Subterranean Biology 48: 117–146 (2024)
doi: 10.3897/subtbiol.48.112984
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

A new species of *Stygobromus* Cope, 1872 (Amphipoda, Crangonyctidae) from a hypotelminorheic seepage spring in Washington, D.C., USA

Matthew L. Niemiller[1], Andrew G. Cannizzaro[2], Thomas R. Sawicki[3], David C. Culver[4]

1 *Department of Biological Sciences, The University of Alabama in Huntsville, Huntsville, AL 35899, USA* **2** *Department of Biology, Miami University, Oxford, OH 45056, USA* **3** *Department of Biological Sciences, Florida Agricultural and Mechanical University, Tallahassee, FL 32307, USA* **4** *Department of Environmental Science, American University, Washington, DC 20016, USA*

Corresponding author: Matthew L. Niemiller (matthew.niemiller@uah.edu, cavemander17@gmail.com)

Academic editor: Denis Copilas-Ciocianu | Received 19 September 2023 | Accepted 17 January 2024 | Published 15 February 2024

https://zoobank.org/727D5BAB-70B9-4C58-AEC5-FD873C6248B0

Citation: Niemiller ML, Cannizzaro AG, Sawicki TR, Culver DC (2024) A new species of *Stygobromus* Cope, 1872 (Amphipoda, Crangonyctidae) from a hypotelminorheic seepage spring in Washington, D.C., USA. Subterranean Biology 48: 117–146. https://doi.org/10.3897/subtbiol.48.112984

Abstract

We describe a new species of subterranean amphipod (Amphipoda: Crangonyctidae) in the genus *Stygobromus* from a hypotelminorheic seepage spring at Shepherd Parkway, part of National Capital East Parks, Washington, D.C., USA, part of the National Park System, using both morphological and genetic approaches. The Anacostia Groundwater Amphipod, *S. anacostensis* **sp. nov.** is a member of the *S. tenuis* species group but differs from related congeners based on body size, serrate blade-like edge of both palms of gnathopods 1 and 2, presence of rastellate setae on the posterodistal margin of the carpus of gnathopod 2, and aspects of the second antennae, mandibular palp, pereopods 5–7, uropods 1 and 2, and telson. Moreover, *S. anacostensis* **sp. nov.** is genetically distinct from *S. tenuis* in the Washington D.C. metropolitan area. The description of *S. anacostensis* **sp. nov.** increases the number of described *Stygobromus* species to eight in the Washington D.C. area and highlights the need for continued biodiversity studies, even in regions that have received considerable attention.

Keywords

Amphipod, crustacean, District of Columbia, groundwater, species delimitation, stygobite, stygobiont, subterranean

Introduction

The Holarctic amphipod genus *Stygobromus* Cope, 1872 is comprised of some 137 described stygobiotic (obligate subterranean) species and several undescribed forms mentioned in the literature, with greatest diversity in the Nearctic (Holsinger 1967, 1974, 1978; Holsinger and Sawicki 2016; Cannizzaro et al. 2019). *Stygobromus* diversity is high in groundwater habitats of the Piedmont and Atlantic Coastal Plain of Maryland, Virginia, and the District of Columbia, from which 13 species have been described, respectively (Holsinger 2009; Holsinger et al. 2011; Culver et al. 2012). *Stygobromus* are extraordinarily diverse in hypotelminorheic habitats and associated seepage springs, a shallow subterranean habitat (SSH; Culver et al. 2006; Culver and Pipan 2011, 2014; Pipan et al. 2012) in the lower Potomac River Basin in and near the Washington D.C. metropolitan area where seven species have been documented (Feller 1997; Hobson 1997; Culver and Šereg 2004; Holsinger 2009; Pipan et al. 2012).

Over 150 seepage springs have been identified in the Washington D.C. metropolitan area (Hutchins and Culver 2008; Culver et al. 2012; Keany 2016; Keany et al. 2019). The study and collection of groundwater fauna from these springs continues to improve our understanding on the distribution and ecology of *Stygobromus* spp. and uncover new diversity. Moreover, cryptic genetic variation and diversity is a common finding of phylogenetic studies of subterranean fauna (Zakšek et al. 2009; Niemiller et al. 2012; Hedin 2015), including amphipods (Lefebure et al. 2006; Finston et al. 2007; Bradford et al. 2010; Delic et al. 2017). Niemiller et al. (2018) discovered substantial genetic variation up to 13.7% uncorrected sequence divergence at the mitochondrial cytochrome oxidase subunit 1 (*co1*) locus among populations of the *S. tenuis* species group in the Atlantic Coastal Plain of Virginia and Washington, D.C., indicating the strong potential for cryptic diversity.

Here we describe *S. anacostensis* sp. nov. from a hypotelminorheic seepage spring at Anacostia Park in metropolitan Washington, District of Columbia based on morphological examination and genetic analyses of five loci commonly used in phylogenetic studies of amphipods (e.g., Englisch and Koenemann 2001; Hou et al. 2007, 2011; Kornobis et al. 2011).

Materials and methods

Collection site and approach

Hypotelminorheic habitats and associated seepage springs are shallow subterranean habitats, characterized by small flows of water in slight depressions lined with decaying leaves (Culver et al. 2006; 2012). Seepage springs drain a small area, often less than a hectare, and the habitat only reaches a few meters in depth. The specimens were collected as part of a census of seepage springs in National Capital East (NACE), a unit of the National Park Service. Over 150 seeps were discovered during

this census (Keany et al. 2018). Specimens were preserved in 100% ethanol and stored at -20 °C for molecular analysis. Specimens examined were deposited in the Smithsonian National Museum of Natural History Invertebrate Zoology Collection in Washington, D.C.

Morphological analyses

To enhance the ability to clearly perceive suture lines and setation patterns, prior to dissection, most specimens were digested overnight in 400 µl of Zymo Research 2× digestion buffer, 40 µl of proteinase K and 360 µl of molecular grade water at 37 °C. Specimens were then stained by being placed into a 2% Lignin Pink solution for at least 2 hours. Specimens were dissected using a Leica M125 stereomicroscope (Leica, Wetzlar, Germany). Slide preparations were made by mounting dissected appendages and other body parts in glycerin. These temporary slide mounts were then examined, and drawings of pertinent structures were prepared using a Leica DM 1000 compound microscope outfitted with a drawing tube. Illustrations were finalized for publication in Adobe Illustrator CC. ImageJ software (Abramoff et al. 2004) was used for body length and appendage measurements. Body length was measured as the distance from the rostrum to the base of the telson following the contour of the body. Dissected parts were later transferred to small vials of ethanol for storage and/or future study.

Nomenclature for setal patterns on the third article of the mandibular palps follow Karaman (1969). The following terms are used. "Defining angle" refers to the posterior margin of the palm and the distalmost point of the posterior margin of the propodus, the area where the tip of the dactylus closes on the propodus; and "clothes-pin setae" refers to two notched robust setae present on the basal segments of the pleopod inner rami as illustrated in Holsinger (2009).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted for select specimens of *S. anacostensis* sp. nov. and other members of the *S. tenuis* species group in the Washington, D.C. area (Table 1) using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Germantown, Maryland, USA) following the manufacturer's protocol. We amplified using polymerase chain reaction (PCR) fragments of five loci: 535-bp of mitochondrial cytochrome oxidase subunit 1 (*co1*), 428-bp of mitochondrial 16S rDNA (*16s*), 329-bp of nuclear histone h3 (*h3*), 611-bp of nuclear 18S rDNA and 835-bp of nuclear 28S rDNA (*28s*). PCR primers used in this study are presented in Table 2.

PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and sequenced in both directions using BigDye chemistry at Eurofins Genomics (Louisville, Kentucky, USA). Low quality reads at the ends of forward and reverse sequences were trimmed and ambiguous base calls verified manually by examining electropherograms. Sequences were assembled into contigs using Chromas

Table 1. *Stygobromus* populations sampled in the Washington, D.C., USA area and GenBank accession numbers for five loci used in the current study. Additional sequences downloaded from GenBank are also included.

Sample	n	Species	State	County/ District	Locality	*co1*	*16s*	*18s*	*28s*	*h3*
SP94	8	*Stygobromus anacostensis*	DC	Washington	Malcolm X Ave seep	OR506977–OR506984	OR530101–OR530107	OR530128–OR530135	OR530153–OR530160	OR506374–OR506381
SP100	3	*Stygobromus anacostensis*	DC	Washington	Malcolm X Ave seep	OR506974–OR506976	OR530108–OR530110	OR530136–OR530138	OR530161–OR530163	OR506372–OR506373
SP31	2	*Stygobromus allegheniensis*	WV	Berkeley	Caskey Spring	KY748254	OR530092	OR530121	OR530142	OR506362
SP26	3	*Stygobromus t. potomacus*	VA	Arlington	Pimmit Run Seepage Spring A	KY748251, OR506961–OR506962	OR530090–OR530091	OR530119–OR530120	OR530145–OR530146	OR506363–OR506364
SP80	3	*Stygobromus t. potomacus*	DC	Washington	Upper Kennedy Street Spring, Rock Creek Park	KY748252, OR506963–OR506964	OR530093–OR530094	OR530122–OR530123	OR530149–OR530150	OR506360–OR506361
SP86	3	*Stygobromus hayi*	DC	Washington	West Rapids Spring, Rock Creek Park	KY748253, OR506965–OR506966	OR530099–OR530100	OR530126–OR530127	OR530143–OR530144	OR506358–OR506359
SP101	1	*Stygobromus t. potomacus*	DC	Washington	Shepherd Park seep	OR506971	–	OR530139	–	OR506371
SP102	2	*Stygobromus t. potomacus*	VA	Arlington	Pimmit Run Seepage Spring B	OR506972–OR506973	OR530097–OR530098	OR530140–OR530141	OR530147–OR530148	OR506369–OR506370
SP104	2	*Stygobromus t. potomacus*	VA	King George	Caledon State Park, Site 4	OR506969–OR506970	OR530111–OR530112	OR530115–OR53016	OR530164–OR530165	OR506367–OR506368
SP105	2	*Stygobromus t. potomacus*	VA	King George	Caledon State Park, Site 1	OR506967–OR506968	OR530113–OR530114	OR530117–OR530118	OR530166–OR530167	OR506365–OR506366
SP95	2	*Stygobromus t tenuis*	MD	Harford	Wilkenson Road seep, Susquehana State Park	OR506985–OR506986	OR530095–OR530096	OR530124–OR530125	OR530151–OR530152	OR506356–OR506357
	1	*Stygobromus t. potomacus*	VA	Caroline	Fort A.P. Hill	KU869712	KU869712	–	–	–
	1	*Stygobromus tenuis*	VA	Caroline	Goldenvale Creek	KP693695	–	–	–	–
	1	*Stygobromus allegheniensis*	NY	Ulster	Xanadu Cave, Mohonk Preserve	–	–	–	–	KP696362
	1	*Stygobromus allegheniensis*	NY	Albany	Clarksville Cave	–	–	–	–	KP696363
	1	*Stygobromus allegheniensis*	NY	Ulster	Ice Cave no. 1, Minnewaska State Park Preserve	–	–	–	–	KP696361

Table 2. Loci and associated PCR primers to infer phylogenetic relationships of *Stygobromus* in the current study.

