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Two new species add to the diversity of *Eoniphargus* in subterranean waters of Japan, with molecular phylogeny of the family Mesogammaridae (Crustacea, Amphipoda)

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Abstract

Amphipod crustaceans are a major group of invertebrates that predominantly occur in groundwater ecosystems. *Eoniphargus* is a mesogammarid genus with only two known species from the groundwater systems of the Japanese archipelago and Korean Peninsula. However, there is a dearth of taxonomic studies on this genus, and the species diversity within *Eoniphargus* is unclear. Here, we describe two new species, *E. iwataorum* **sp. nov.** and *E. toriii* **sp. nov.**, collected from the interstitial waters in Tochigi and Shizuoka Prefectures in the Japanese archipelago. These two new species are distinguished from their congeners by the following features: head, urosomite 3, first and second antennae, mandibles, and maxilla 1. *Eoniphargus kojimai* is redescribed here based on material collected near the type locality. Molecular phylogenetic analyses based on the nuclear 28S rRNA and mitochondrial COI genes revealed that *E. kojimai* is sister to *E. iwataorum* **sp. nov.** In this study, we also briefly discuss the phylogenetic relationships of Mesogammaridae based on the molecular phylogenetic analyses.

Keywords

Cryptic species, interstitial water, molecular phylogeny, new species, taxonomy

Introduction

Compared to epigean ecosystems, subterranean environments are generally stable (Badino 2010). Owing to the absence of light, subterranean habitats are almost entirely dependent on external energy, which is mostly scarce (Poulson and Lavoie 2000). Therefore, subsurface habitats usually have low biomass and species diversity (Holsinger 1988; Cardoso 2012). Several species show limited distribution as a result of isolation and adaptation to underground environments (Culver et al. 2000). Crustaceans, segmented worms, flatworms, rotifers, water mites, and larvae of aquatic insects are predominant in subterranean waters, but insects are rarely found in cave waters (Boulton et al. 1998; Sket 1999). Amphipod crustaceans constitute a major portion of the aquatic organisms of groundwater ecosystems and have been reported from a variety of subterranean environments, including caves, interstitial riverbed waters, and springs (Holsinger 1993).

Eoniphargus Uéno, 1955 was established by Uéno (1955) as a new subgenus of *Neoniphargus* Stebbing, 1899. This subgenus was subsequently elevated to the genus level by Straškraba (1964). The affiliation of this genus to a family is controversial. Straškraba (1964) included *Eoniphargus* in Gammaridae Leach, 1814. Bousfield (1977) moved this genus to the newly established Neoniphargidae family. However, neither Barnard and Barnard (1983) nor Holsinger (1994) assigned *Eoniphargus* to a specific family, owing to the unclear nature of its diagnostic traits. A taxonomic revision of *Eoniphargus* by Tomikawa et al. (2007), based on molecular phylogenetic analyses, included this genus in Mesogammaridae Bousfield, 1977. Mesogammaridae currently include two marine genera, *Mesogammarus* Tzvetkova, 1965 and *Paramesogammarus* Bousfield, 1979, and four subterranean genera that occur in freshwaters, *Eoniphargus*, *Indoniphargus* Straškraba, 1967, *Octopupilla* Tomikawa, 2007, *Potiberaba* Fišer, Zagmajster & Ferreira, 2013; however, the monophyly of the family remains to be tested using molecular methods (see Sidorov et al. 2018 for alternative opinion on *Indoniphargus*). *Eoniphargus* currently comprises two species: *E. kojimai* Uéno, 1955 from the subterranean waters in Tokyo and Shizuoka and *E. glandulatus* Stock & Jo, 1990 from cave pools in the Korean Peninsula (Uéno 1955; Stock and Jo 1990; Tomikawa et al. 2007). In recent years, molecular phylogenetic analyses using DNA sequences have greatly advanced our understanding of the phylogenetic relationships of amphipods (Hou et al. 2011, 2014; Copilaş-Ciocianu et al. 2020). However, the phylogenetic position of Mesogammaridae and phylogenetic relationships within the family are not fully understood.

The authors recently obtained several specimens of *Eoniphargus* from interstitial waters in Japan. Detailed morphological observations and molecular phylogenetic analyses of these samples have revealed the presence of two previously undescribed species. Here, we describe these two novel species and redescribe *E. kojimai* obtained near the type locality.

Methods

Sampling and morphological observation

Specimens of *Eoniphargus* were collected from four localities in Japan (Fig. 1). The specimens were collected by washing out gravel from the bottom of rivers and springs using fine-mesh hand nets and fixed in 99% ethanol on-site.

All appendages were dissected by fine needles in 80% ethanol under a stereomicroscope (Olympus SZX7) and mounted in gum-chloral medium on glass slides. Slides were examined using a light microscope (Nikon Eclipse Ni), with appendages illustrated using a camera lucida.

Type specimens are deposited at the National Museum of Nature and Science, Tsukuba (**NSMT**).

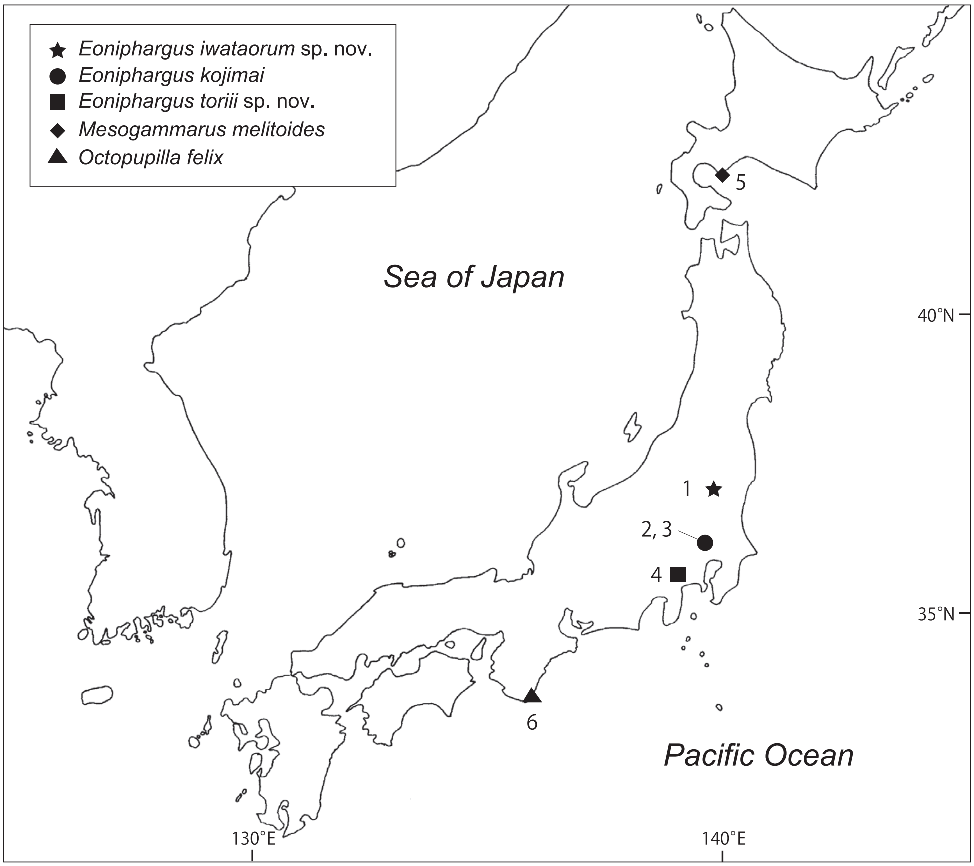

Figure 1. Map showing the collection localities of the specimens examined in this study. Names of localities are shown in Table 1.

Table 1. Samples used for molecular analyses with voucher/isolate number, collection locality, and NCBI GenBank accession number. Sequences marked with an asterisk (*) were obtained for the first time in this study.

