lateral margins parallel, posterior margin with medial concavity (Fig. 2), *cephalon* trapezoidal, deeply inserted into pereonite 1, 1.6 times as long as greatest width, anterior margin rounded, posterior margin weakly concave with sparse fringe of short, simple setae (Fig. 2); *eyes* absent.

Antennula extending to middle of pereonite 1, peduncle articles 1 and 2 subequal in length, approximately 5 times length of article 3, peduncle articles with 3 or 4 plumose setae, flagellum with 6 articles, each with 1–3 pappose setae (Fig. 3); *antenna* extending to pereonite 5, peduncle articles 1 vestigial, 2 reduced, 3 and 4 subequal in length, approximately half as long as article 5, peduncle article 5 slightly shorter than 6, articles 5 and 6 with 2–3 plumose setae and 2–6 short, simple setae, flagellum with 20 articles subequal in length with clusters of 3–5 short simple setae near distal ends, pappose setae lacking.

Frontal lamina narrow, extending to distal margin of antennule peduncle article 1 (Figs 2, 3); *clypeus* transversely rectangular, concave posterior margin with sparse fringe of short simple setae (Fig. 3); *labrum* directed ventrally, rectangular, with dorsomedial margin produced anteriorly (Fig. 3).

Left mandible molar process anteriorly with 24 sharp to peg-shaped teeth, distally with tiny finger-like process; lacinia mobilis with approximately 14 curved spiniform setae; incisor hyaline, with jagged cutting surface, cusps indistinct, dorsal margin produced as rounded lobe; palp segment 2 approximately two times as long as segment 1 or 3, with 6 short and 3 long serrate setae, 6 short spiniform setae distally; palp segment 3 approximately 2.5 times as long as wide, tapering distally, with approximately 26 serrate setae (Fig. 3). *Right mandible* molar process similar to left mandible; lacinia mobilis with 12 curved spiniform setae; incisor hyaline, with 3 cusps decreasing in size dorsally; palp similar to left mandible but segment 2 with 12 serrate setae, segment 3 with 20 serrate setae (Fig. 3). *Maxillula* endopod with 3 large, circumplumose setae, 2 small, simple setae, exopod with 8 robust serrate and 4 robust cuspidate setae (Fig. 3). *Maxilla* basis with 1 simple seta distally; lateral and medial lobes subequal in length, with 4 and 7 long, serrate setae, respectively; mesial lobe with 13 long, serrate setae distally, 1 simple setae near articulation with medial lobe, patch of small, simple setae distomedially (Fig. 3). *Maxilliped* endite with 2 coupling hooks, 5 long, plumose setae; palp articles 2 and 3 with well-developed lobes mesially, article 1 with 2 plumose setae on lateral margin and 4 plumose setae on mesial margin, article 2 with 6 plumose setae on lateral margin and 14 plumose setae on mesial margin, article 3 with 3 plumose setae on lateral margin and 13 plumose setae on distomesial lobe, article 4 with 3 plumose setae on lateral margin and 12 plumose setae distally (Fig. 3).

Pereopod 1 (Fig. 4) robust, haptorial; basis subequal in length to propodus, rectangular, with 1 plumose seta at base, and few short, simple setae; ischium triangular, sub equal in length to merus and carpus, with row of 3–6 short spiniform setae on ventral margin; merus short, rectangular, with 2 pairs of robust spiniform setae and 1 simple seta on ventral margin; carpus with 1 spiniform seta and 2 simple setae on distal, ventral corner; propodus length to width ratio 1.6:1, palmar margin straight with 3 large serrations corresponding to bases of 3 robust spiniform setae with rasp-like tips; dactylus simple with spiniform seta at base of unguis.

Pereopod 2 (Fig. 4); haptorial but not as robust as P1; basis rectangular, with short simple setae; ischium triangular, with robust spiniform seta on ventral and dorsal disto-apical corners; merus rectangular, subequal in length to ischium with 8–9 robust spiniform seta on ventral margin and dorsal-apical corner; carpus rectangular, slightly shorter than merus, with 6 robust spiniform seta on ventral margin and ventral-distal corner; propodus sub-rectangular, length to width ratio 2.6:1, palmar margin straight with 4 robust spiniform setae, distal-most tooth serrate or rasp-like, remaining simple, all 4 with simple accessory setae, single plumose seta near anterior-distal corner; dactylus simple, curved, with spiniform setae at base of unguis.