Locus	Name	Genome	Length	Primers	Reference(s)
co1	cytochrome oxidase subunit 1	mtDNA	535	*jgLCO1490* – TITCIACIAAYCAYAARGAYATTGG *jgHCO2198* – TAIACYTCIGGRTGICCRAARAAYCA	Geller et al. (2013)
16s	16S ribosomal DNA	mtDNA	428	*16STf* – GGTAWHYTRACYGTGCTAAG *16Sbr* – CCGGTTTGAACTCAGATCATGT	Palumbi et al. (1991), Macdonald et al. (2005)
18s	18S ribosomal DNA	nuclear	611	*18Sf* – CCTAYCTGGTTGATCCTGCCAGT *18S700r* – CGCGGCTGCTGGCACCAGAC	Englisch and Koenemann (2001)
28s	28S ribosomal DNA	nuclear	835	*28Sf* – TTAGTAGGGGCGACCGAACAGGGAT *28S1000r* – GACCGATGGGCTTGGACTTTACACC	Hou et al. (2007)
h3	histone H3	nuclear	329	*H3f* – AAATAGCYCGTACYAAGCAGAC *H3r* – ATTGAATRTCYTTGGGCATGAT	Corrigan et al. (2014)

v2.6.6 (Technelysium, South Brisbane, Queensland, Australia), then aligned using MUSCLE (Edgar 2004) in the program MEGA v.7.0.26 (Kumar et al. 2016). All new sequences generated during this study were accessioned into GenBank (Table 1). We also included additional sequences available for the *S. tenuis* species group on GenBank accessioned from previous studies (e.g., Aunins et al. 2016; Niemiller et al. 2018; Benito et al. 2021; Table 1).

Phylogenetic analyses

Uncorrected p-distances for each locus were calculated in MEGA. Optimal models of nucleotide substitution for each locus, including first, second, and third codon positions for *co1*, were determined in jModelTest2 (Darriba et al. 2012) using corrected Akaike's Information Criterion (AICc). Five molecular datasets were assessed: *co1*, *16s*, mtDNA (*co1+16s*), nucDNA (*18s+28s+h3*), mtDNA+nucDNA (*co1+16s+18s+28s+h3*). Maximum likelihood (ML) analyses were conducted in RAxML v.8 (Stamatakis 2014). A consensus tree was generated for each dataset using rapid bootstraps for 1,000 replicates under a GTR+Γ model of evolution. Bayesian inference (BI) analyses were conducted in MrBayes v.3.2.6 (Ronquist et al. 2012) using a random starting tree with three heated and one cold chain under a temperature profile of 0.2. BI analyses were run independently twice for 50,000,000 generations and sampled every 1,000 generations under the models of evolution determined by jModelTest2. Stationarity was determined by examining the average standard deviation, assuming stationarity was achieved if the average standard deviation was < 0.005. In general, the first 12.5 million generations (25%) were discarded as burn-in. Convergence of runs was assessed utilizing Tracer v. 1.4 (Rambaut and Drummond 2007). The remaining trees from the stationarity distribution were sampled to generate a 50% majority-rule consensus tree.

Haplotype networks for nuclear loci were constructed using the median-joining network algorithm (Bandelt et al. 1999) using the program PopART v1.7 (Leigh and Bryant 2015).

Species delimitation

We employed three species delimitation approaches on the mtDNA dataset to define molecular operational taxonomic units (MOTUs): Automatic Barcode Gap Discovery (ABGD; Puillandre et al. 2012) and Poisson Tree Processes (PTP; Zhang et al. 2013), and Multi-rate Poisson Tree Processes (mPTP; Kapli et al. 2017). ABGD partitions sequences into candidate species based on a statistically inferred barcode gap defined as a significant disparity between pairwise genetic distances, presumably between intraspecific and interspecific distances. This process is applied recursively to newly obtained groupings of sequences to assess the potential of internal division. This method was employed excluding outgroup taxa via the ABGD web server (http://wwwabi.snv.jusieu.fr/public/abgd/abgdweb.html) using the Kimura two-parameter (Kimura 1980) model with a standard X (relative gap width) = 1.5. The initial development of the multispecies coalescent PTP model assumed one exponential distribution for speciation events and one for all coalescent events (Zhang et al. 2013). The mPTP approach fits speciation events for candidate species to a unique exponential distribution (Kapli et al. 2017) rather than assuming one exponential distribution for speciation events and one for all coalescent events in PTP models (Zhang et al. 2013). Both the PTP and mPTP methods were employed using rooted ML trees for each dataset for 10 million generations, with a burn-in discarding the first 25% in mptp (Kapli et al. 2017).

Conservation assessment

We conducted IUCN Red List and NatureServe conservation assessments following IUCN (2001) and Master et al. (2009). Both assessments rank taxa into one of seven unique categories on a continuum of increasing extinction risk. Risk categories were calculated using the RAMAS Red List 3.0 (Akcakaya et al. 2007) and the NatureServe Rank Calculator v3.186 (Faber-Langendoen et al. 2012) for the IUCN Red List and NatureServe assessments, respectively. Geographic range size was calculated using two different measures for the extent of occurrence (EOO) and area of occupancy (AOO).

Results

Class Crustacea Brünnich, 1772
Order Amphipoda Latreille, 1816
Infraorder Gammarida Latreille, 1802
Superfamily Crangonyctoidea Bousfield, 1973

Family Crangonyctidae Bousfield, 1973; emended by Holsinger, 1977
Genus *Stygobromus* Cope, 1872

***Stygobromus anacostensis* Cannizzaro, Sawicki, & Niemiller, sp. nov.**
https://zoobank.org/D66AA3F8-A53B-41A2-B16C-CA9486A39BC1
Figs 1–8

Type material. *Holotype*: male 5.9 mm, from USA, Washington, District of Columbia, Anacostia Park, (38.83059°N, -76.9995°W), deposited in the collection of the United States National Museum of Natural History, Smithsonian Institution, Washington, D.C (USNM 1606902); female allotype 5.3 mm (USNM 1606903). Holotype male and female allotype collected 18 October 2021 by Lizzy Sartain.

***Paratypes*:** 1 male (USNM 1606904) and 2 females (USNM 1606905–1606906) collected on 18 October 2021 by Lizzy Sartain from type locality; 1 female collected on 20 September 2021 by Lizzy Sartain from the type locality (USNM 1606907).

Etymology. The specific epithet *anacostensis* refers to its occurrence in Anacostia neighborhood in Washington, D.C., USA. It is part of the Anacostia River drainage.

Type locality. USA. Washington, District of Columbia, hypotelminorheic seepage spring in a highly urbanized area that emerges from a small, 2-m high rockface ca. 5 m from Malcolm X Avenue SE in Shepherd Parkway (Figs 9, 10, 38.83059°N, -76.9995°W). Shepherd Parkway is part of National Capital Parks East. Most individuals were collected in the water flowing over moss-covered rocks. A few individuals were also present in decaying leaves at the base, a more usual hypotelminorheic habitat (Culver et al. 2006, 2012). The site is at the extreme tip of Shepherd Parkway, a unit of National Capital East (National Park Service). The width of park land is about 20 m and is bordered by Malcolm X Avenue. The site was discovered when a park ranger noticed extensive ice on the adjacent sidewalk resulting from flow from the seep.

Diagnosis. Small stygomorphic species distinguished from other members of the *tenuis* group by size, largest male 5.9 mm, largest female 5.3 mm and as follows: *S. tenuis tenuis* – by antenna 2 subequal to or shorter than antenna 1; *S. tenuis potomacus* – only 2 C-setae on mandibular palp and up to 8 E-setae; *S. allegheniensis* – pereopods 5–7 basis posterior margin weakly convex, and telson tapering distally; *S. hayi* – by significantly less spinose uropods 1 and 2, and telson with significantly fewer apical robust setae. Further distinguished from all *tenuis* group species by gnathopods 1 and 2 with a serrate blade-like edge running the length of both palms, and by the posterodistal margin of gnathopod 2 carpus possessing rastellate seta(e).

Description. Male: holotype, USNM 1606902 (Fig. 1A); Size 5.9 mm.

***Antennae*.** Antenna 1 (Fig. 2A): 45% body length, 60% length of antenna 2 (in paratype (USNM 1606904); primary flagellum with 18 segments, aesthetascs on most segments, aesthetascs shorter than respective segments; accessory flagellum 2-segmented, reaching beyond first segment in length.

Antenna 2 (Fig. 2B): damaged in holotype, description based on paratype (USNM 1606904); gland cone distinct; peduncle 80% length of flagellum, with weak plumose

Figure 1. *Stygobromus anacostensis* sp. nov., habitus: **A** holotype male, 5.9 mm (USNM 1606902) **B** Allotype female, 5.3 mm (USNM 1606903). Scale bar: 1 mm.

setules concentrated on postero and anterodistal margins of segments 4 and 5, peduncle segment 4 subequal in length to 5; flagellum 12-segmented, segment 5 with robust seta on anterodistal margin and segments 6 and 7 with robust seta placed along posterodistal margins, small calceoli-like structures apically on flagellar segments 5–12.

Mouthparts (Figs 2C, D, 3). Mandibles: left mandible (Fig. 2C) incisor 5-dentate, lacinia mobilis 5-dentate, with 7 robust serrate and numerous plumose accessory setae; molar process reduced with simple seta; palp with 3 segments, second segment 85% length of third, with inner margin bearing 8 setae and sparse fine setae; segment 3 with 2 C-setae, 5 E-setae, 1 B-seta, and 8 plumose D-setae, lacking A-setae; face of article covered in numerous, fine pubescent setae.