Species	Voucher or isolate #	Locality	NCBI GenBank acc. nos.	
			28S rRNA	COI
Acanthogammaridae				
Dorogostaiskia parasitica	MZH:112037	Lake Baikal, Russia	KF586548	KF586540
Anisogammaridae				
Jesogammarus hebeiensis	294	Beijing, China	EF582998	KT180186
Gammaracanthidae				
Gammaracanthus lacustris	SLOCHN141	Savonranta Munic, Finland	JF965829	JF965997
Gammaracanthu loricatus	SLOCHN171	Spitsbergen, Norway	JF965830	JF965998
Gammarellus angulosus	RBINS-INV.132647	Westkapelle, Netherland	KT808715	FJ581638
Gammaridae				
Anopogammarus revazi	SLOCHN245	Martvili, Georgia	KF478431	KF478522
Barnardiorum shadini	SLOCHN263	Hodža Obi-Garm, Tajikistan	JF965826	JF965994
Chaetogammarus ischnus	SLOCHN051	Babadag, Romania	KF478441	KF478532
Dikerogammarus villosus	SLOCHN052	Babadag, Romania	KF478442	KF478533
Echinogammarus acarinatus	SLOCHN082	Mostar, Bosnia and Herzegovina	KF478458	KF478548
Gammarus lacustris			EF582964	EF570317
G. mukudai	G857	Katsumoto, Iki, Nagasaki, Japan	AB893233	AB893343
G. nipponensis	G621	Ukyo, Kyoto, Japan	AB893226	AB893336
G. tigrinus	609	Netherland	EF582994	EF570348
Jugogammarus kusceri	SLOCHN073	Krka, Slovenia	KF478462	KF478552
Rhipidogammarus rhipidiophorus	SLOCHN162	Dorgali, Sardegna, Italy	JF965823	JF965991
Mesogammaridae				
Eoniphargus iwataorum sp. nov.	NSMT-Cr 30783	Sabi River, Imaizumi, Ohtawara, Tochigi Prefecture, Japan (1)	LC709238*	LC709248*
E. kojimai	G1750	Mamashita Spring, Kunitachi, Tokyo, Japan (2)	LC709239*	LC709249*
E. kojimai	NSMT-Cr 30787	Hinochūōtoshokan Spring, Hino, Tokyo, Japan (3)	LC709240*	LC709250*
E. kojimai	NSMT-Cr 30788	Hinochūōtoshokan Spring, Hino, Tokyo, Japan (3)	LC709241*	LC709251*
E. toriii sp. nov.	G56	Seto River, Terajima, Fujieda, Shizuoka, Japan (4)	LC709242*	LC709252*
E. toriii sp. nov.	G71	Seto River, Terajima, Fujieda, Shizuoka, Japan (4)	LC709243*	LC709253*
Mesogammarus melitoides	G86	Muroran, Hokkaido, Japan (5)	LC719002*	NA
Octopupilla felix	G54	Koza River, Wakayama, Japan (6)	LC719003*	LC719248*
Pontogammaridae				
Obesogammarus crassus	SLOCHN055	Babadag, Romania	KF478445	KF478536
Paraniphargoides motasi	SLOCHN188	Gilan, Iran	KF478485	KF478571
Pontogammarus robustoides	SLOCHN255	Delta Volgi, Russia	JF965822	JF965990
Stenogammarus similis	SLOCHN187	Gilan, Iran	KF478484	KF478570
Turcogammarus spandli	SLOCHN026	Thessaloniki, Greece	KF478437	KF478528
Typhlogammaridae				
Metohia carinata	SLOCHN019	Rijeka Crnojevica, Montenegro	KF478498	KF478584
Typhlogammarus mrazeki	SLOCHN113	Cetinje, Montenegro	KF478504	KF478590
Zenkevitchia admirabilis	SLOCHN200	Gudauta, Georgia	KF478514	KF478599
Outgroup				
Pseudocrangonyx yezonis	KUZ Z1969	Daisen, Akita, Japan	LC17151	LC171519

Molecular phylogenetic analyses

Genomic DNA was extracted from the appendage muscle of the specimens following procedures detailed by Tomikawa et al. (2014a, b). The primer sets for PCR and cycle sequencing reactions used in this study were as follows: for 28S rRNA (28S), 28SF and 28SR (Tomikawa et al. 2012); and for cytochrome *c* oxidase subunit I (COI), LCO1490 and HCO2198 (Folmer et al. 1994). PCR and DNA sequencing were performed using the method detailed by Tomikawa (2015). In total, 15 sequences were newly obtained and deposited in the International Nucleotide Sequence Databases (**INSD**) through the DNA Data Bank of Japan (**DDBJ**) (Table 1).

In addition to the newly obtained sequences, 50 sequences of 24 gammaroid species and one crangonyctoid *Pseudocrangonyx yezonis* Akatsuka & Komai, 1922, which was selected as the outgroup, were obtained from the INSD in accordance with the previous studies (Hou et al. 2011, 2014; Copilaş-Ciocianu et al. 2020), and were included in the present phylogenetic analyses (Table 1). The phylogenetic analyses were conducted based on sequences of nuclear 28S and mitochondrial COI. The alignment of COI was trivial, as no indels were observed. The 28S sequences were aligned using the Muscle algorithm implemented in MEGA X (Kumar et al. 2018). The lengths of the 28S and COI were 892 and 658 bp.

Phylogenetic relationships were reconstructed via Maximum Likelihood (ML) and Bayesian Inference (BI). The best evolutionary models were selected based on the corrected Akaike Information Criterion (AIC) for ML using MEGA X (Kumar et al. 2018). ML phylogenies were conducted using MEGA X (Kumar et al. 2018) under the substitution model GTR+G, and 1000 bootstrap replications (Felsenstein 1985) were performed to estimate statistical support for branching patterns. BI and Bayesian posterior probabilities (PPs) were estimated using MrBayes v. 3.2.5 (Ronquist et al. 2012). The best-fit partition scheme and models for each partition were selected with the Bayesian information criterion using PartitionFinder with the “greedy” algorithm: for 28S, SYM+G; for COI 1st position, HKY+G; for COI 2nd position, HKY+I+G; for COI 3rd positions, GTR+I+G. Two independent runs of four Markov chains were conducted for 10 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer v. 1.7.1 (Rambaut et al. 2018), and the first 50001 trees were discarded based on the results.

Results

Family Mesogammaridae Bousfield, 1977

Genus *Eoniphargus* Uéno, 1955
Japanese name: Chikayokoebi-zoku

Neoniphargus (*Eoniphargus*) Uéno, 1955: 148.

Eoniphargus: Straškraba, 1964, 136, 138; Straškraba 1967, 127; Bousfield 1977, 301; Barnard and Barnard 1983, 581–582; Holsinger 1994, 157; Tomikawa et al. 2007, 646.

Diagnosis. Head without eyes, rostrum short, inferior antennal sinus distinct. Pleonites 1–3 with dorsal setae, lacking processes. Urosomite 2 with dorsal robust setae. Antenna 1 longer than antenna 2; accessory flagellum three- or four-articulate. Male antenna 2 with calceoli. Mandible with triturative molar with seta; left incisors five- or six-dentate; left lacinia mobilis four- or five-dentate; palp article 3 with A-, D- and E-setae. Maxilla 1 with inner plate bearing plumose setae; outer plate with 11 serrate robust setae. Inner plate of maxilla 2 with oblique row of facial setae. Gnathopods subchelate. Coxa of pereopod 4 with posterior concavity. Coxal gills with stalks, on gnathopod 2 and pereopods 3–6. Uropod 1 with basofacial robust setae on peduncle. Uropod 3 with small, scale-like inner ramus; outer ramus one- or two-articulate. Telson cleft with apical robust setae.

Type species. *Neoniphargus* (*Eoniphargus*) *kojimai* Uéno, 1955, original designation.

Remarks. *Eoniphargus* is morphologically similar to *Octopupilla* in the presence of pleonites without dorsal processes, five-dentate incisor of the left mandible, mandibular palp article 3 with A-setae, inner plate of maxilla 2 with oblique row of facial setae, stalked coxal gills, peduncle of uropod 1 with basofacial robust setae, and telson lobes tapering distally. However, *Eoniphargus* differs from *Octopupilla* in the following features (features of *Octopupilla* in parentheses): eyes absent (rudimentary eyes), incisor of right mandible five- to six-dentate (four-dentate), inner lobes of the lower lip absent (vestigial), and inner ramus of uropod 3 shorter than 0.3 × the outer ramus (0.7 ×). *Eoniphargus* also resembles *Indoniphargus* Straškraba, 1967, but the familial affiliation of the latter has been controversial. Tomikawa et al. (2007) and Fišer et al. (2013) included *Indoniphargus* in Mesogammaridae. Sidorov et al. (2018) regarded *Indoniphargus* as a member of Austroniphargidae Iannilli, Krapp, & Ruffo, 2011. However, since the taxonomic position of *Indoniphargus* is beyond the scope of this paper, it will not be discussed here further. *Eoniphargus* is distinguished from *Indoniphargus* by the presence of facial setae in an oblique row of the inner plate of maxilla 2 (absent in *Indoniphargus*) and the elongate propodi of gnathopods 1 and 2 (mitten-form in *Indoniphargus*).

***Eoniphargus toriii* sp. nov.**
https://zoobank.org/2A3DFB47-9FEC-46E4-A003-923CCD93E20D
Figs 2–5
New Japanese name: Torii-chikayokoebi

Eoniphargus kojimai: Tomikawa et al. 2007, 647, figs 2–6.

Type material. ***Holotype*:** ♂ 4.3 mm (NSMT-Cr 16652), Seto River (34.880555°N, 138.218888°E), Terajima, Fujieda, Shizuoka Prefecture, Japan, collected by T. Torii on 3 June 2004. ***Paratypes*:** 2 ♀♀, 4.6 mm and 4.3 mm (NSMT-Cr 16653 and 16654), data same as for the holotype.

Diagnosis. Urosomite 3 without dorsal setae. Epimeral plates 2–3 without ventral setae. Peduncular article 1 of antenna 1 with robust seta on posterodistal corner. Antenna 2 with peduncular article 2 gland cone not exceeding end of article 3; calceoli present in male. Mandible with 5-dentate left incisor; left lacinia mobilis 4-dentate, right one bifid with three or four teeth. Inner plate of maxilla 1 with six plumose setae. Inner plate of maxilla 2 with seven facial seta in oblique row. Peduncle of pleopod 3 with seta. Uropod 1 with peduncle bearing basofacial robust setae. Uropod 3 with inner ramus 0.3 times as long as outer ramus; outer ramus two-ariticulate, with plumose setae on medial margin. Telson length 1.1 times width, cleft for 67% of length.

Etymology. The new species is named after Dr. Takaaki Torii, who collected the species.