Pereopod 3 (Fig. 4) slightly haptorial, but not as strongly as P1–2; basis rectangular, with short simple setae and 1 plumose seta near mid-anterior margin; ischium triangular, subequal in length to merus, with 3 robust spiniform seta on distal-apical margin; merus quadrate, slightly longer than carpus, with 4 robust spiniform seta ventrally, 7 robust spiniform seta on distal-apical margin; carpus rectangular with pair of robust spiniform seta on ventral margin, 5–7 on apical margin; propodus length to width ratio 3.0:1, ventral margin straight with 4 robust spiniform seta (first 3 serrate, distal-most rasp-like), additional short spiniform setae on ventral surface; dactylus simple, curved, with 1 or 2 spiniform setae at base of unguis.

Pereopod 4 (Fig. 4) ambulatory; basis elongate, rectangular, 2 plumose setae on posterior margin, short simple setae on posterior and anterior margins; ischium narrowly triangular, stout spiniform setae on distal margin; merus quadrate with stout spiniform setae on distal and posterior margins; carpus quadrate with stout, simple and denticulate spiniform setae on distal and posterior margins; propodus narrow, 3 simple setae and 1 plumose seta on distal margin, posterior margin with 3 pairs of stout spiniform setae, single spiniform seta near anterior end, with 4 pairs of stout spiniform setae on margin; dactylus simple, curved, with 1 or 2 spiniform setae at base of unguis.

Pereopod 5 (Fig. 4) ambulatory; basis elongate, rectangular, 2 plumose setae on posterior margin, short simple setae on posterior and anterior margins; ischium narrowly triangular, stout spiniform setae on distal margin, 2 spiniform setae on posterior margin; merus quadrate with spiniform setae on distal margin, 1 denticulate and 1 spiniform seta on posterior margin; carpus narrow, quadrate, 2 pairs of spiniform setae on posterior margin, stout spiniform setae on distal margins; propodus narrow, rectangular, 3 simple setae and 1 plumose seta on distal margin, posterior margin with 2 pairs of stout spiniform setae, additional single spiniform seta near anterior end between 2 spiniform seta pairs, with 4 pairs of stout spiniform setae on margin; dactylus simple, curved, with 1 to 2 spiniform setae at base of unguis.

Pereopod 6 (Fig. 4) ambulatory; basis sub-rectangular, 3 plumose setae on anterior margin, short simple setae on posterior anterior and distal margins; ischium narrowly triangular, stout spiniform setae on distal margin, 4 spiniform setae on posterior margin; merus rectangular, 2 simple and 1 denticulate spiniform setae on posterior margin, a cluster of robust simple and 1 denticulate spiniform setae on distal end; carpus narrow, quadrate, 3 pairs of spiniform setae on posterior margin, stout spiniform setae and 2 denticulate spiniform setae on distal margins with 1 plumose setae near distal end; propodus narrow, rectangular, 4 simple and 1 plumose setae on distal margin, posterior margin with 1 pair of stout spiniform setae and 3 single stout spiniform setae, with 4 pairs of stout spiniform setae on margin; dactylus simple, curved, with 1 or 2 accessory spiniform setae at base of unguis.

Pereopod 7 (Fig. 4) ambulatory; basis with 2 plumose setae on anterior margin, short simple setae on posterior and anterior margins; ischium narrow, rectangular, with 4 short simple setae on posterior margin, robust simple and denticulate spiniform setae on distal margin; merus rectangular, 3 spiniform setae on posterior margin, robust simple and 1 denticulate spiniform seta on distal margin; carpus narrow, quadrate, 2 pairs of stout spiniform setae on posterior margin, stout spiniform setae and 3 denticulate spiniform setae on distal margin with 1 plumose setae near distal end; propodus narrow, rectangular, 4 simple setae and 1 plumose seta on distal margin; posterior margin with 2 pairs of stout spiniform setae and 3 stout single spiniform setae, with 5 pairs of stout spiniform setae on margin; dactylus simple, curved, with 1 or 2 accessory spiniform setae at base of unguis.