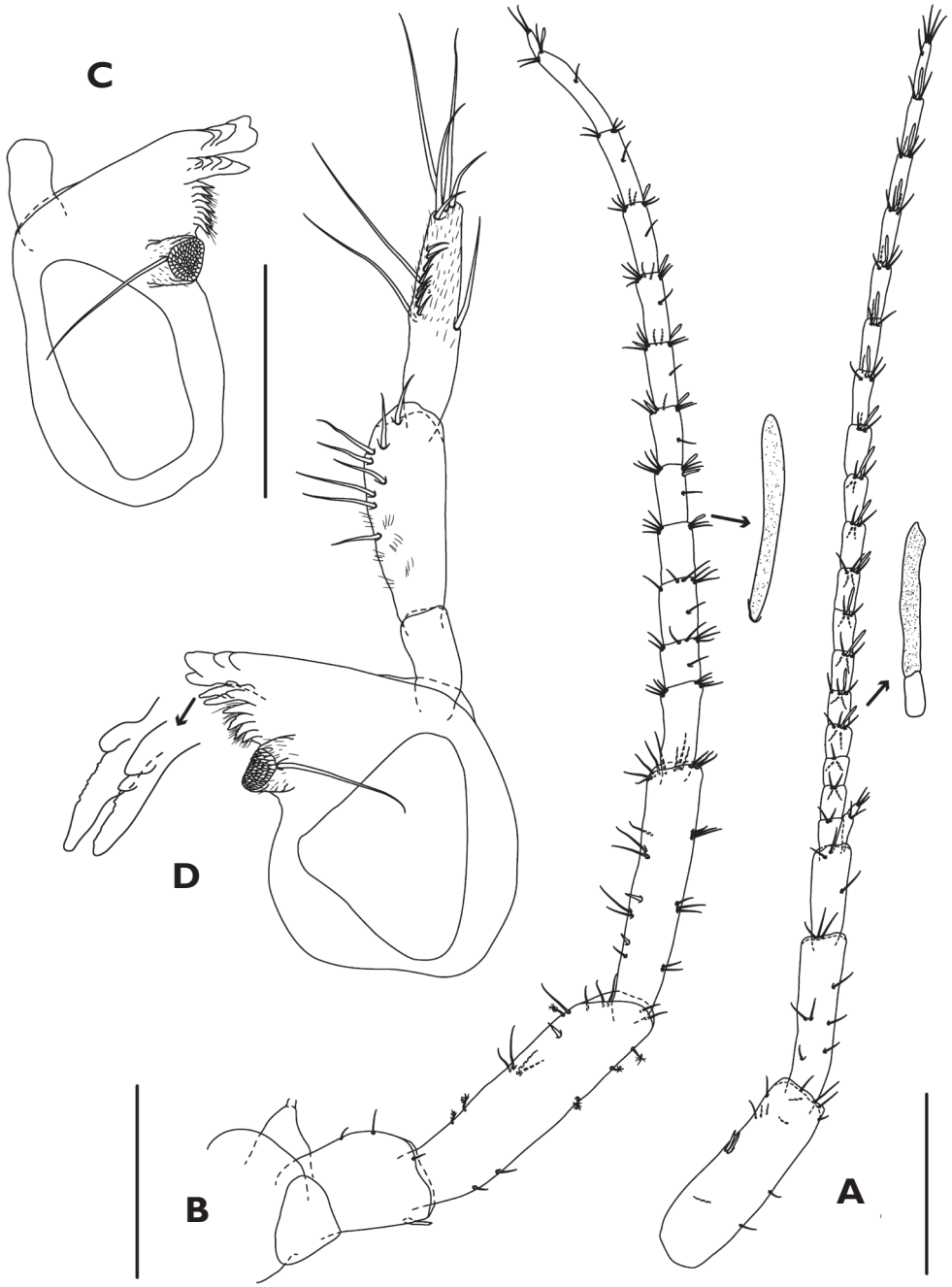

Figure 2. *Stygobromus anacostensis* sp. nov., Holotype male, 5.9 mm (USNM 1606902): **A** antenna 1 (single aesthetasc enlarged) **C** left mandible (palp omitted) **D** right mandible (lacinia mobilis enlarged). Paratype male, 5.7 mm (USNM 1606904): **B** antenna 2 (single calceolus enlarged). Scale bars: 0.5 mm (**A, B**); 0.25 mm (**C, D**).

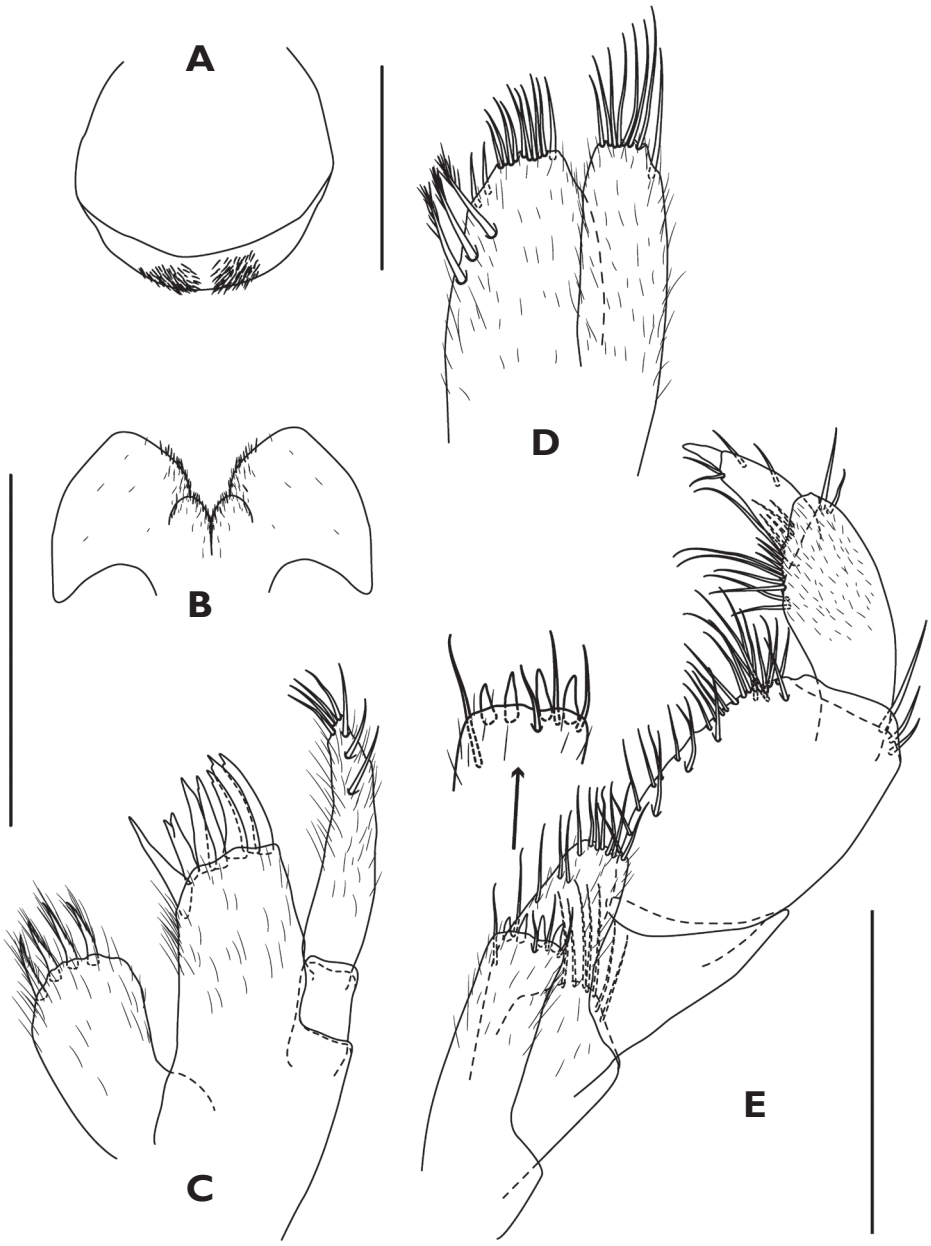

Figure 3. *Stygobromus anacostensis* sp. nov., Paratype male, 5.7 mm (USNM 1606904): **A** upper lip **D** maxilla 2. Holotype male, 5.9 mm (USNM 1606902): **B** lower lip **C** maxilla 1 **E** maxilliped (distal margin of inner plate enlarged). Scale bars: 0.25 mm.

Right Mandible (Fig. 2D): incisor 4-dentate, lacinia mobilis bifurcate, both lobes with numerous protuberances; accessory setae row with 4 robust, serrate setae and numerous plumose setae; molar process reduced with simple seta. Palp with 3 articles, relative articles length and setation patterns as in left mandible.

Upper Lip (Fig. 3A): rounded, apical margin of labrum with numerous fine setae. Lower Lip (Fig. 3B): inner lobes distinct; outer margin of outer lobe sparsely covered in fine setae; inner margin of outer lobe heavily setose.

Maxilla 1 (Fig. 3C): missing in holotype, description based on paratype (USNM 1606904); inner plate with 4 plumose marginal setae and numerous fine, pubescent setae covering entire plate; outer plate with 7 apical comb spines, pubescence covering inner margin, decreasing laterally and proximally; palp with 2 segments, distal segment covered in pubescence; subapical margin of distal article with 3 long setae, apical margin with 4 setae.

Maxilla 2 (Fig. 3D): missing in holotype, description based on paratype (USNM 1606904); both inner and outer plates covered in pubescent setae; outer plate not as wide as inner plate, not narrowing distally, with numerous distal setae; inner plate narrowing slightly distally, with numerous apical setae and 3 large plumose facial setae.

Maxilliped (Fig. 3E): inner plate shorter than outer plate, with 4 naked cuspidate setae 3 setae along apical margin, surface of plate covered in fine pubescence; outer plate armed with numerous setae covering inner and apical margins; palp second segment with numerous marginal setae, third article with numerous marginal/submarginal setae; dactyl with 2 outer setae and 2 inner setae.

Gnathopods. Gnathopod 1 (Fig. 4A): coxal plate with 3 apical setae; basis with long setae inserted along anterior, and posterior margins, small patch of pubescence on posterodistal corner; ischium with 4 setae and pubescence along posterior margin; merus weakly pubescent along posterior surface, numerous distal setae, and robust seta along anterior margin; carpus approximately 50% length of propodus with robust seta along anterior margin and a group of setae on anterodistal margin, one of which is approximately 50% length of propodus, posterior margin with single group of plumose setae and 6 submarginal setae directed distally; propodus 1.3× longer than broad, with 1 marginal anterior seta, 4 superior medial setae, with middle group of medial setae paired, 4 setae inserted at anterodistal corner, 6 inferior medial setae and numerous plumose posterior setae; palm oblique, concave, with serrate blade-like edge running the length, 7 outer and 7 inner bifid robust setae, 6 outer setae, and inner seta; inner margin of defining angle with 3 bifid robust setae, outer margin with 4 bifid robust setae; dactylus with outer seta and 7 short setae covering the entire inner margin and 3 setae placed along the inner margin at base of nail.