Description. Male holotype, NSMT-Cr 16652. Head (Fig. 2A) as long as pereonites 1 and 2 combined; eyes absent; inferior antennal sinus distinct with rounded angle. Dorsal margins of pleonites 1–3 (Figs 2A, 5A–C) each with four setae. Epimeral plates 1–3 (Figs 2A, 5N–P) with weakly pointed posterodistal corners; ventral margins without setae; posterior margins of plates 1–3 with one, zero, one seta, respectively. Dorsal margins of urosomites 1 and 2 with robust seta, urosomite 3 dorsally bare.

Antenna 1 (Figs 2A, 3A) length 0.6 times as long as body; length ratio of peduncular articles 1–3 in 1.0 : 0.8 : 0.4; posterodistal corner of peduncular article 1 with robust seta; accessory flagellum three-articulate (Fig. 3B); primary flagellum with approximately 22 articles, each article with one aesthetasc (Fig. 3C). Antenna 2 (Figs 2A, 3D) length 0.6 times as long as antenna 1; peduncular article 4 length 0.9 times as long as article 5; right peduncular article 5 with calceoli, left one lacking calceoli; flagellum 16-articulate, with calceoli (Fig. 3E).

Upper lip (Fig. 2B) with rounded apical margin bearing fine setae. Mandibles (Fig. 2C–E) with left and right incisors comprising five and five–six teeth, respectively; left lacinia mobilis comprising four teeth, right lacinia mobilis bifid with three teeth; molar process triturative with plumose seta; length ratio of palp articles 1–3 in 1.0 : 2.4 : 1.9; palp article 1 without setae; palp article 2 with eight marginal setae; palp article 3 with pair of A-setae, many D-setae and E-setae, lateral face with fine setae. Lower lip (Fig. 2G) lacking inner lobes; outer lobes broad, shoulder rounded, with fine setae. Maxilla 1 (Fig. 2H–J) with triangular inner lobe, bearing six plumose setae on medial margin; outer plate rectangular, with 11 serrate robust setae apically; palp 2-articulate, article 1 without setae, article 2 with six robust setae apically. Maxilla 2 (Fig. 2K) with inner plate bearing seven plumose setae in oblique row. Maxilliped (Fig. 2L–O) with inner plate reaching end of palp article 1, subquadrate, bearing three subapical robust setae and medial robust seta; outer plate ovate, reaching middle of palp article 2, with row of robust setae along apical to medial margins; palp 4-articulate, article 2 longest with medial setae, nail of article 4 distinct.

Gnathopod 1 (Fig. 3J, K) with subquadrate coxa bearing setae along anterior to ventral margins; anterior and posterior margins with long setae; carpus length 0.9 times as long as length of propodus and 1.5 times width of carpus; propodus length 1.9 times width, palmar margin weakly serrate, oblique, with three medial and two lateral

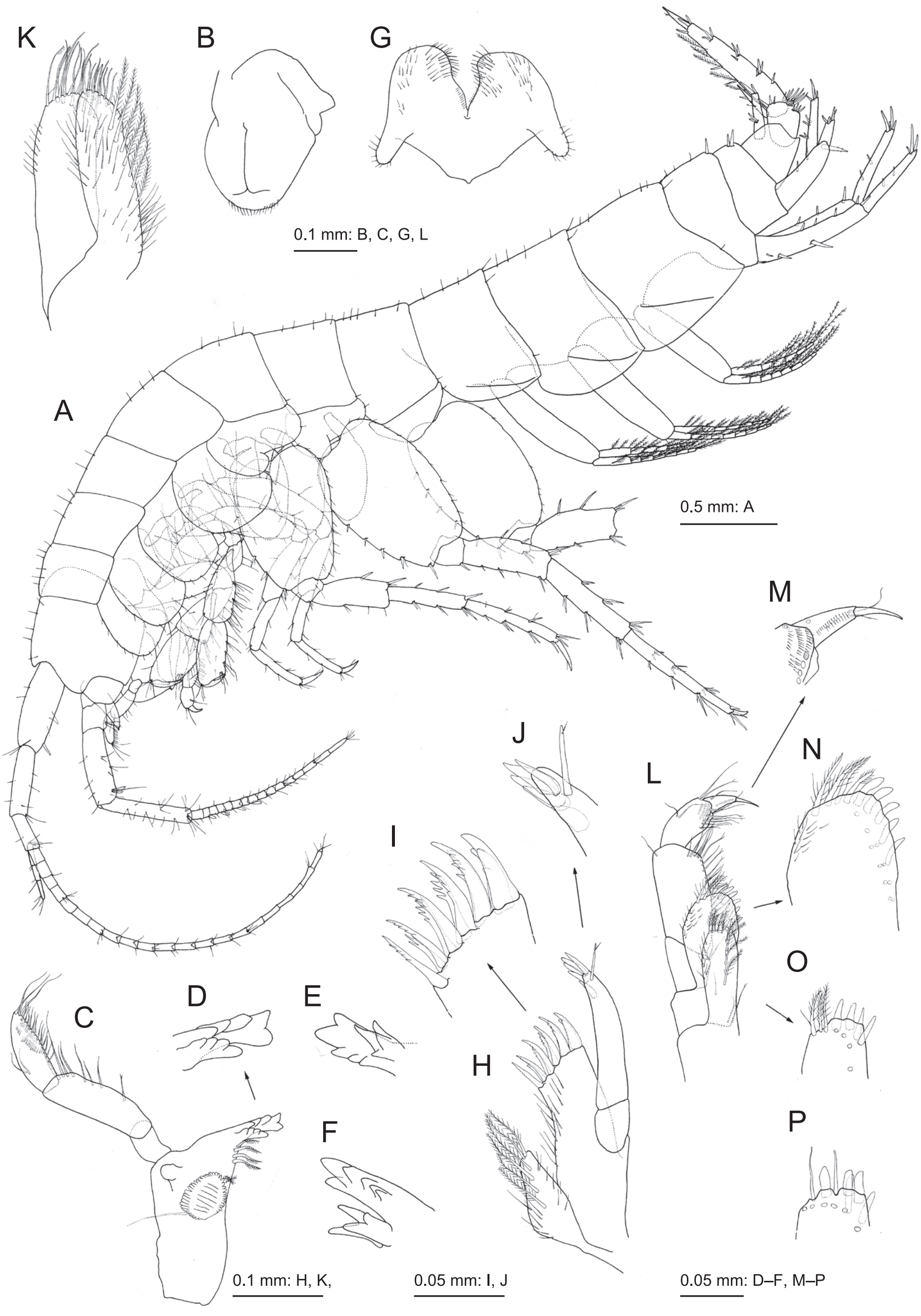

Figure 2. *Eoniphargus toriii* sp. nov., male holotype (NSMT-Cr 16652), female paratype (NSMT-Cr 16653) **A** habitus, lateral view **B** upper lip, anterior view **C** left mandible, medial view **D** incisor and lacinia mobilis of left mandible, medial view **E** and **F** incisor and lacinia mobilis of right mandible, medial view **G** lower lip, anterior view **H** maxilla 1, dorsal view **I** outer plate of maxilla 1, dorsal view **J** palp of maxilla 1, dorsal view **K** maxilla 2, dorsal view **L** maxilliped, dorsal view **M** palp article 4 of maxilliped, dorsal view **N** outer plate of maxilliped, dorsal view **O, P** inner plate of maxilliped, dorsal view **F, P** female; others, male. Modified from Tomikawa et al. (2007).