Pleopod 1 protopod with 7 coupling hooks (Fig. 5); endopod 3.7 times as long as wide, lateral margin slightly concave, mesial margin nearly straight, rounded distally, with 30 plumose setae on distal margin, no suture; exopod trapezoidal, 1.6 times as long as wide, 62–66 plumose setae on distal margin, 1 setae proximally on lateral margin, no suture. *Pleopod 2* protopod with 7 coupling hooks (Fig. 5); endopod 2.6 times as long as wide, lateral margin slightly concave, mesial margin straight, rounded distally, with 23 plumose setae on distal margin, no suture; exopod trapezoidal, 1.6 times as long as wide, 60 plumose setae distal to transverse margin, 2 plumose setae on lateral margin proximal to suture. *Pleopod 3* protopod with 7 coupling hooks (Fig. 5); endopod 1.9 times as long as wide, ovate, with proximal transverse suture; exopod quadrate, 1.4 times as long as wide, 49 plumose setae distal to transverse suture, 3 plumose setae on lateral margin proximal to suture. *Pleopod 4* protopod with 7 coupling hooks (Fig. 5); endopod 1.7 times as long as wide, ovate, with proximal transverse suture; exopod ovate, 1.4 times as long as wide, 42 plumose setae distal to transverse suture, 4 plumose setae on lateral margin proximal to suture. *Pleopod 5* peduncle without coupling hooks, 1 setae on lateral margin (Fig. 5); endopod 1.5 times as long as wide, sub-ovate with proximal transverse suture; exopod ovate, 1.3 times as long as wide, with 14 plumose setae distal to oblique suture, with 5 plumose setae on lateral margin proximal to suture.

Uropod protopod triangular (Fig. 5), produced as an acute point distomedially, slightly longer than wide, extending to middle of pleotelson and proximal fourth of exopod, 6 plumose setae distomedially, 2 lanceolate setae distolaterally; endopod broadly lanceolate, 1.6 times as long as wide, 11 plumose setae on medial margin, 5 plumose setae on lateral margin, 14 robust simple and plumose setae distally; exopod narrowly lanceolate, 3 times as long as wide, 7 simple setae on lateral margin, 4 simple setae distally.

Male. Slightly larger than female. *Pereopod 1–7, pleopods 1–5* (except *pleopod 2*), *habitus, uropod, antenna,* and *antennula* (Fig. 6) as in females. *Pleopod 2* (Fig. 6): *appendix masculina* smooth, curved, 1.3x as long as endopod, end narrowly rounded distally; inserted distally from base of endopod (~20% of length, from base); exopod ovate, 1.4 times as long as wide, 47 plumose setae on distal margin, distal to transverse suture, 1 plumose setae on lateral margin proximal to suture; endopod 2.4 times as long as wide, 21 plumose setae on distal margin; 1 circumplumose seta and 6 coupling hooks on protopod; this contrasts with 7 coupling hooks on female *pleopod 2. Penial processes* (Fig. 2): flat, thin, paddle-shaped with slight indentations in squared distal ends, tapering slightly distally, 2.3x longer than wide, centerline of processes separated by 15% width of sternite 7 (Fig. 2).

Size and morphological differences. Mature male *C. wassenichae* **sp. nov.** (mean = 9.5 mm, range = 8.4–10.7 mm) are slightly larger than mature females (mean = 8.8 mm range = 5.3–12.3 mm). Additionally, *C. wassenichae* **sp. nov.** is slightly smaller than mature *C. texensis* males (mean length = 11.1 mm, range = 7.4–12.4 mm) and females (mean length = 10.4 mm, range = 5.5–16.4 mm). Across all body sizes, *C. wassenichae* **sp. nov.** consistently has ~35% as many flagellar segments on A1 and A2 as *C. texensis* (Fig. 7). There are no differences in relationships between body size and number of flagellar segments between sexes in either species.

Etymology. The species epithet *wassenichae* honors Mrs. Dianne Wassenich, Program Director of the San Marcos River Foundation (SMRF) and long-time advocate for San Marcos River and Edwards Aquifer conservation. We advocate adoption of the common name, Wassenich's isopod.