Gnathopod 2 (Fig. 4B): coxal plate with 4 apical setae and facial seta; basis with long setae inserted along anterior, and posterior margins, small patch of pubescence on posterodistal corner; ischium with 3 setae and pubescence along posterior margin; merus with pubescence covering posterior surface and 4 posterodistal setae and robust seta along anterior margin; carpus approximately 75% length of propodus, with robust seta along anterior margin and two setae on anterodistal margin, one of which is approximately 33% length of propodus, posterior margin with 4 groups of plumose setae, distal-most bearing 3 rastellate setae, and 3 submarginal setae directed distally; propodus 1.3× longer than broad, with marginal anterior seta, 5 superior medial setae, distal-most paired, 5 setae inserted at anterodistal corner, 5 inferior medial setae, proximal-most paired, and 8 groups of plumose setae along posterior margin; palm

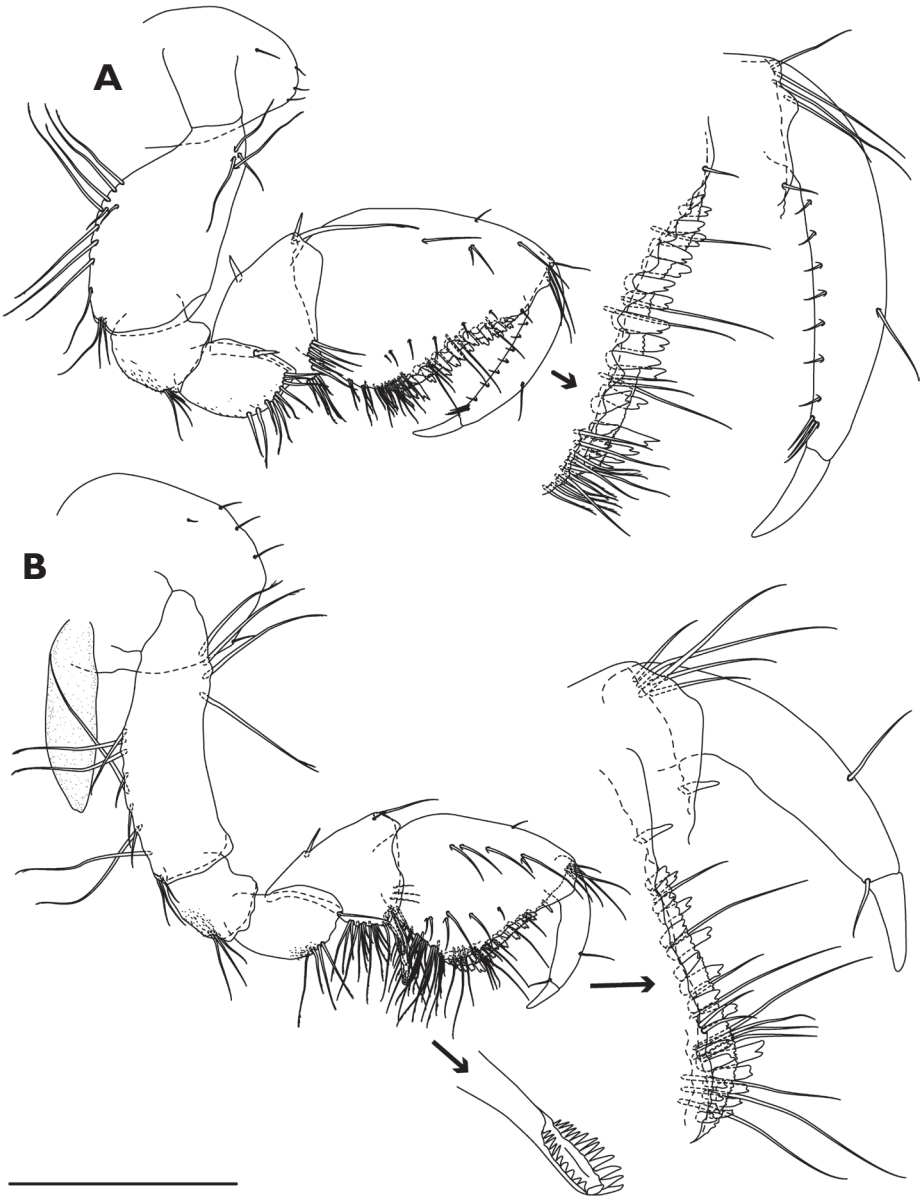

Figure 4. *Stygobromus anacostensis* sp. nov., Holotype male, 5.9 mm (USNM 1606902): **A** gnathopod 1 (palm and dactyl enlarged) **B** gnathopod 2 (rastellate seta, palm and dactyl enlarged). Scale bar: 0.5 mm.

oblique, straight, with serrate blade-like edge running the length, 5 outer and 5 inner bifid robust setae, 5 outer setae, and 2 inner setae; inner margin of defining angle with 6 bifid robust setae, outer margin with 5 bifid robust setae; dactylus with outer seta and seta placed along the inner margin at base of nail.

Pereopods. Pereopod 3 (Fig. 5A): coxal plate with 5 apical setae; merus 1.4× longer than carpus, carpus approximately 85% of propodus in length; dactylus approximately

50% length of propodus, with plumose seta on posterior margin, 2 setae along anterior margin followed by thin seta on medial margin.

Pereopod 4 (Fig. 5B): subequal to pereopod 3 in length; coxal plate armed with 4 anterior and 3 posterior apical setae; merus approximately 1.6× longer than carpus; carpus approximately 60% length of propodus; dactylus approximately 40% length of propodus, setation as in pereopod 3.

Pereopod 5 (Fig. 5C): coxal plate large, bilobate with distinct anterior and posterior lobes, posterior lobe with 4 robust setae on distal corner; basis posterior margin weakly convex with 9 shallow serrations, anterior margin with 6 split-tipped robust setae and 3 distal split-tipped setae; merus subequal in length to carpus; carpus subequal to propodus, dactylus approximately 50% length of propodus, setation as in pereopod 4.

Pereopod 6 (Fig. 5D): coxal plate bilobate, with weakly produced anterior lobe, posterior lobe bearing 2 robust apical setae; basis posterior margin weakly convex with 8 serrations, anterior margin with 5 split-tipped robust setae, and 3 robust setae at anterodistal corner; merus approximately 1.2× length of carpus; carpus approximately 90% of propodus in length, dactylus approximately 50% length of propodus, setation as in pereopod 5.

Pereopod 7 (Fig. 5E): coxal plate small, subtriangular, with 4 posterior setae; basis posterior margin weakly convex with 10 serrations and straight distal corner, anterior margin with 8 split-tipped robust setae, and 2 robust setae at anterodistal corner; merus subequal in length to carpus; carpus approximately 80% length of propodus, dactylus approximately 40% length of propodus, setation as in pereopods 5, 6.

Gills (Fig. 5F). coxal gills on somites 2–6, somites 6 and 7 with bifurcate sternal gills.

Pleon. Epimera (Fig. 6A): first epimeron ventral margin with robust seta, distoposterior corner rounded, posterior margin with 2 setae. Second epimeron ventral margin with 3 robust setae, distoposterior corner rounded, posterior margin with 2 setae. Third epimeron ventral margin with 3 robust setae, distoposterior corner rounded, posterior margin with 2 setae.

Pleopods: pleopod 1 (Fig. 6B) peduncle lacking setae, with 2 coupling hooks; outer, inner rami with 8 and 11 segments respectively, basal segment of outer ramus with clothes-pin setae. Pleopod 2 peduncle lacking setae, with 2 coupling hooks; outer, inner rami with 7, 11 segments respectively, basal segment of outer ramus with clothes-pin setae. Pleopod 3 outer, inner rami with 7, 7 segments respectively, basal segment of outer ramus with clothes-pin setae.

Urosome. Mostly bare, with sparse setae covering dorsal surface. Uropod 1 (Fig. 6C): peduncle 1.4× inner ramus in length, with 8 outer robust setae and inner robust seta(e), posteromedial margin with distinct protuberance approximately 20% of inner ramus in length, dorsal margin weakly serrate; outer ramus approximately 80% length of inner, with 2 inner and outer robust setae and 4 apical robust setae; inner ramus possessing 3 outer and two inner robust setae, and 5 apical robust setae.

Uropod 2 (Fig. 6D): peduncle subequal in length to inner ramus, with 2 outer robust setae and inner robust seta; outer ramus approximately 88% length of inner ramus without robust setae along the inner and outer margins, and 4 apical robust setae; inner ramus with 2 outer and 2 inner robust setae, with 5 apical robust setae.