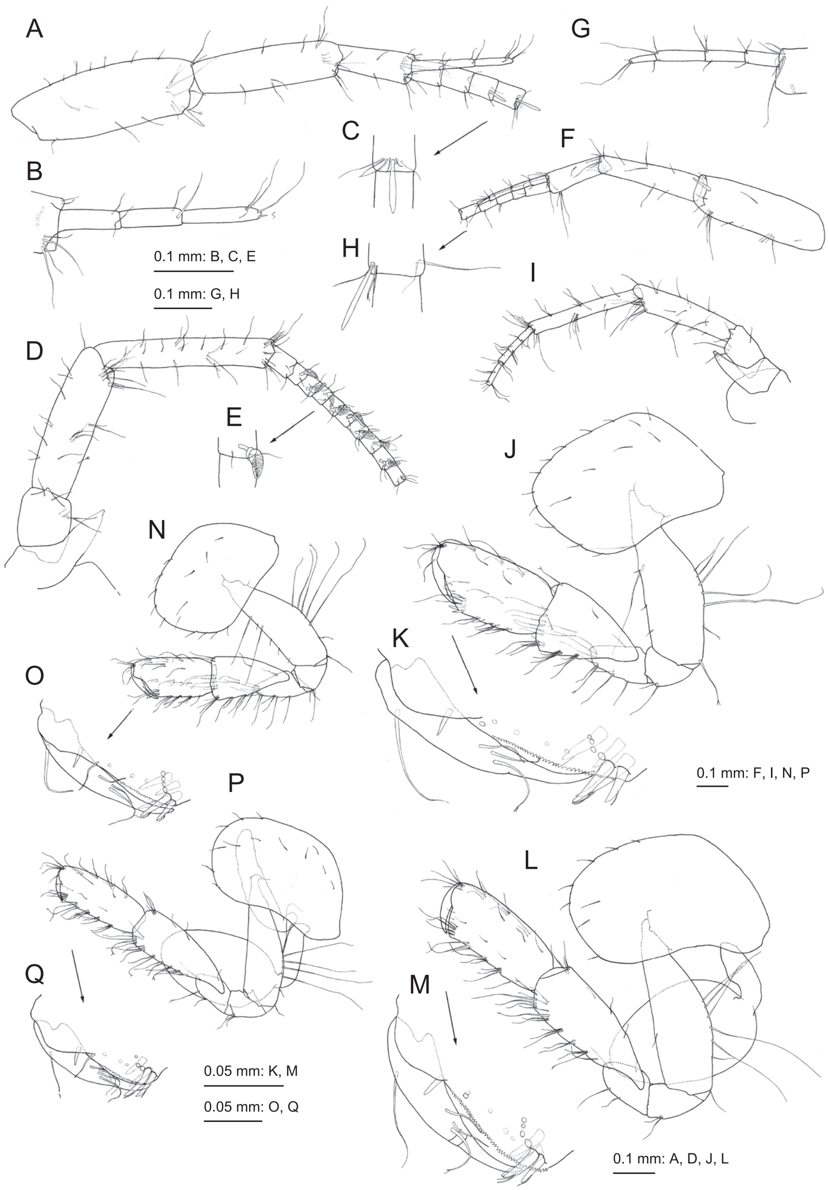

Figure 3. *Eoniphargus toriii* sp. nov., male holotype (NSMT-Cr 16652), female paratype (NSMT-Cr 16653) **A** antenna 1, medial view **B** accessory flagellum of antenna 1, medial view **C** aesthetasc on flagellar article of antenna 1, medial view **D** antenna 2 (some flagellar articles are omitted), medial view **E** calceolus on flagellar article of antenna 2, medial view **F** antenna 1 (female, some flagellar articles are omitted), medial view **G** accessory flagellum of antenna 1 (female), medial view **H** aesthetasc on flagellar article of antenna 1 (female), medial view **I** antenna 2 (female, some flagellar articles are omitted), medial view **J** gnathopod 1, lateral view **K** palmar margin and dactylus of gnathopod 1 (some setae are omitted), lateral view **L** gnathopod 2, lateral view **M** palmar margin and dactylus of gnathopod 2 (some setae are omitted), lateral view **N** gnathopod 1 (female), lateral view **O** palmar margin and dactylus of gnathopod 1 (female), lateral view **P** gnathopod 2 (female), lateral view **Q** palmar margin and dactylus of gnathopod 2 (female, some setae are omitted), lateral view **F–H, N–Q** female; others, male. Modified from Tomikawa et al. (2007).

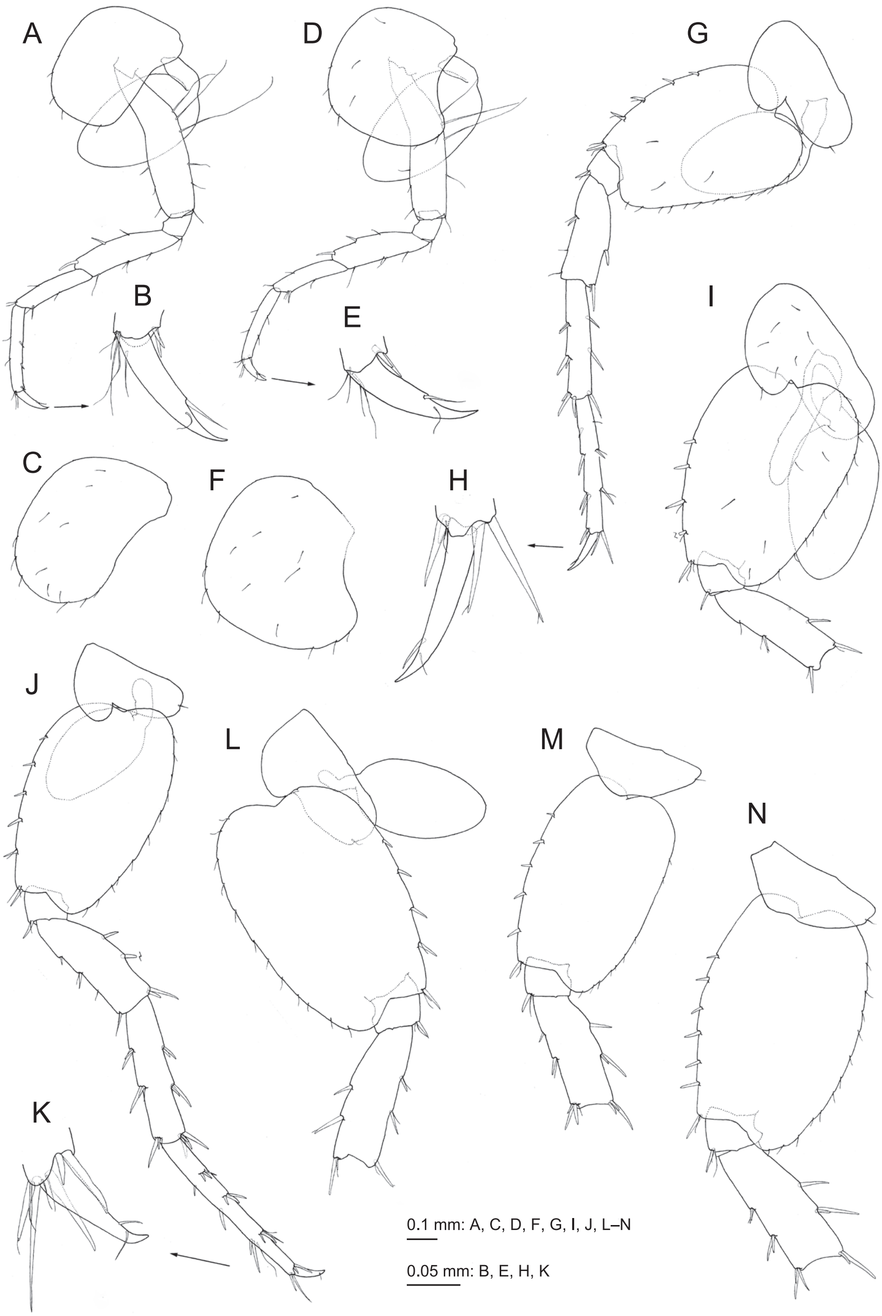

Figure 4. *Eoniphargus toriii* sp. nov., male holotype (NSMT-Cr 16652), female paratype (NSMT-Cr 16653) **A** pereopod 3, lateral view **B** dactylus of pereopod 3, lateral view **C** coxa of pereopod 3 (female) , lateral view **D** pereopod 4, lateral view **E** dactylus of pereopod 4, lateral view **F** coxa of pereopod 4 (female) , lateral view **G** pereopod 5, lateral view **H** dactylus of pereopod 5, lateral view **I** coxa–merus of pereopod 5 (female), lateral view **J** pereopod 6, lateral view **K** dactylus of pereopod 6, lateral view **L** coxa–merus of pereopod 6 (female) , lateral view **M** coxa–merus of pereopod 7, lateral view **N** coxa–merus of pereopod 7 (female), lateral view **C, F, I, L, N** female; others, male. Modified from Tomikawa et al. (2007).