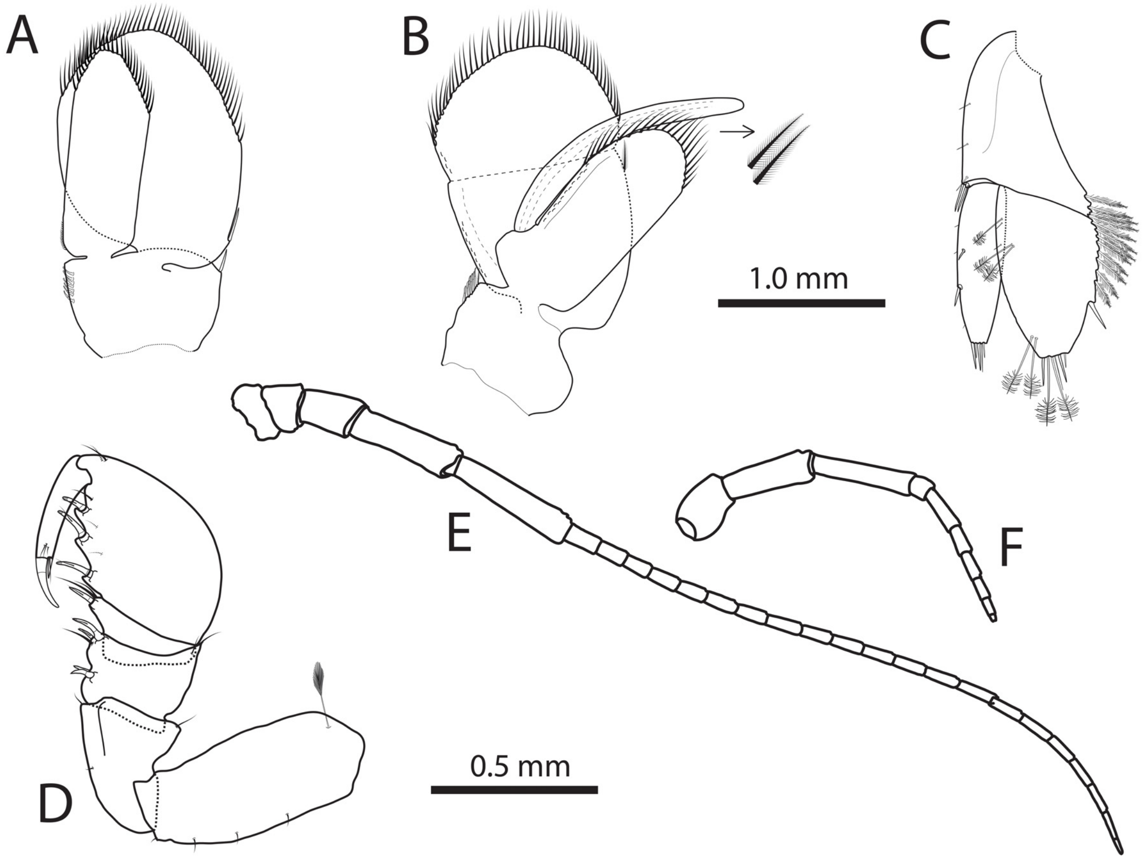

FIGURE 6. ♂ (10.2 mm): A, pleopod 1; B, pleopod 2; C, uropod; D, pereopod 1; E, antenna; F, antennula.

Genetics. The *Co1* phylogeny (see Fig. 8) recovered two deeply divergent lineages within *Cirolanides* (0.117 ± 0.0061 uncorrected *p;* see Table 2). One lineage corresponds to described morphologies of *C. texensis;* the other corresponds to the new species, *C. wassenichae* **sp. nov.** Despite deep divergence between lineages, within lineage divergence was shallow (*C. texensis* 0.002 ± 0.0042 uncorrected *p*, *C. wassenichae* **sp. nov.** 0.0058 ± 0.0039 uncorrected *p*). *28S* displayed a similar pattern of divergence, with higher levels of between-lineage divergence (0.0097 ± 0.0016 uncorrected *p*) than within-lineage (*C. texensis*: 0.002 ± 0.0024 uncorrected *p*; *C. wassenichae* **sp. nov.**: 0.00017 ± 0.00061 uncorrected *p*). Both nuclear and mitochondrial phylogenies showed strong posterior support values for two clades within *Cirolanides* (*C. texensis*, *C. wassenichae* **sp. nov.**; Fig. 8). Divergence was higher for mitochondrial than nuclear genes, which is expected (Brown *et al.* 1979).