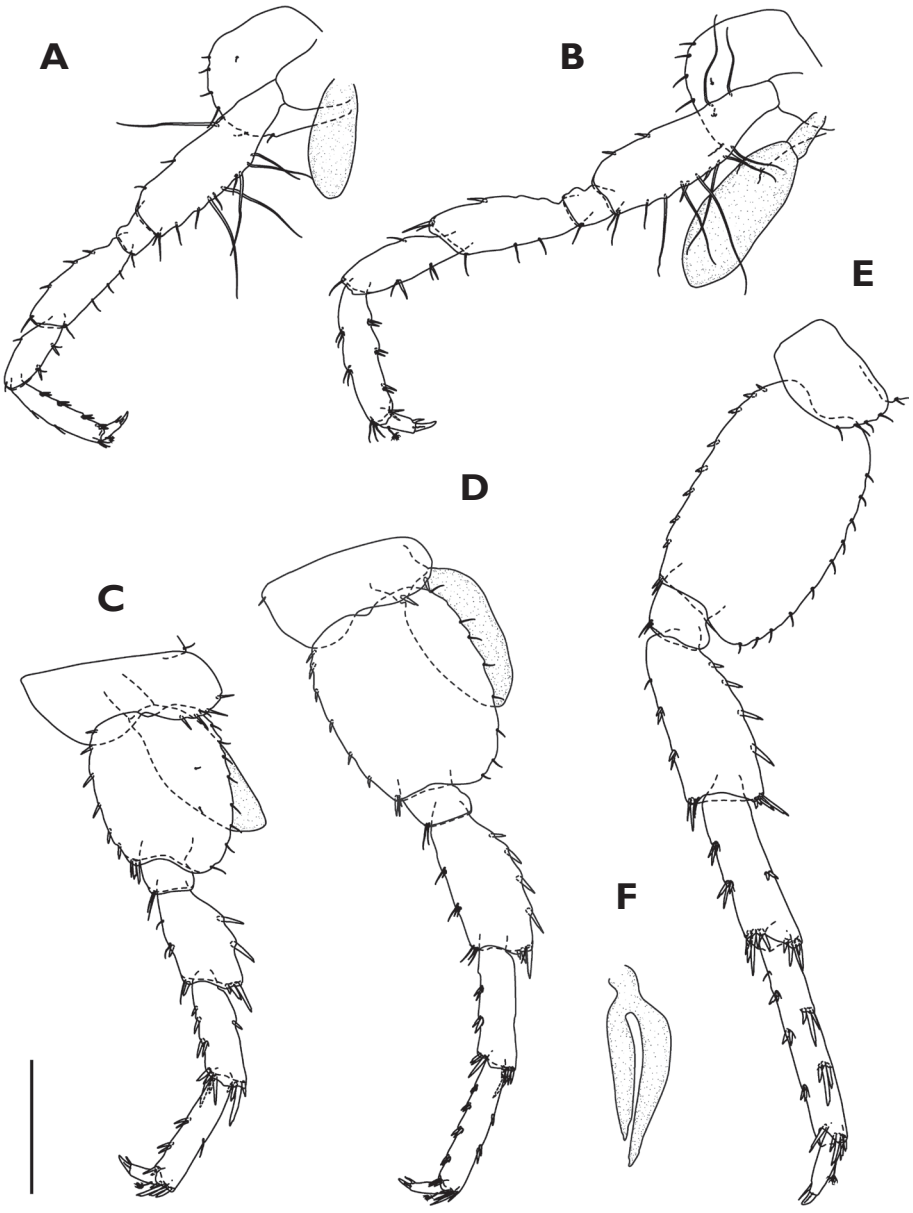

Figure 5. *Stygobromus anacostensis* sp. nov., Holotype male, 5.9 mm (USNM 1606902): **A** pereopod 3 **B** pereopod 4 **C** pereopod 5 **D** pereopod 6 **E** pereopod 7 **F** bifurcate sternal gill located on somites 6 and 7. Scale bar: 0.5 mm.

Uropod 3 (Fig. 6E): small, shorter than telson, uniramous; peduncle 2× length of ramus; ramus with 3 apical robust setae.

Telson (Fig. 6F). Telson entire, elongated, 1.5× longer than broad, weakly tapering distally; apex with 10 robust setae, and plumose seta, 2 plumose setae arise dorsolaterally from both outer margins.

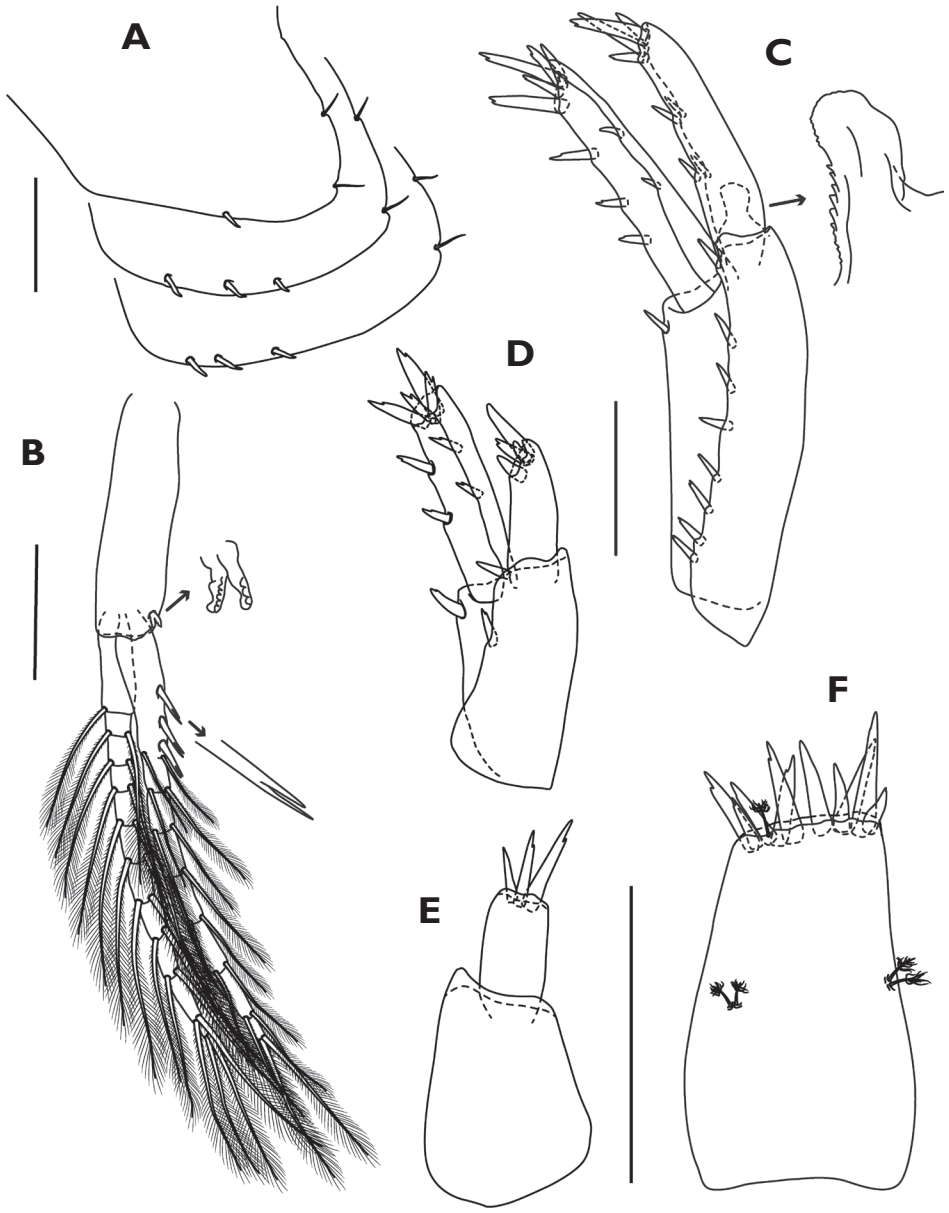

Figure 6. *Stygobromus anacostensis* sp. nov., Holotype male, 5.9 mm (USNM 1606902): **A** epimera 1–3 **B** pleopod 1 (coupling spines and clothes pin seta enlarged) **C** uropod 1 (posteromedial protuberance enlarged) **D** uropod 2 **E** uropod 3 **F** telson. Scale bars: 0.25 mm.

Female: allotype USNM 1606903 (Fig. 1B); Size 5.3 mm. Differing from male in several points, including, antennae; gnathopod shape and setation; uropods 1 and 2 shape and setation. Structures not described below are as in male.

***Antennae*.** Antenna 1 (not illustrated, but see Fig. 1B): 50% body length, 1.5× longer than antenna 2; peduncle, flagellum lacking robust setae; primary flagellum

with 16 segments. Antenna 2 (Fig. 7A): gland cone distinct; peduncle 1.5× longer than flagellum, with robust setae anteriorly, laterally on segments 3, 4, peduncle segment 4 subequal in length to segment 5; flagellum 7-segmented, without small calceoli-like structures apically on distal flagellar segments.

Gnathopods. Gnathopod 1 (Fig. 7B): coxal plate with 3 apical and 2 facial setae; ischium with 2 setae and pubescence along posterior margin; carpus approximately 40% length of propodus with robust seta along anterior margin and a group of setae on anterodistal margin, one of which is approximately 50% length of propodus, posterior margin with single group of plumose setae and 4 submarginal setae directed distally; propodus 1.25× longer than broad, with 1 marginal anterior seta, 3 superior medial setae, 3 setae inserted at anterodistal corner, 3 inferior medial setae and numerous plumose posterior setae; palm oblique, straight, with serrate blade-like edge running the length, 5 outer and 5 inner bifid robust setae, 4 outer setae, and inner seta; inner margin of defining angle with 3 bifid robust setae, outer margin with 4 bifid robust setae; dactylus with outer seta and 4 short setae covering the inner margin and 2 setae placed along the inner margin at base of nail.

Gnathopod 2 (Fig. 7C): coxal plate with 6 apical setae and 2 facial setae; ischium with 2 setae and pubescence along posterior margin; merus with pubescence covering posterior surface and 4 posterodistal setae, without robust seta along anterior margin, and two robust setae along distal margin; carpus subequal in length to propodus, with robust seta along anterior margin and two setae on anterodistal margin, one of which is approximately 33% length of propodus, posterior margin with 3 groups of plumose setae, distal-most bearing rastellate seta, and 3 submarginal setae directed distally; propodus 1.1× longer than broad, with marginal anterior seta, 3 superior medial setae, 4 setae inserted at anterodistal corner, 4 inferior medial setae, and 5 groups of plumose setae along posterior margin; palm oblique, straight, with serrate blade-like edge running the length, 3 outer and 3 inner bifid robust setae, 3 outer setae, and 1 inner seta; inner margin of defining angle with 4 bifid robust setae, outer margin with 4 bifid robust setae; dactylus with outer seta and 4 short setae covering the inner margin and seta placed along the inner margin at base of nail.

Gills and brood plates. Gills as in male with coxal gills on somites 2–6, somites 6 and 7 with bifurcate sternal gills (Fig. 8A illustrates somite 7). Brood plates early in development in allotype, present on somites 2–5.

Urosome. Uropod 1 (Fig. 8B): peduncle 1.5× length of inner ramus, with 6 outer robust setae and inner robust seta(e), posteromedial margin lacking protuberance; outer ramus approximately 90% length of inner, with 1 inner and outer robust seta, and 4 apical robust setae; inner ramus possessing 2 outer and inner robust seta(e), and 5 apical robust setae.

Uropod 2 (Fig. 8C): peduncle subequal in length to inner ramus, with 2 outer robust setae and inner robust seta; outer ramus approximately 66% length of inner ramus with outer robust seta, and 4 apical robust setae, inner robust setae lacking.

Uropod 3 (Fig. 8D): small, shorter than telson, uniramous; peduncle 1.5× length of ramus; ramus with 4 apical robust setae.