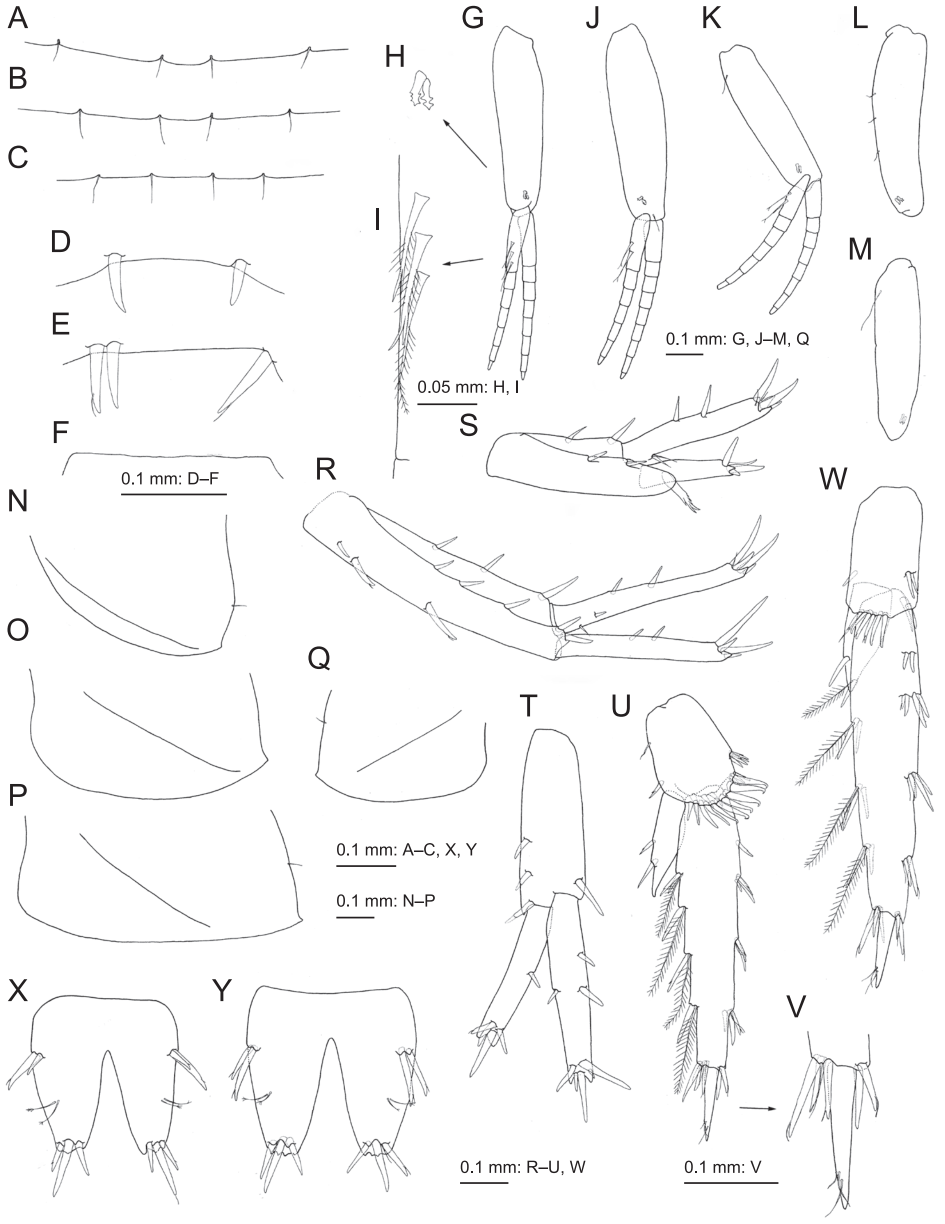

Figure 5. *Eoniphargus toriii* sp. nov., male holotype (NSMT-Cr 16652), female paratype (NSMT-Cr 16653) **A–C** dorsal margins of pleonites 1–3, dorsal views **D–F** dorsal margins of urosomites 1–3, dorsal views **G** pleopod 1 (plumose setae on rami are omitted), medial view **H** retinacula of pleopod 1, medial view **I** bifid setae of pleopod 1, medial view **J** pleopod 2 (plumose setae on rami are omitted), medial view **K** pleopod 3 (plumose setae on rami are omitted), medial view **L** pleopod 1 (female, rami are omitted), medial view **M** pleopod 2 (female, rami are omitted), lateral view **N–P** epimeral plates 1–3, lateral views **Q** epimeral plate 2 (female), lateral view **R** uropod 1, dorsal view **S** uropod 2, dorsal view **T** uropod 2 (female) , dorsal view **U** uropod 3, ventral view **V** terminal article of outer ramus of uropod 3, ventral view **W** uropod 3 (female), ventral view **X** telson, dorsal view **Y** telson (female), dorsal view **L, M, Q, T, W, Y** female; others, male. Modified from Tomikawa et al. (2007).

robust setae; dactylus not reaching posterodistal corner of propodus. Gnathopod 2 (Fig. 3L, M) with coxa expanded proximally bearing setae along anterior to ventral margins; posterior margin of basis with long setae; carpus length 1.2 times as long as length of propodus and 2.8 times width of carpus; propodus length 2.4 times width, palmar margin weakly serrate, almost vertical, with two medial and two lateral robust setae; dactylus not reaching posterodistal corner of propodus.

Pereopod 3 (Fig. 4A, B) with subquadrate coxa, proximally expanded, bearing setae along anterior to ventral margins; posterior margin of basis with long setae. Pereopod 4 (Fig. 4D, E) with coxa bearing setae along anterior to ventral margins, posteroproximally concave; posterior margin of basis with long setae. Pereopod 5 (Fig. 4G, H) with bilobed coxa bearing seta on anterior and posterior lobes; anterior margin of basis with robust setae, posterodistal corner weakly lobate. Pereopod 6 (Fig. 4J, K) with bilobed coxa bearing seta on posterior lobe; basis ovate, with robust setae on anterior margin, posterodistal corner weakly lobate. Pereopod 7 (Fig. 4M) with subtriangular coxa bearing seta on posterior margin; basis elliptical, with robust setae on anterior margin, posterodistal corner weakly lobate.

Coxal gills (Figs 3L, 4A, D, G, J) ovate with stalks on gnathopod 2 and pereopods 3–6.

Pleopods 1–3 (Fig. 5G–K) with peduncles longer than rami, bearing distal seta on pleopod 2 and proximal seta on pleopod 3; retinacula paired (Fig. 5H); mediobasal margin of inner ramus with bifid plumose setae (Fig. 5I); rami well developed.

Uropod 1 (Fig. 5R) with peduncle bearing three basofacial robust setae and dorsal robust setae; inner ramus length 0.7 times as long as peduncle, with two medial and one lateral robust setae, and ventroproximal robust seta; outer ramus length 0.9 times as long as inner ramus, with two robust setae on medial margin. Uropod 2 (Fig. 5S) with peduncle bearing dorsal robust setae; inner ramus length 0.8 times as long as peduncle, bearing two medial robust setae and two ventroproximal slender setae; outer ramus length 0.6 times as long as inner ramus, without marginal setae. Uropod 3 (Fig. 5U, V) with peduncle length 0.3 times as long as outer ramus, bearing bent robust setae on distal edge; inner ramus length 0.3 times as long as outer ramus, with two robust setae on medial margin; outer ramus 2-articulate, proximal article with plumose setae on medial margin and robust setae on medial and lateral margins, terminal article length 0.3 times as long as proximal article with subapical setae.

Telson (Fig. 5X) length 1.1 times width, each lobe with two lateral and three apical robust setae and two dorsolateral penicillate setae, cleft for 67%.

Female paratype, NSMT-Cr 16653. Antenna 1 (Fig. 3F–H) with peduncles 1–3 of which length ratio in 1.0 : 0.8 : 0.5; accessory flagellum 4-articulate (Fig. 3G). Antenna 2 (Fig. 3I) without calceoli.

Incisor of right mandible 6-dentate (Fig. 2F); right lacinia mobilis 4-dentate, weakly bifid. Apical robust setae of inner plate of maxilliped stiffer than those of male (Fig. 2P).

Gnathopod 1 (Fig. 3N, O) with carpus length 1.8 times width; length of propodus 2.0 times width; dactylus reaching posterodistal corner of propodus. Gnathopod 2 (Fig. 3P, Q) with dactylus reaching posterodistal corner of propodus.

Brood plates (Figs 3P, 4I) narrow, lacking setae, on gnathopod 2 and pereopods 3–5.

Peduncles of pleopods 1 and 3 with three and one setae, respectively (Fig. 5L, M).

Uropod 2 (Fig. 5T) with inner ramus bearing two medial and one lateral robust setae; outer ramus with medial robust seta. Uropod 3 (Fig. 5W) with terminal article of outer ramus length 0.2 times as long as proximal article.

Telson (Fig. 5Y) as long as wide, cleft for 71%.

Distribution and environment. The species is known only from its type locality in the Seto River, Shizuoka Prefecture, Japan. Specimens were collected at a depth of 20 cm, from the bank of the river.

Remarks. *Eoniphargus toriii* sp. nov. is similar to *E. kojimai* and *E. iwataorum* sp. nov., with a head bearing deep antennal sinus, antenna 1 peduncular article 1 with a robust seta on the posterodistal corner, antenna 2 peduncular article 2 with a gland cone not exceeding peduncular article 3, uropod 1 peduncle with basofacial robust setae, and uropod 3 with 2-articulate outer ramus. However, *E. toriii* sp. nov. differs from *E. kojimai* and *E. iwataorum* sp. nov. in the following features: right mandible with lacinia mobilis bearing three or four teeth (more than four teeth in *E. kojimai* and *E. iwataorum* sp. nov.) and maxilla 1 having six medial setae on the inner plate (eight medial setae in *E. kojimai* and *E. iwataorum* sp. nov.).

Eoniphargus iwataorum sp. nov.

https://zoobank.org/4234A07E-026A-4DB1-A2E8-2DE845E6295E

Figs 6–8

New Japanese name: Iwata-chikayokoebi

Type material. ***Holotype*:** ♀ 5.6 mm (NSMT-Cr 30782), Sabi River (36.898181°N, 140.012153°E), Imaizumi, Ohtawara, Tochigi Prefecture, Japan, collected by Y. Iwata on 27 January 2019. ***Paratype*:** ♀ 5.3 mm (NSMT-Cr 30783; G1751), ♀ 6.0 mm (NSMT-Cr 30784), data same as for the holotype.

Diagnosis. Urosomite 3 without dorsal setae. Epimeral plates 2–3 each with ventral robust seta. Peduncular article 1 of antenna 1 with robust seta on posterodistal corner. Antenna 2 with peduncular article 2 gland cone not exceeding end of article 3. Mandible with 5-dentate left incisor; left lacinia mobilis 4-dentate, right one bifid with many teeth. Inner plate of maxilla 1 with eight plumose setae. Inner plate of maxilla 2 with seven facial seta in oblique row. Peduncle of pleopod 3 with seta. Uropod 1 with peduncle bearing basofacial robust setae. Uropod 3 with inner ramus 0.25 times as long as outer ramus; outer ramus 2-ariticulate, with plumose setae on medial margin. Telson almost as long as width, cleft for 67% of length.

Etymology. The specific epithet was derived from the names of Mr. Yasuyuki Iwata and Mr. Tomofumi Iwata, who collected the specimens of this new species.

Description. Female holotype, NSMT-Cr 30782. Head (Fig. 6A) as long as pereonites 1 and 2 combined; eyes absent; inferior antennal sinus distinct with rounded angle. Dorsal margins of pleonites 1–3 (Fig. 6B–D) each with four setae. Posterodistal corners of epimeral plates 1–3 (Fig. 6E–G) weakly produced; ventral margin of epimeral plate 1 without setae, epimeral plates 2 and 3 each with ventral robust seta; posterior margins of epimeral plates 1–3 each with two setae. Dorsal margin of urosomite 1 (Fig. 6H) with slender setae; urosomite 2 (Fig. 6I) with dorsal robust setae; urosomite 3 (Fig. 6J) without dorsal setae.