Distribution. The type locality, known for stygobiotic diversity, is a well completed in the confined zone in a relatively shallow portion of the San Antonio Pool of the Edwards Aquifer. At 59.5 m depth, the well intersects a 1.5 m tall conduit (Holsinger & Longley 1980), from which the water and biological materials are presumed to discharge. Water quality is high and has been dye-traced to San Marcos Springs, less than 1 km to the northeast (see details in Ogden *et al*. 1986). In November 2013, water temperature at the well averaged 22.3°C (SD ±0.01), dissolved oxygen averaged 5.3 mg/L (SD ±0.01), and electrical conductivity averaged 608 µS/cm (SD ±0.50). *Cirolanides wassenichae* **sp. nov.** is known from nine sites, in addition to the type locality (Table 3). All sites are springs, caves, or wells that intersect the phreatic zone of the karstic Edwards Aquifer, in a narrow 200 km long band that parallels the Balcones Escarpment in central Texas (Fig. 1). The new species is sympatric with *C. texensis* at seven sites (Table 3).

Remarks. *Cirolanides wassenichae* **sp. nov.** differs from the current diagnosis of the genus in that the 2nd pereopod is haptorial and not ambulatory and the 3rd pereopod is intermediate between ambulatory and haptorial rather than ambulatory. As discussed below, however, a generic revision is premature at this time. *Cirolanides wassenichae* **sp. nov.** is readily distinguished from *C. texensis* by the following features: smaller size (see statistics above), flagellum of antennula with fewer articles (3–5 *vs.* 9–15); flagellum of antenna with fewer articles (12–21 *vs.* 21–39); pereopod 2 haptorial *vs.* ambulatory; pereopod 3 semi-haptorial *vs.* ambulatory; pereopod 1 longer than pereopods 2 and 3 (which are subequal in length) *vs.* pereopod 1 shorter than pereopods 2–3 in *C. texensis*; body more slender with lateral margins nearly parallel *vs.* ovate; posterior margin of pleotelson truncate with medial concavity *vs.* well-rounded; uropod endopod wider and overlapping exopod more; uropod endopod and exopod shorter and extending only slightly past posterior margin of pleotelson *vs.* well past.