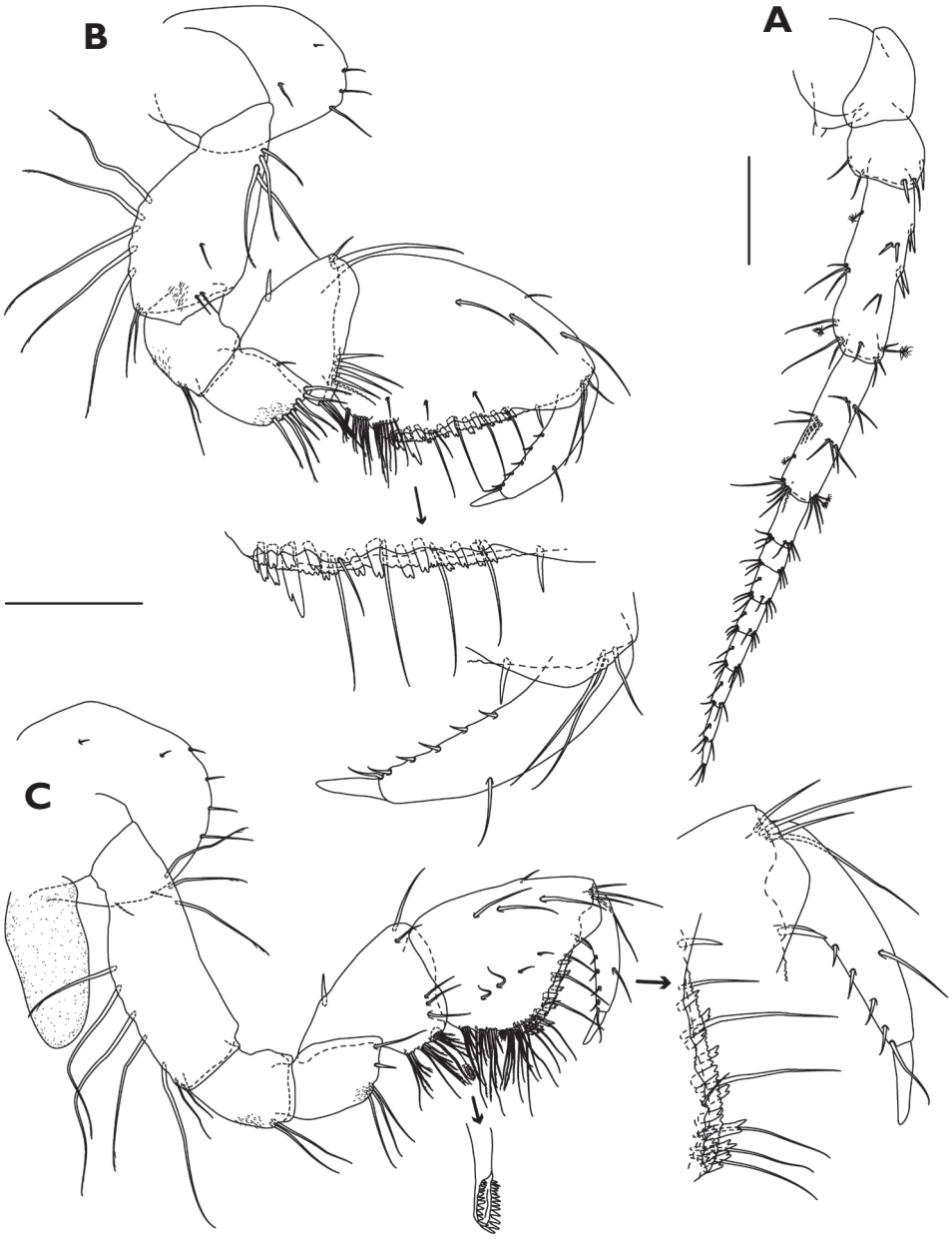

Figure 7. *Stygobromus anacostensis* sp. nov., Allotype female, 5.3 mm (USNM 1606903): **A** antenna 2 **B** gnathopod 1 (palm and dactyl enlarged) **C** gnathopod 2 (rastellate seta, palm and dactyl enlarged). Scale bars: 0.25 mm (**A**); 0.5 mm (**B, C**).

Telson (Fig. 8E). Telson entire, elongated, 1.5× longer than broad, weakly tapering distally; apex with 9 robust setae, 2 plumose setae arise dorsolaterally from both outer margins.

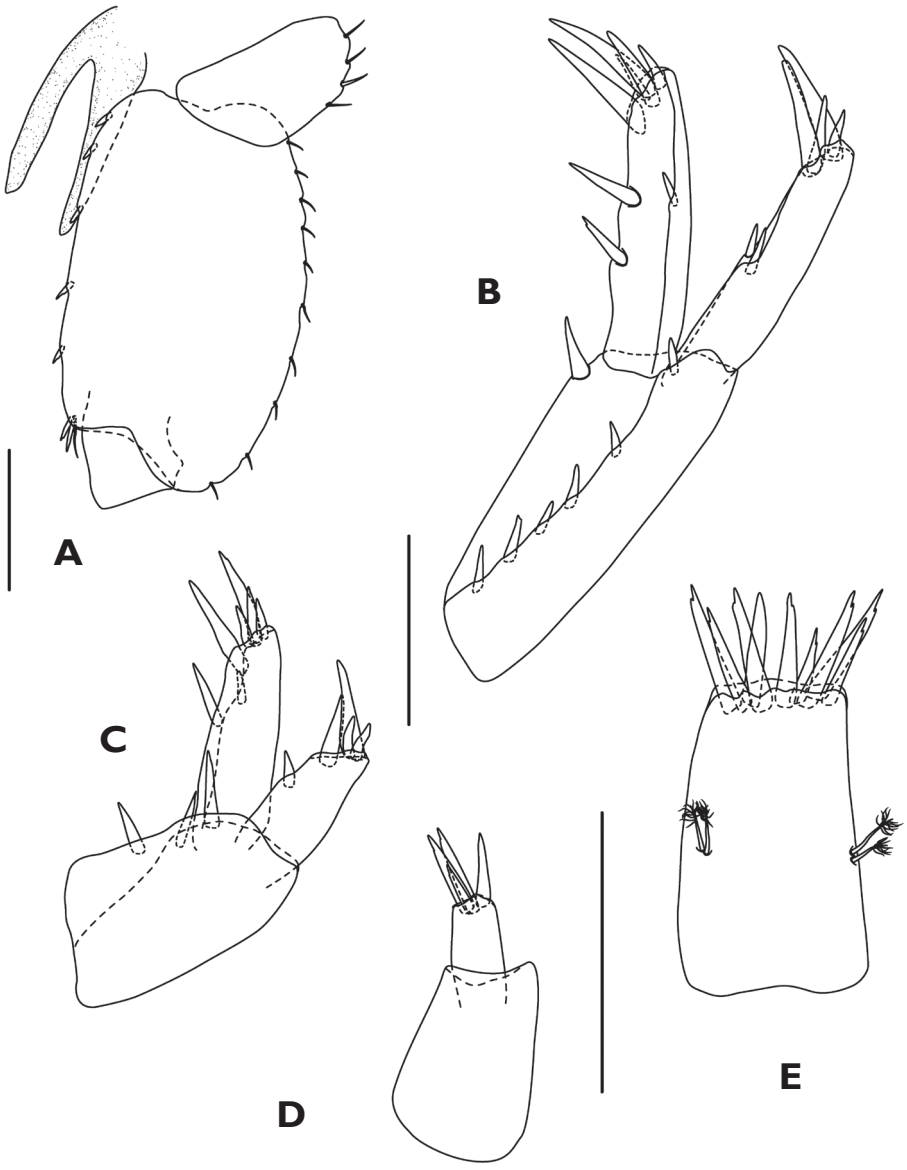

Figure 8. *Stygobromus anacostensis* sp. nov., Allotype female, 5.3 mm (USNM 1606903): **A** coxa and basis of pereopod 7 showing placement of bifurcate sternal gill **B** uropod 1 **C** uropod 2 **D** uropod 3 **E** telson. Scale bars: 0.5 mm (**A–C**); 0.25 mm (**D, E**).

Variation. The new species was shown to vary slightly in several morphological characteristics, particularly between males and females (Table 3).

Molecular diagnosis. Average uncorrected pairwise genetic distance at the mitochondrial *co1* locus between *S. anacostensis* and the most closely related populations of *S. t. potomacus* sampled at Caledon State Park is 6.5%, with 32 fixed mutations separating the two taxa. Between *S. anacostensis* and the closest *S. t. potomacus* population

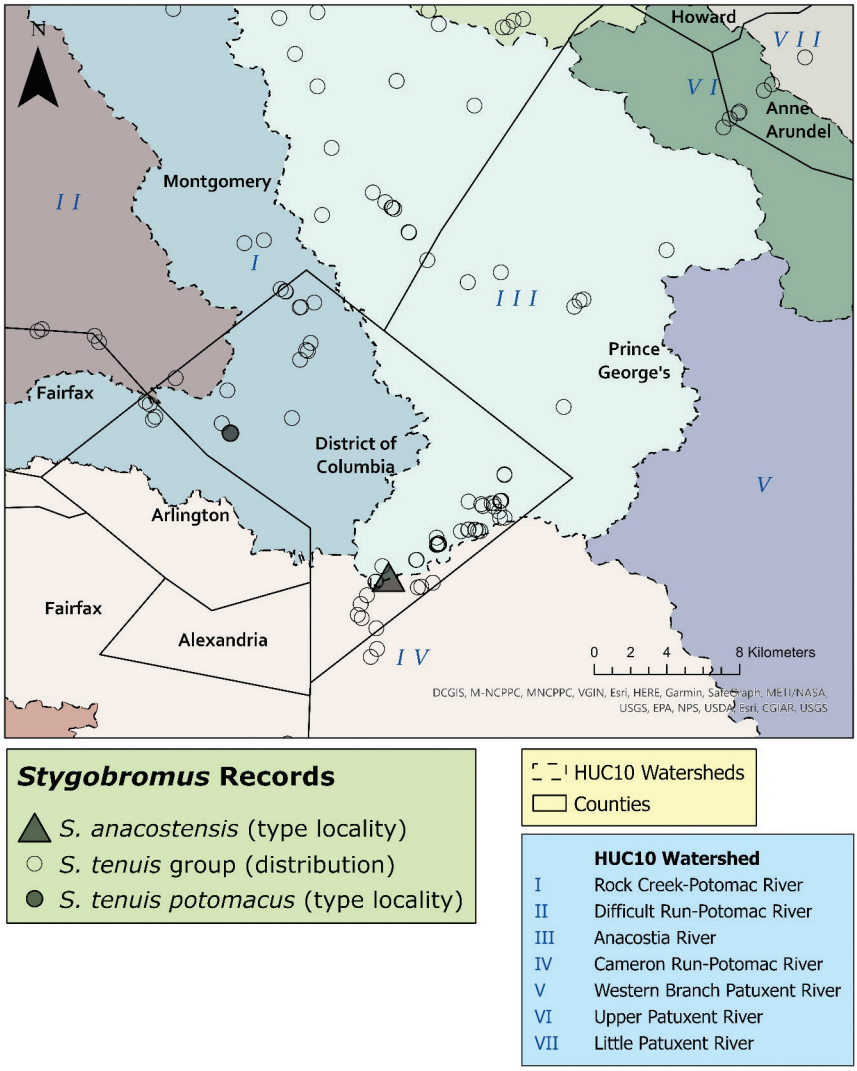

Figure 9. Distribution of *Stygobromus anacostensis* sp. nov. and other *S. tenuis* group species in the Washington D.C. area, USA

(seepage spring near Malcolm X Ave in Anacostia Park; SP101), p-distance is 12.6%, with 67 fixed mutations. P-distance at the mitochondrial *16s* locus between *S. anacostensis* and the populations of *S. t. potomacus* sampled at Caledon State Park is 2.3%, with eight fixed mutations. Nuclear loci exhibited low levels of variation among all *S. tenuis* species group taxa sampled; however, some diagnostic genetic variation was noted. Two fixed mutations in the *h3* locus and one fixed mutation in the *18s* locus exist between *S. anacostensis* and the closest *S. t. potomacus* population (SP101).