Antenna 1 (Fig. 6K) length 0.25 times as long as body; length ratio of peduncular articles 1–3 in 1.0 : 0.8 : 0.4; posterodistal corner of peduncular article 1 with robust seta; accessory flagellum 4-articulate; primary flagellum 27-articulate. Antenna 2 (Fig. 6L) length 0.7 times as long as antenna 1; peduncular article 4 slightly shorter than article 5; flagellum 22-articulate; calceoli absent.

Upper lip (Fig. 6M) with rounded apical margin bearing fine setae. Mandibles (Fig. 6N–P) with left and right incisors comprising five and six teeth, respectively; left lacinia mobilis comprising four teeth, right lacinia mobilis bifid with many teeth; molar process triturative with plumose seta; length ratio of palp articles 1–3 in 1.0 : 2.0 : 1.5; palp article 1 without setae; palp article 2 with eight marginal setae; palp article 3 with pair of A-setae, several D-setae and E-setae, lateral face with fine setae. Lower lip (Fig. 6Q) lacking inner lobes; outer lobes broad, shoulder rounded, with fine setae. Maxilla 1 (Fig. 6R, S) with elliptical inner lobe, bearing eight plumose setae on medial margin; outer plate rectangular, with 11 serrate robust setae apically; palp 2-articulate, article 1 without setae, article 2 with five robust setae and slender plumose seta apically. Maxilla 2 (Fig. 6T) with inner plate bearing seven plumose setae in oblique row. Maxilliped (Fig. 7A–C) with inner plate exceeding end of palp article 1, subquadrate, bearing three subapical robust setae and medial robust seta; outer plate ovate, reaching middle of palp article 2, with row of robust setae along apical to medial margins; palp 4-articulate, article 2 longest with medial setae, nail of article 4 distinct.

Gnathopod 1 (Fig. 7D, E) with subquadrate coxa bearing setae along anterior to ventral margins; anterior and posterior margins of basis with long setae; carpus length 0.9 times as long as length of propodus and 1.4 times width of carpus; propodus length 1.7 times width, palmar margin weakly serrate, oblique, with three medial and one lateral robust setae; dactylus reaching posterodistal corner of propodus. Gnathopod 2 (Fig. 7F, G) with coxa expanded proximally bearing setae along anterior to ventral margins; posterior margin of basis with long setae; carpus length 1.2 times as long as length of propodus and 2.8 times width of carpus; propodus length 2.2 times width, palmar margin weakly serrate, almost vertical, with two medial and two lateral robust setae; dactylus reaching posterodistal corner of propodus.

Pereopod 3 (Fig. 7H, I) with subquadrate coxa, proximally expanded, bearing setae along anterior to ventral margins; posterior margin of basis with long setae. Pereopod 4 (Fig. 7J) with coxa bearing setae along anterior to ventral margins, posteroproximally concave; posterior margin of basis with long setae. Pereopod 5 (Fig. 7K) with bilobed coxa bearing seta on posterior lobe; anterior margin of basis with robust setae, posterodistal corner weakly lobate. Pereopod 6 (Fig. 7L) with bilobed coxa bearing seta on anterior and posterior lobes; basis with robust setae on anterior margin, posterodistal corner weakly lobate. Pereopod 7 (Fig. 8A–C) with elliptical basis bearing robust setae on anterior margin, posterodistal corner weakly lobate.

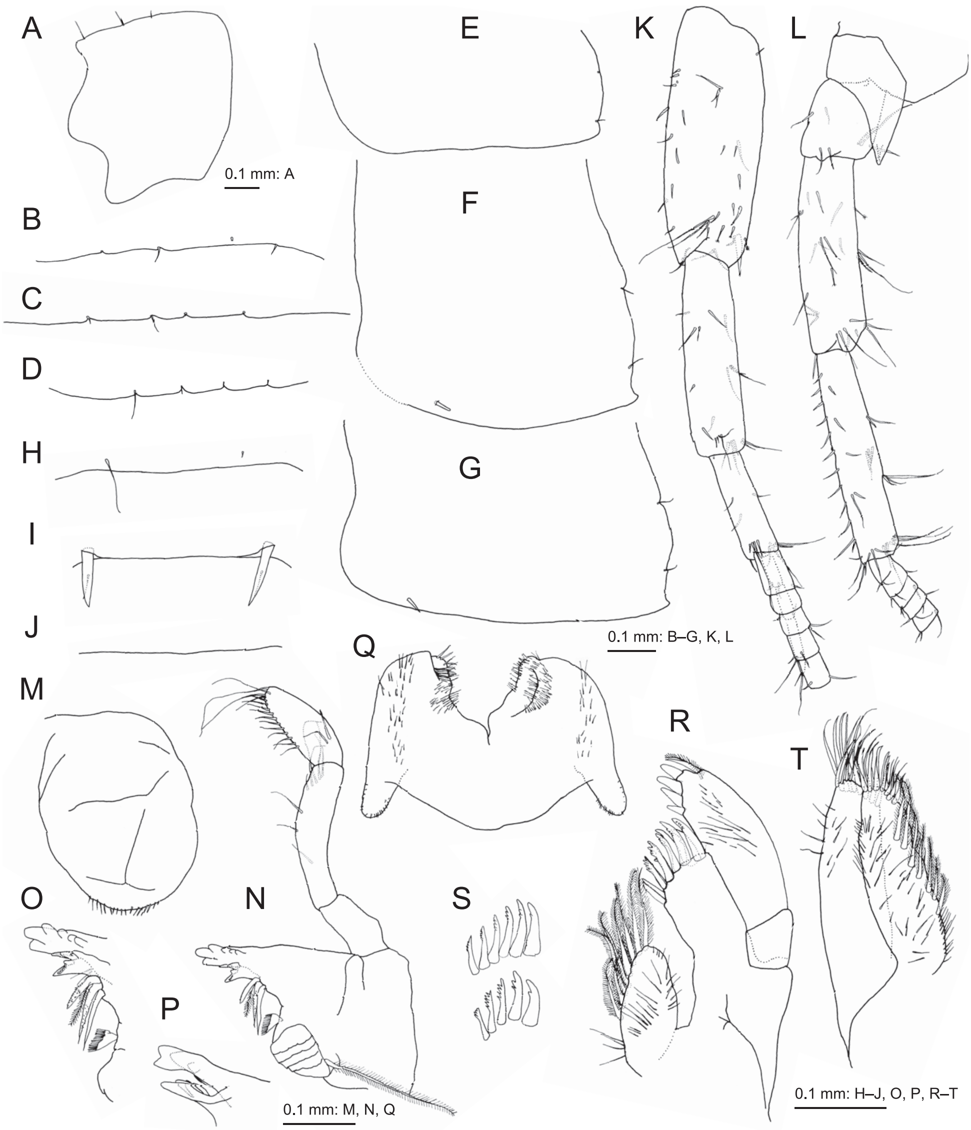

Figure 6. *Eoniphargus iwataorum* sp. nov., female holotype (NSMT-Cr 30782) **A** head, lateral view **B–D** dorsal margins of pleonites 1–3, dorsal views **E–G** epimeral plates 1–3, lateral views **H–J** dorsal margins of urosomites 1–3, dorsal views **K** antenna 1 (some flagellar articles are omitted), lateral view **L** antenna 2 (some flagellar articles are omitted), lateral view **M** upper lip, anterior view **N** right mandible, medial view **O** incisor and lacinia mobilis of right mandible, medial view **P** incisor and lacinia mobilis of left mandible, lateral view **Q** lower lip, anterior view **R** maxilla 1, dorsal view **S** apical robust setae on outer plate of maxilla 1, dorsal view **T** maxilla 2, dorsal view.

Coxal gills (Fig. 8D, E) ovate with stalks on gnathopod 2 and pereopods 3–6.

Pleopods 1–3 (Fig. 8F–I) with peduncles longer than rami, bearing distal seta on pleopods 1 and 2 and proximal seta on pleopod 3; retinacula paired (Fig. 8I); mediobasal margin of inner ramus with bifid plumose setae; rami well developed.