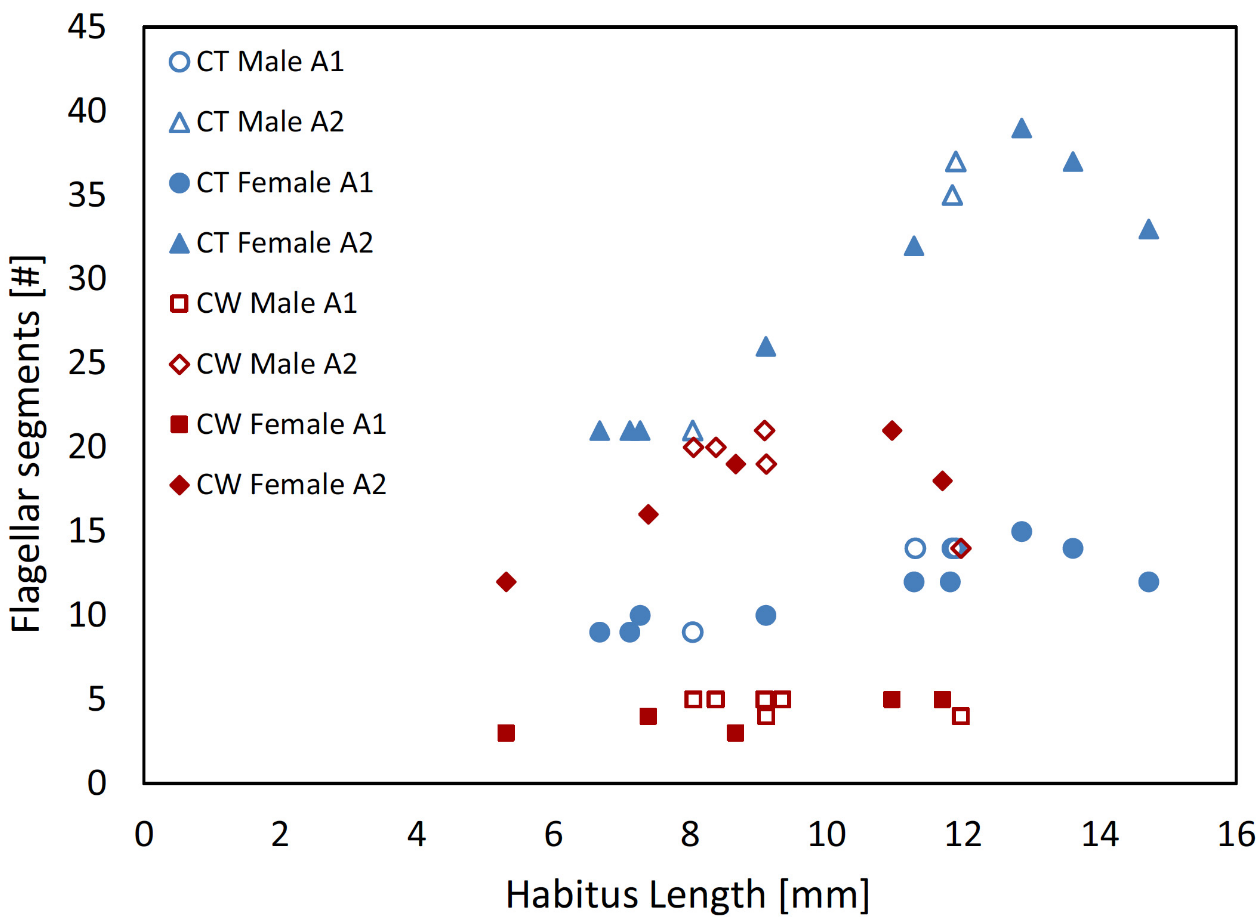

FIGURE 7. Relationships between body length and flagellar segments *Cirolanides wassenichae* **sp. nov.** and *Cirolanides texensis*. Red symbols = *C. wassenichae* **sp. nov**. Blue symbols = *C. texensis.* Open symbols = Male; filled symbols = Female. Squares and circles = A1; circles and triangles = A2.

TABLE 2. Genetic distance within *C. texensis*, *C. wassenichae* **sp. nov.**, and between species for *Co1* and *28S*.

Genetic distance (uncorrected *p* distance) in *Cirolanides*		
C. texensis	*C. wassenichae* **sp. nov.**	*C. texensis* x *C. wassenichae* **sp. nov.**
28S		
Average = 0.002028 ± 0.002415	Average = 0.00017 ± 0.000612	Average = 0.009707 ± 0.001591
Maximum = 0.0102	Maximum = 0.00236	Maximum = 0.01567
Maximum = 0	Maximum = 0	Maximum = 0.00483
Co1		
Average = 0.002 ± 0.004160676	Average = 0.005831 ± 0.00392764	Average = 0.116846 ± 0.006136438
Maximum = 0.01237	Maximum = 0.01373	Maximum = 0.12325
Minimum = 0	Minimum = 0	Minimum = 0.00159

TABLE 3. Names and locations of *C. wassenichae* **sp. nov.** sites. * Locations where *C. wassenichae* **sp. nov.** is sympatric with *C. texensis*. Location data use the WGS 84 datum.

Site	Latitude (°N)	Longitude (°W)
* Artesian Well (SMAW)	29.88963	97.93645
Bill Stockton Well H-4-62	29.20020	99.84400
* Comal Springs #3	29.71352	98.13707
* Comal Springs #7	29.71533	98.13487
* Ezell's Cave	29.87382	97.95939
Mission Valley Bowling Well	29.72056	98.17945
* Panther Canyon Well	29.71361	98.13833
R. K. Dunbar Well	29.23139	99.86139
* Deep Hole Spring	29.89237	97.93225
* Spring Lake Outflow Well	29.89292	97.93144

Discussion

Support for species level designation. Three lines of evidence support the validity of *C. wassenichae* **sp. nov**. (1) Two distinctive morphologies are now described in *Cirolanides* of the Edwards aquifer, (2) both mitochondrial and nuclear genes support divergent lineages corresponding to these morphologies, and (3) these lineages are sympatric at most known *C. wassenichae* **sp. nov.** sites, with no evidence of introgression. This work represents the second documented species in the genus and increases our understanding of *Cirolanides* diversity in central Texas, USA. Our *Co1* phylogeny supports a sister relationship between *C. texensis* and *C. wassenichae* **sp. nov.** and we have placed the new species within the genus *Cirolanides*.