Geographical distribution. The species is known to date only from the type locality in Shepherd Parkway, which is a 1200-acre national park located on the southern

Table 3. Variation in morphological characters among select specimens of *Stygobromus anacostensis* sp. nov. examined.

Character	Holotype Male USNM 1606902	Paratype Male USNM 1606904	Allotype Female USNM 1606903	Paratype Female USNM 1606906	Paratype Female USNM 1606905
Body size	5.9 mm	6.7 mm	5.3 mm	4.8 mm	4.8 mm
Antenna 1					
Flagellar segments	18	21	16	16	12
Accessory flagellum	> than 1st flagellar segment	> than 2nd flagellar segment	> than 1st flagellar segment	> than 2nd flagellar segment	sub equal to second flagellar segment
Antenna 2					
Peduncle Segments 4 to 5 length	1.04×	97%	1.15×	1.12×	1.13×
Flagellar segments	unknown	12	7	7	7
Left Mandible					
Palp segment 2 setae	8	7	5	3	4
E-setae	5	5	5	4	3
D-setae	8	8	8	8	5
Right Mantible					
Palp 2nd segment setae	8	5	5	4	4
E-setae	5	5	4	4	3
D-setae	8	8	8	7	5
Maxilla 1					
Inner plate marginal setae	unknown	4	4	3	3
Palp subapical, apical setae	unknown	3, 4	2, 4	2, 4	4, 2
Maxilla 2					
Mx 2 inner plate facial setae	unknown	3	2 or 3	2 or 3	2
Ganthopod 1					
Ischium posterior setae	4	4	2	3	3
Carpus to propdus length	40%	40%	43%	46%	45%
Carpus submarginal setae	6	5	4	4	5
Propodus superior, inferior medial setae	4, 6	2, 4	3, 3	3, 3	2, 4
Palm inner, outer bifid setae	7, 7	7, 6	5, 5	4, 4	5, 3
Ganthopod 2					
Coxal plate apical, facial setae	4,1	4,1	6,2	4,1	3,0
Ischium posterior setae	3	4	2	2	3
Merus anterior margin robust seta	1	1	0	0	0
Carpus to propdus length	71%	64%	82%	79%	84%
Carpus rastellate setae	3	3	1	3	0
Propodus superior, inferior medial setae	5, 5	3, 4	3, 4	3, 3	3, 3
Palm inner, outer bifid setae	5, 5	5, 6	3, 3	3, 3	3, 4
Dactylus inner setae	1	1	5	4	1
Pereopod 5					
Coxal plate anterior apical setae	0	0	2	1 to 2	2
Basis posterior serrations	9	11	8	8	8
Pereopod 7					
Coxal plate posterior apical setae	4	3	4	3	2
Basis anterior setae	8	7	5	5	4
Epimera					
Epimeron 2 ventral, posterior setae	3, 2	unknown	3, 2	2, 2	2, 4

Character	Holotype Male USNM 1606902	Paratype Male USNM 1606904	Allotype Female USNM 1606903	Paratype Female USNM 1606906	Paratype Female USNM 1606905
Uropods					
Uropod 1 peduncle outer, inner setae	8, 1	10, 1	6, 1	9, 1	8, 1
Uropod 2 peduncle outer, inner setae	2, 2	3, 1	2, 1	2, 1	2, 1
Uropod 2 outer ramus outer, inner setae	0, 0	1, 0	1, 0	1, 0	0, 0
Uropod 2 outer ramus apical setae	4	3	4	4	5
Uropod 2 inner ramus outer, inner setae	2, 2	2, 1	1, 1	1, 1	1, 1
Uropod 3 ramus setae	3	4	4	3	2
Telson apical robust setae	10	10	9	9	8

Figure 10. The type locality of *S. anacostensis* is a small hypotelminorheic seepage spring just off of Malcolm X Avenue, Shepherd Parkway, Washington, D.C., USA. Photograph by Jenna Keany.

bank of the Anacostia River just upstream from where the river flows into the Potomac River (Fig. 9). Shepherd Parkway is part of National Capital Parks East (NACE).

Habitat and ecology. Like other species of *Stygobromus*, *S. anacostensis* is a stygobiotic species occurring in groundwater habitats. All specimens have been collected from a seepage spring just off Malcolm X Avenue SE that marks the resurgence of hypotelminorheic groundwater at the surface (Fig. 10). Amphipods have been observed and collected from underneath moss-covered rocks, moss, and leaf litter on the small, 2-m high rockface as well as the small pool of the seepage spring. The seepage spring possesses water throughout most of the year Little is known regarding the ecology and life history currently. *Stygobromus anacostensis* co-occurs with the groundwater isopod *Conasellus* (=*Caecidotea*) *kenki* (Bowman, 1967).

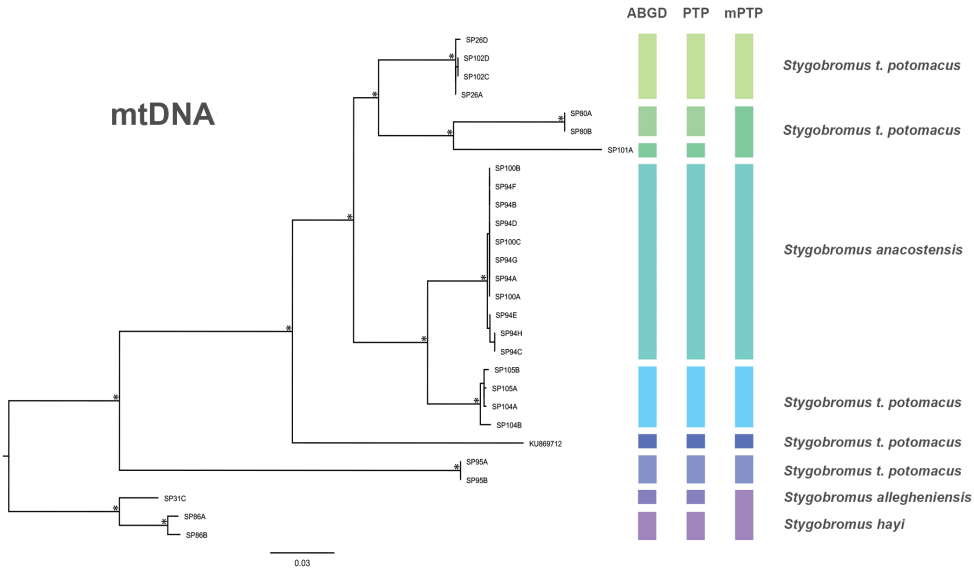

Figure 11. Maximum-likelihood phylogeny and species delimitations of *Stygobromus anacostensis* and other *S. tenuis* species group taxa for the mtDNA dataset (*co1+16s* loci). Asterisk represents bootstrap node support greater than 90. Colored bars represented hypothesized MOTU groupings (i.e., species) based on corresponding delimitation analyses.

Conservation. *Stygobromus anacostensis* is known only from the type locality. The NatureServe conservation rank calculated is Critically Imperiled (G1). Under IUCN Red List criteria, *S. anacostensis* was assessed as Critically Endangered (CR B1) because of an extremely small EOO and AOO (known from a single small seep) in an urban area. Major threats to the species include increased risk of human intrusion and disturbance, habitat degradation, and pollution. The type locality population is offered some protection by occurring on National Park Service land, but the area controlled by the NPS is very narrow, and the site is highly vulnerable to road salt as well as any attempt to "improve" the drainage in the vicinity of the sidewalk.

Genetic and phylogenetic analyses

We amplified in total 2,738 bp of five loci. Uncorrected mtDNA p-distance between *S. anacostensis* and populations of *S. tenuis potomacus* at Caledon State Park (SP104 and SP105) was 6.5% and 12.6% between *S. anacostensis* and the nearest *S. t. potomacus* population sampled in Anacostia Park (SP101). Average uncorrected nucDNA p-distance was substantially lower, averaging 0.001 between *S. anacostensis* and *S. tenuis potomacus* at Caledon State Park (SP104 and SP105), and 0.004 between *S. anacostensis* and the nearest *S. t. potomacus* population sampled in Anacostia Park (SP101).