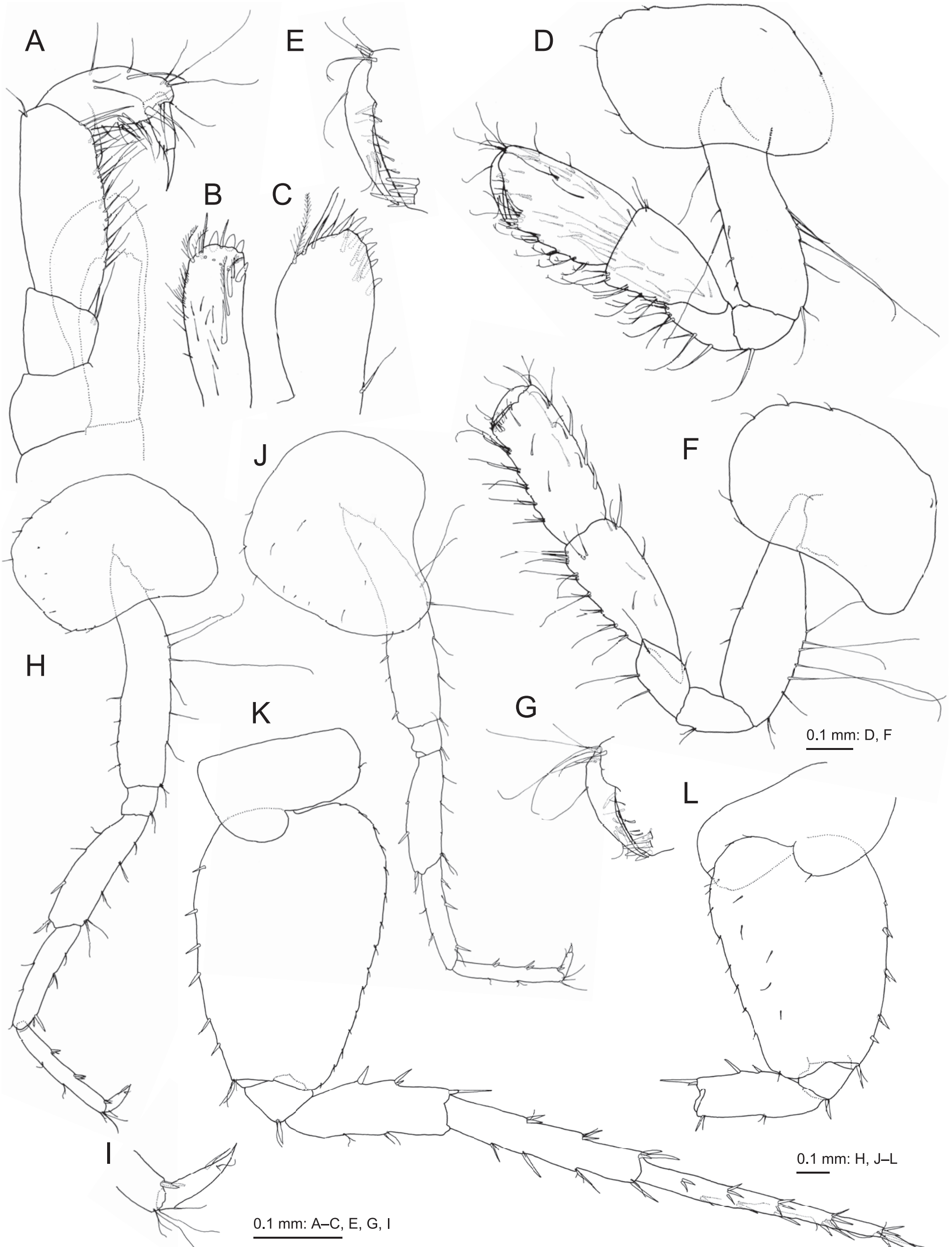

Figure 7. *Eoniphargus iwataorum* sp. nov., female holotype (NSMT-Cr 30782) **A** maxilliped, dorsal view **B** inner plate of maxilliped, dorsal view **C** outer plate of maxilliped, dorsal view **D** gnathopod 1, lateral view **E** palmar margin and dactylus of gnathopod 1, medial view **F** gnathopod 2, lateral view **G** palmar margin and dactylus of gnathopod 2, medial view **H** pereopod 3, lateral view **I** dactylus of pereopod 3, lateral view **J** pereopod 4, lateral view **K** pereopod 5, lateral view **L** pereopod 6, lateral view.

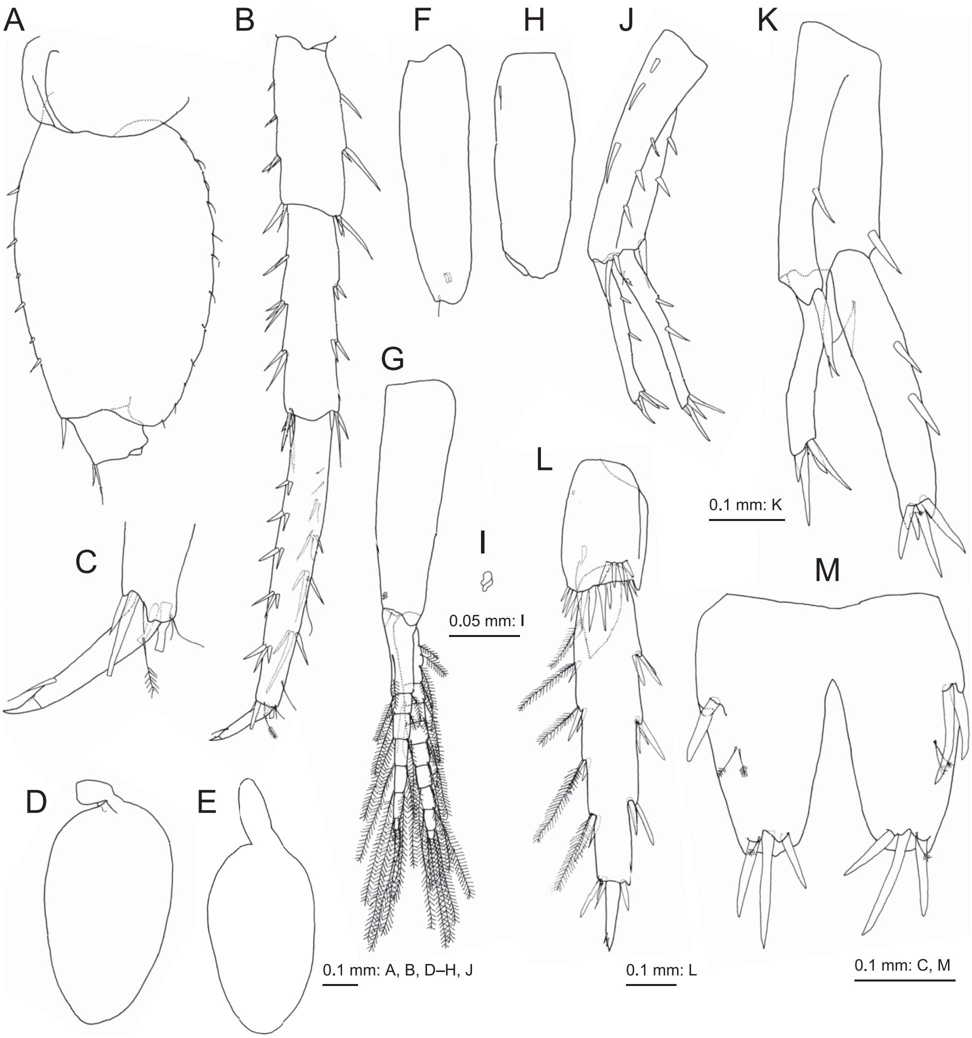

Figure 8. *Eoniphargus iwataorum* sp. nov., female holotype (NSMT-Cr 30782) **A, B** pereopod 7, lateral views **C** dactylus of pereopod 3, lateral view **D** coxal gill 4, lateral view **E** coxal gill 6, lateral view **F** peduncle of pleopod 1, lateral view **G** pleopod 2, medial view **H** peduncle of pleopod 3, lateral view **I** retinacula of pleopod 2, medial view **J** uropod 1, dorsal view **K** uropod 2, dorsal view **L** uropod 3, ventral view **M** telson, dorsal view.

Uropod 1 (Fig. 8J) with peduncle bearing three basofacial robust setae and dorsal robust setae; inner ramus length 0.7 times as long as peduncle, with two medial and one lateral robust setae, and ventroproximal seta; outer ramus length 0.9 times as long as inner ramus, with two robust setae on medial margin. Uropod 2 (Fig. 8K) with peduncle bearing dorsal robust setae; inner ramus as long as peduncle, bearing two medial robust setae and ventroproximal slender seta; outer ramus length 0.7 times as long as inner ramus, without marginal setae. Uropod 3 (Fig. 8L) with peduncle length 0.3

times as long as outer ramus, bearing robust setae on distal edge; inner ramus length 0.2 times as long as outer ramus, with seta on medial margin; outer ramus 2-articulate, proximal article with plumose setae on medial margin and robust setae on medial and lateral margins, terminal article length 0.2 times as long as proximal article with subapical setae.

Telson (Fig. 8M) length slightly shorter than wide, each lobe with two lateral and three apical robust setae and two dorsolateral penicillate setae, cleft for 67%.

Distribution and environment. The species is known only from its type locality in the Sabi River, Tochigi Prefecture, Japan.

Remarks. *Eoniphargus iwataorum* sp. nov. is similar to *E. kojimai* but differs from the latter in the following features (features of *E. kojimai* in parentheses): urosomite 3 without robust setae on dorsal margin (bearing robust setae), maxilla 2 with inner plate bearing seven setae in oblique row (nine setae), and uropod 2 without robust seta on lateral margin of outer ramus (bearing robust seta).

Eoniphargus kojimai Uéno, 1955

Figs 9–12
Japanese name: Kojima-chikayokoebi

Neoniphargus (*Eoniphargus*) *kojimai* Uéno, 1955: 148, figs 1–3.
Eoniphargus kojimai: Straškraba, 1964, 138; Straškraba 1967, 127; Bousfield 1977, 301; Barnard and Barnard 1983, 581.

Material examined. ♀ 6.3 mm (NSMT-Cr 30785, G1905), ♂ 4.8 mm (NSMT-Cr 30786), Mamashita Spring (35.680066°N, 139.428283°E), Kunitachi, Tokyo, Japan, collected by K. Tomikawa on 25 June 2016. ♀ 5.0 mm (NSMT-Cr 30787, G 1930), ♀ 4.5 mm (NSMT-Cr 30788, G 1931), Hinochūōtoshokan Spring (35.655783°N, 139.382133°E), Hino, Tokyo, Japan, collected by K. Tomikawa on 25 June 2016.