Size, rarity, and taxonomic oversight. Despite gross morphologic differences (Fig. 9) that are apparent under low magnification, *Cirolanides wassenichae* **sp. nov.** was unrecognized for at least 45 years (based on confirmed specimens in historic collections archived at the EARDC), and probably for more than a century, based on intensive earlier collection efforts at known *C. wassenichae* **sp. nov.** sites. The smaller size and relative rarity of *C. wassenichae* **sp. nov.** have likely contributed to this oversight; of 304 *Cirolanides* collected at the type locality by the authors between February 2013 and November 2015, *C. wassenichae* **sp. nov.** is represented by 19 individuals (6%).

Unresolved taxonomy within Cirolanides. Previous analyses have suggested that stygobionts with ranges larger than 200 kilometers in length are extremely rare and are probably complexes of cryptic species (Trontelj *et al.* 2009). As it is currently understood, the range of *C. texensis texensis* is >600 km from east to west (and >400 km north to south, if the subspecies *C. texensis mexicensis* is included). *Cirolanides texensis* is the largest ranging groundwater obligate species in Texas (Krejca 2009), recorded from more than 50 sites, and with a range of >70,000 km^2. If the subspecies *C. texensis mexicensis* is included, the range is >120,000 km^2, incorporating two Mexican states (Botosaneanu & Iliffe 2002).

Genetic data (Krejca 2005) suggests substantial divergence among presumed *Cirolanides texensis* populations, and morphologic variation across the species' range has been previously described (Botosaneanu & Iliffe 2002) and additionally observed by the authors, particularly when presumed juveniles are examined. Populations putatively identified as *C. texensis* likely represent distinct species and, potentially, additional sympatric Cirolanid species.

This is especially true for *C. texensis mexicensis*, which is geographically isolated and morphologically distinct. Based on the Co1 phylogeny (Fig. 8), our description of *C. wassenichae* **sp. nov.** renders *C. texensis* paraphyletic. Given that *C. t. mexicensis* has previously been described as morphologically distinct (Botosaneanu & Iliffe, 2002), and we show it is genetically divergent from *C. texensis* and *C. wassenichae* **sp. nov.** (Fig. 8), sub-species status of *C. t. mexicensis* should be critically evaluated in future work. Species level diversity of *Cirolanides* in Texas remains unresolved and a revision of the genus will require additional morphologic and molecular investigation of populations through Texas and northern Mexico.

Speocirolana hardeni Bowman 1992

Speocirolana hardeni Bowman 1992: 23–29, figs 26–28.

All specimens curated at the Texas State University Aquifer Research and Data Center. Texas: Uvalde Co., Leon Walton Farm Well (29.24786°N, 99.8725°W, coll. R.C. Wiedenfeld, February 18, 1980 (EARDC Cat# LWW 800218-1).

Remarks. This previously unreported specimen was found in EARDC archived samples.

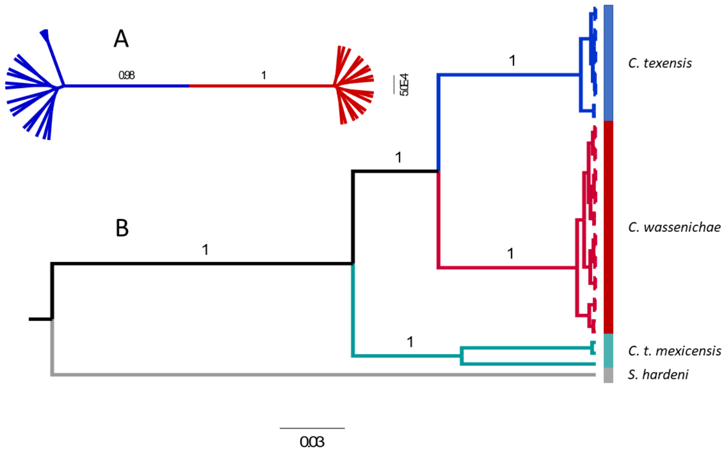

FIGURE 8. Genetic divergence between *Cirolanides texensis* (blue) and *Cirolanides wassenichae* **sp. nov.** (red) based on *28S* (A) and *Co1* (B).