The optimal substitution models for first, second, and third positions of *co1* were TrNef+I (Tamura and Nei 1993), F81 (Felsenstein 1981), and K81+I (Kimura 1981), respectively. The optimal substitution model was HKY + I + G (Hasegawa et al. 1985)

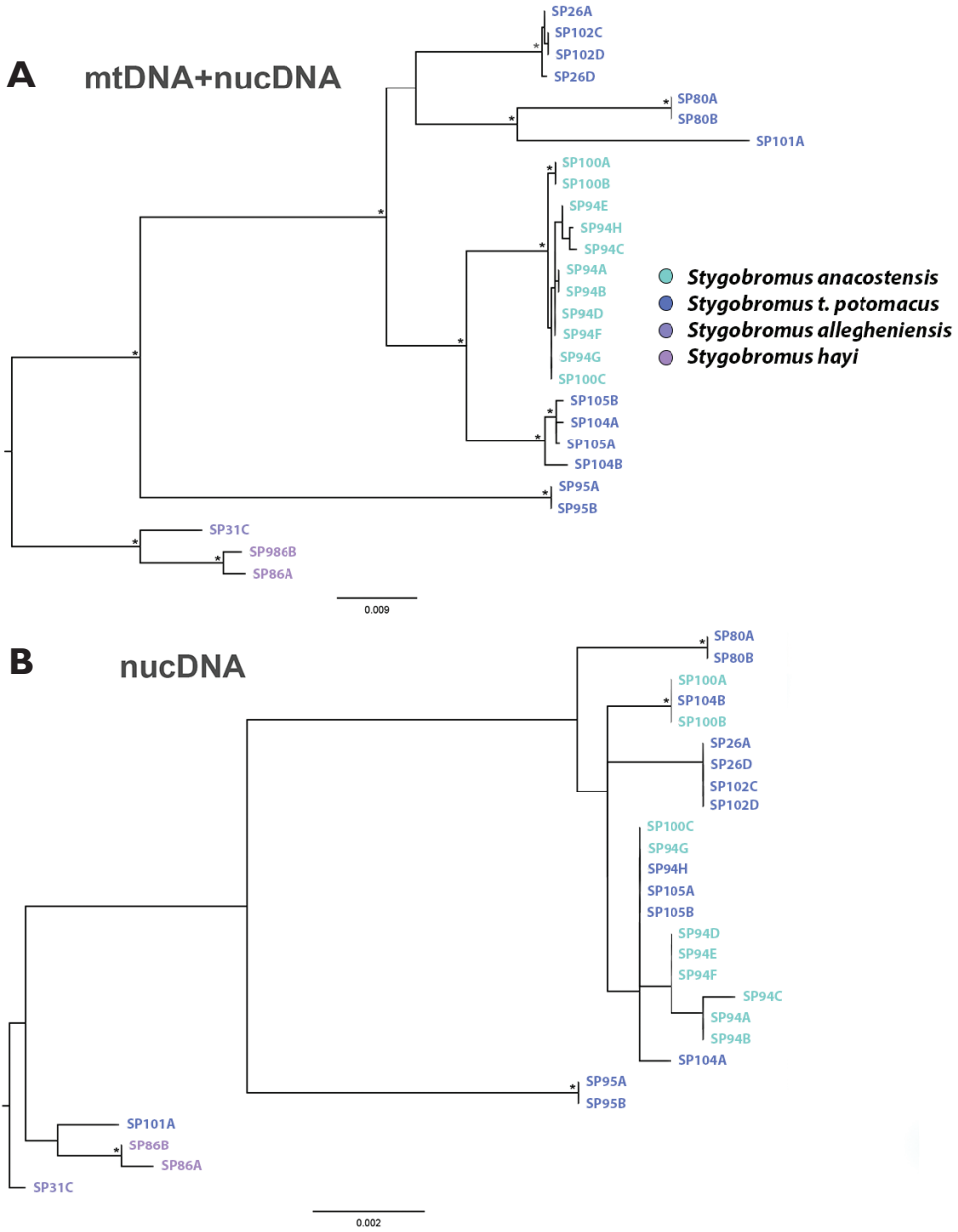

Figure 12. Maximum-likelihood phylogenies of of *Stygobromus anacostensis* and other *S. tenuis* species group taxa for the (**A**) mtDNA+nucDNA dataset (*co1+16s+18s+28s+h3* loci) and (**B**) nucDNA dataset (*18s+28s+h3* loci). Asterisk represents bootstrap node support greater than 90.

for *16s*, K80 (Kimura 1980) for *18s*, TIM2+I for *28s*, and JC (Jukes and Cantor 1980) for *h3*. Phylogenetic tree topologies obtained for ML and Bayesian inference were highly similar. Phylogenetic trees reconstructed using both ML and Bayesian inference for the mtDNA (*co1+16s*; Fig. 11) and mtDNA+nucDNA datasets (Fig. 12A) delimited

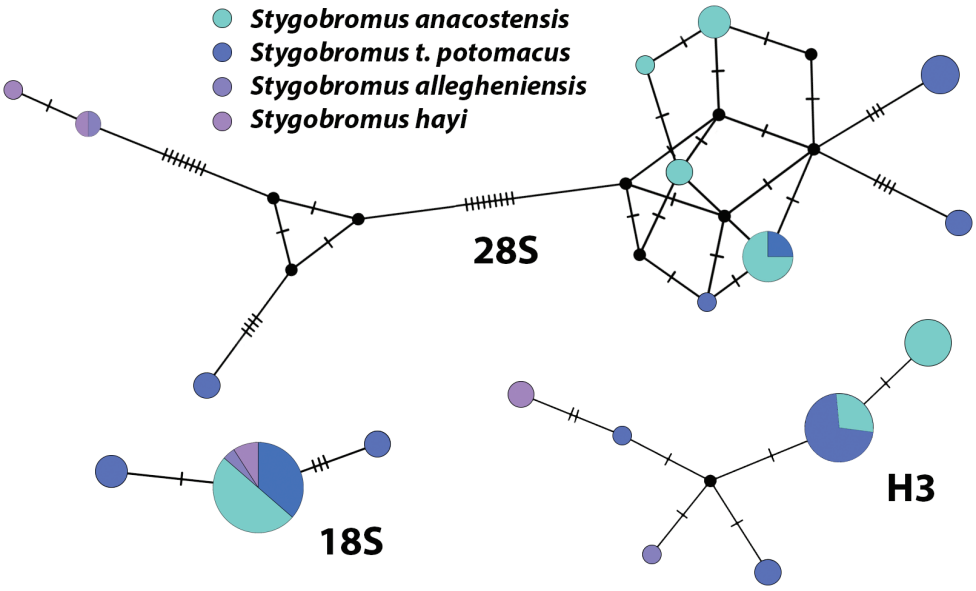

Figure 13. Median joining networks for nuclear loci (*18s*, *28s*, and *h3*) generated in PopART v1.7.

individuals of *Stygobromus anacostensis* from the type locality as distinct from other populations of the *S. tenuis* species group sampled with high bootstrap support. Populations of *S. tenuis potomacus* did not form a monophyletic group for any dataset. Nuclear loci exhibited low levels of variation among all *S. tenuis* species group taxa sampled (Figs 12B, 13). Two fixed mutations in the *h3* locus and one fixed mutation in the *18s* locus exist between *S. anacostensis* and the closest *S. t. potomacus* population (SP101).

Species delimitation

For the mtDNA dataset (Fig. 11), the ABGD approach resulted in nine MOTUs, with convergence of initial and recursive partitions at prior intraspecific divergence (P) = 0.028, which remained stable until P = 0.0359. The PTP approach yielded the same MOTU delimitations. All *S. anacostensis* samples formed a MOTU, while several *S. tenuis potomacus* populations were delimited as distinct MOTUs. The mPTP approach estimated seven MOTUs, with highly similar designations to the ABGD delimitations. *Stygobromus anacostensis* individuals were grouped as a single MOTU, as were several *S. tenuis potomacus* populations. *Stygobromus allegheniensis* and *S. hayi* were grouped into a single MOTU.

Discussion

Stygobromus anacostensis is morphologically and genetically most similar to *S. tenuis potomacus*, which overlaps in distribution with the new species. However, several morphological characters readily distinguish the two species in the Washington D.C.

area, including by having only 2 C-setae on the mandibular palp and up to 8 E-setae. *Stygobromus anacostensis* also shares a similar overall morphology with other members of the *S. tenuis* species group as defined by Holsinger (1978) in the region, but the new species can be distinguished morphologically from other members of the species complex by possessing a serrate blade-like edge along the length of both palms of gnathopods 1 and 2 and by possessing rastellate setae on the posterodistal margin of the carpus of gnathopod 2. It should be noted that the serrate blade-like edge along the length of both gnathopod palms was most discernable after the digestion protocol noted in the materials and methods. However, this feature was also easily visible on nondigested specimens, including juveniles. Thus, the characteristic is not an artifact of the digestion protocol. It is possible that this feature may be found on other *Stygobromus* species but has never been documented prior to this analysis. If so, this characteristic may be diagnostic not by its presence, but by its degree, as it was so highly visible. A reexamination of the palms of other *Stygobromus* species will help to determine the status of this characteristic.

With the description of *S. anacostensis*, the total number of described stygobiotic amphipods from the Piedmont and Atlantic Coastal Plain of the Maryland, Virginia, and District of Columbia area is now 14 species, with eight species now known from hypotelminorheic habitats in and near the Washington D.C. metropolitan area. Interestingly, unlike many other seepage springs in the region (Culver et al. 2012), *S. anacostensis* is not known to co-occur with any other *Stygobromus* species. *Stygobromus tenuis potomacus* is known from a seepage spring one km from the type locality in Anacostia Park, although in a different HUC10 drainage, but the possibility exists that this species co-occurs with *S. anacostensis*.

The discovery of a new species of *Stygobromus* amphipod from the Piedmont and Atlantic Coastal Plain is not surprising given high species richness of the genus not only in the region but also throughout North America, and the description of several species in recent years throughout the United States (Holsinger et al. 2011; Holsinger and Ansell 2014; Holsinger and Sawicki 2016; Cannizzaro et al. 2019; Gibson et al. 2021). Moreover, uncovering cryptic diversity is an increasingly common finding of population genetic and phylogenetic studies in groundwater fauna (Lefébure et al. 2006; Murphy et al. 2009; Zakšek et al. 2009; Niemiller et al. 2012, 2013; Devitt et al. 2019), including crangonyctid amphipods (Etheridge et al. 2013; Niemiller et al. 2018; Cannizzaro et al. 2020). Niemiller et al. (2018) uncovered cryptic genetic variation at the mitochondrial *co1* locus among populations of *S. tenuis potomacus* sampled in the Washington D.C. area, including up to 9.2% sequence divergence among populations separated by only 7.2 km straight-line distance. We uncovered similar levels of genetic variation among and within species of the *S. tenuis* species group highlighted by 12.6% mtDNA sequence divergence between populations of *S. anacostensis* and *S. t. potomacus* separated by just 10 km. Such levels of divergence support the view that many groundwater species are dispersal limited and that morphological species with broader distributions are likely comprised of multiple morphologically similar but genetically distinct lineages (Niemiller et al. 2012, 2018; Ethridge et al. 2013). *Stygobromus anacostensis* is one of likely several additional undescribed species that await morphological and genetic investigation and formal description within the *S. tenuis* species group.

Acknowledgments

We thank Elizabeth Burch, Heather Gilbert, Chris Hobson, Jenna Keany, and Mikaila Milton for assistance with field collection. We thank Katie Dooley and Joseph Benito for their assistance with molecular genetic work. We thank Mike Antonioni (NPS), William Moser (Smithsonian Institution), and Kathryn Ahlfield (Smithsonian Institution) for assistance with accessioning specimens. We thank the U.S. Department of the Interior, National Park Service for funding (A Survey of the Fauna of Seepage Springs in National Capital Parks-East (NACE) - P18AC01102/A19-005) to DCC; subaward to MLN). M.L.N. was also supported by the National Science Foundation (award no. 2047939).

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