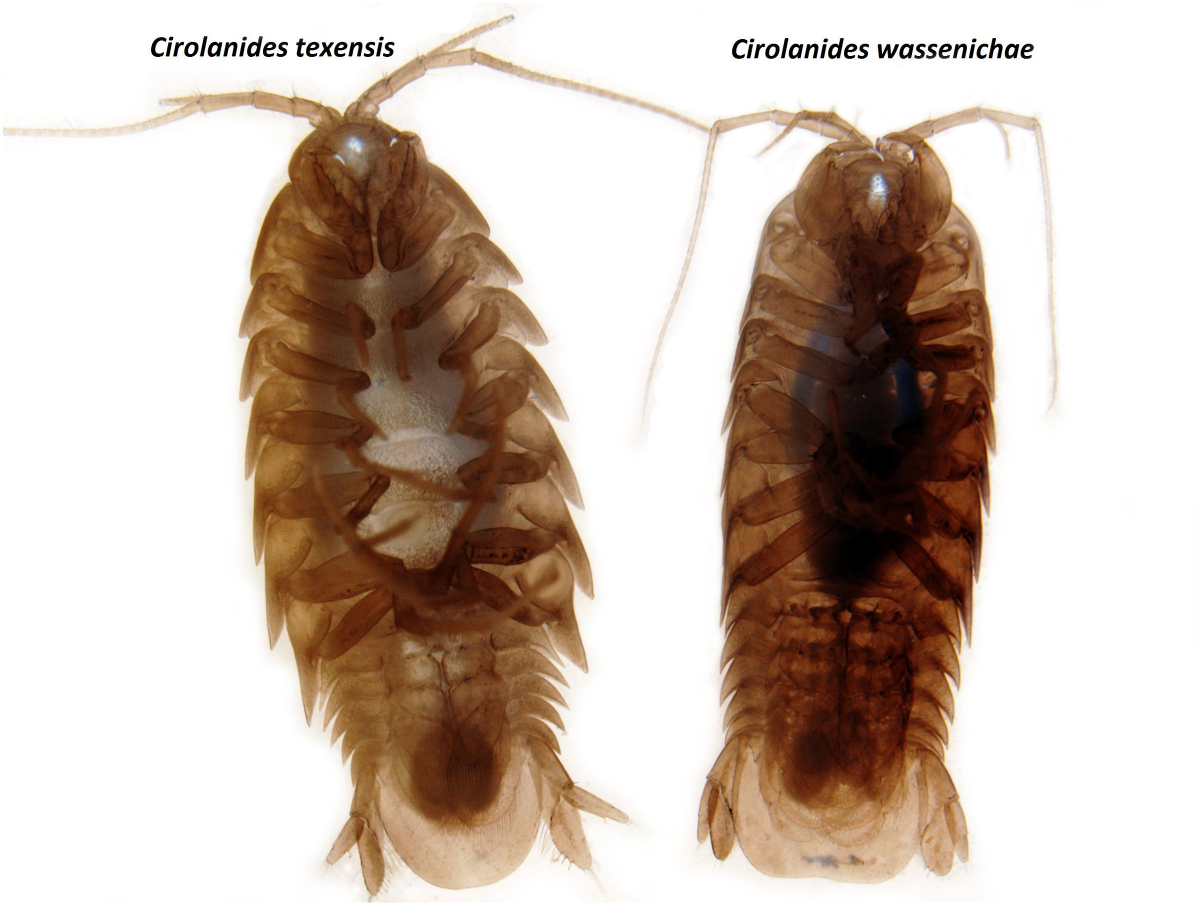

FIGURE 9. Negative photograph of freshly collected *C. texensis* (left) and *C. wassenichae* **sp. nov.** (right, and ♀ type specimen). Both specimens were collected on the same day from their type locality of San Marcos Artesian Well. Negative colors are used to highlight differences in body parts that are otherwise difficult to discern.

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