Diagnosis. Urosomite 3 with dorsal robust setae. Epimeral plates 2–3 each with ventral robust seta. Peduncular article 1 of antenna 1 with robust seta on posterodistal corner. Antenna 2 with peduncular article 2 gland cone not exceeding end of article 3; calceoli present in male. Mandible with 5- or 6-dentate left incisor; 4- or 5-dentate left lacinia mobilis, right one bifid with many teeth. Inner plate of maxilla 1 with eight plumose setae. Inner plate of maxilla 2 with nine facial seta in oblique row. Peduncle of pleopod 3 without seta. Uropod 1 with peduncle bearing basofacial robust setae. Uropod 3 with inner ramus 0.25 times as long as outer ramus in female and 0.27 times in male; outer ramus 2-ariticulate, with plumose setae on medial margin. Telson length 0.9 times width, cleft for 69% of length.

Description. Female (NSMT-Cr 30785). Head (Fig. 9A) as long as pereonites 1 and 2 combined; eyes absent; inferior antennal sinus distinct with rounded angle. Dorsal margins of pleonites 1–3 (Fig. 9B–D) each with 4 setae. Posterodistal corners of epimeral plates 1–3 (Fig. 9E–G) weakly produced; ventral margin of epimeral plate 1 without setae, epimeral plates 2 and 3 each with ventral robust seta; posterior margins of epimeral

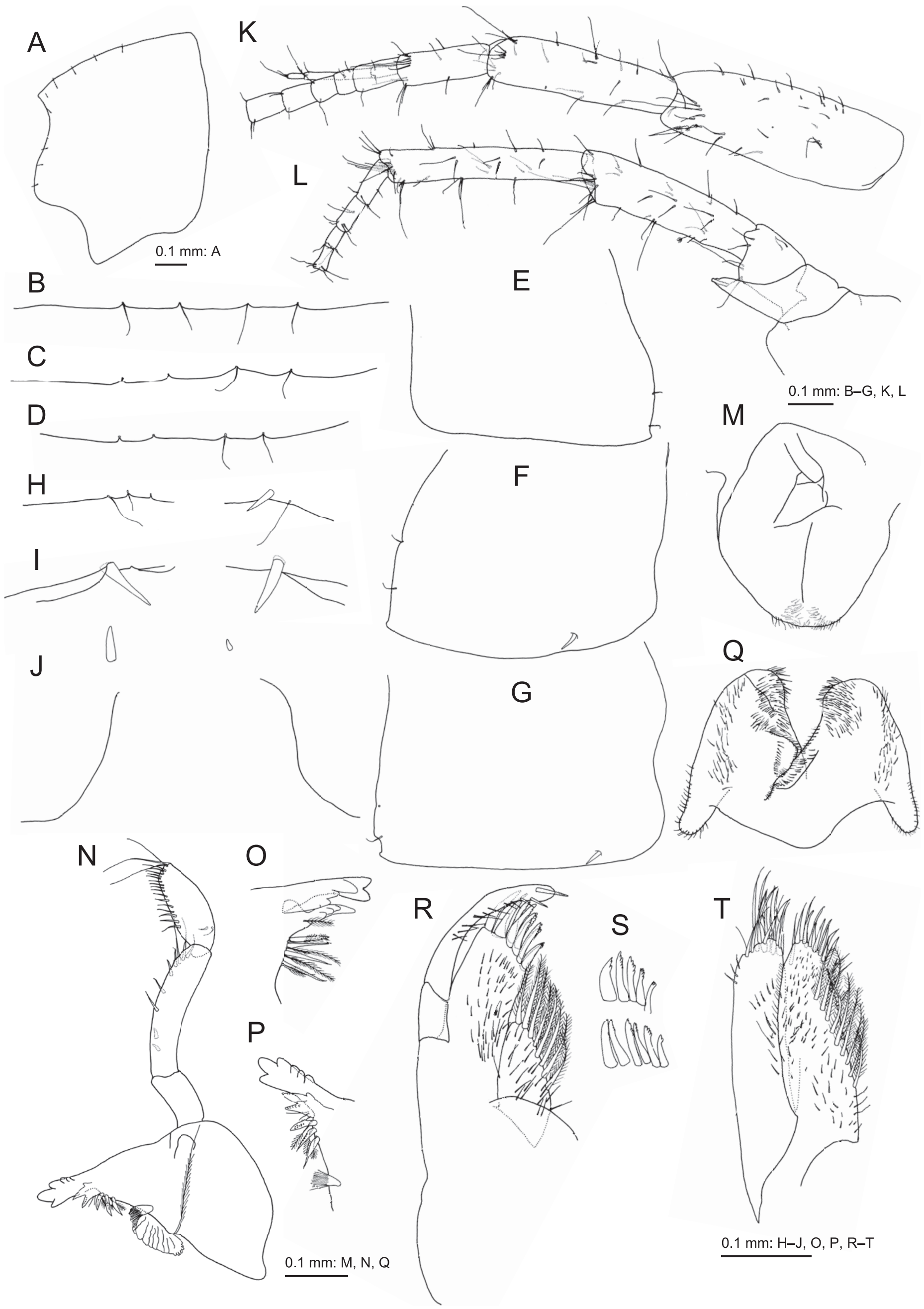

Figure 9. *Eoniphargus kojimai* Uéno, 1955, female (NSMT-Cr 30785) **A** head, lateral view **B–D** dorsal margins of pleonites 1–3, dorsal views **E–G** epimeral plates 1–3, lateral views **H–J** dorsal margins of urosomites 1–3, dorsal views **K** antenna 1 (some flagellar articles are omitted), lateral view **L** antenna 2 (some flagellar articles are omitted), medial view **M** upper lip, posterior view **N** right mandible, medial view **O** incisor and lacinia mobilis of left mandible, medial view **P** incisor and lacinia mobilis of right mandible, medial view **Q** lower lip, anterior view **R** maxilla 1, dorsal view **S** apical robust setae on outer plate of maxilla 1, dorsal view **T** maxilla 2, dorsal view.

plates 1–3 each with two setae. Dorsal margin of urosomite 1 (Fig. 9H) with slender setae and robust seta; urosomites 2 and 3 (Fig. 9I, J) with pair of dorsal robust setae.

Antenna 1 (Fig. 9K) length 0.22 times as long as body; length ratio of peduncular articles 1–3 in 1.0 : 0.8 : 0.4; posterodistal corner of peduncular article 1 with robust seta; accessory flagellum 4-articulate; primary flagellum 26-articulate. Antenna 2 (Fig. 9L) length 0.6 times as long as antenna 1; peduncular article 4 length 0.9 times as long as article 5; flagellum 13-articulate; calceoli absent.

Upper lip (Fig. 9M) with rounded apical margin bearing fine setae. Mandibles (Fig. 9N–P) with left and right incisors comprising five and six teeth, respectively; left lacinia mobilis comprising four teeth, right lacinia mobilis bifid with many teeth; molar process triturative with plumose seta; length ratio of palp articles 1–3 in 1.0 : 2.3 : 1.7; palp article 1 without setae; palp article 2 with nine marginal setae; palp article 3 with pair of A-setae, several D-setae and three E-setae, lateral face with fine setae. Lower lip (Fig. 9Q) lacking inner lobes; outer lobes broad, shoulder rounded, with fine setae. Maxilla 1 (Fig. 9R, S) with subtriangular inner lobe, bearing eight plumose setae on medial margin; outer plate rectangular, with 11 serrate robust setae apically; palp 2-articulate, article 1 without setae, article 2 with three robust setae and slender plumose seta along apically to medial margins. Maxilla 2 (Fig. 9T) with inner plate bearing nine plumose setae in oblique row. Maxilliped (Fig. 10A–C) with inner plate exceeding end of palp article 1, subquadrate, bearing three subapical robust setae and medial robust seta; outer plate ovate, reaching middle of palp article 2, with row of robust setae along apical to medial margins; palp 4-articulate, article 2 longest with medial setae, nail of article 4 distinct.

Gnathopod 1 (Fig. 10D, E) with subquadrate coxa bearing setae along anterior to ventral margins; anterior and posterior margins of basis with long setae; carpus length 1.1 times as long as length of propodus and 1.7 times width of carpus; propodus length 1.9 times width, palmar margin weakly serrate, oblique, with three medial and two lateral robust setae; dactylus almost reaching posterodistal corner of propodus. Gnathopod 2 (Fig. 10F, G) with coxa expanded proximally bearing setae along anterior to ventral margins; posterior margin of basis with long setae; carpus length 1.2 times as long as length of propodus and 3.3 times width of carpus; propodus length 2.8 times width, palmar margin weakly serrate, almost vertical, with two medial and one lateral robust setae; dactylus reaching posterodistal corner of propodus.

Pereopod 3 (Fig. 10H, I) with subquadrate coxa, proximally expanded, bearing setae along anterior to ventral margins; anteroproximal and posterior margins of basis with long setae. Pereopod 4 (Fig. 10J) with coxa bearing setae along anterior to ventral margins, posteroproximally concave; anteroproximal and posterior margins of basis with long setae. Pereopod 5 (Fig. 10K) with bilobed coxa bearing seta on anterior and posterior lobes; anterior margin of basis with robust setae, posterodistal corner weakly lobate. Pereopod 6 (Fig. 11A) with bilobed coxa bearing two setae on posterior lobe; basis with robust setae on anterior margin, posterodistal corner weakly lobate. Pereopod 7 (Fig. 11B) with elliptical basis bearing robust setae on anterior margin, posterodistal corner weakly lobate.

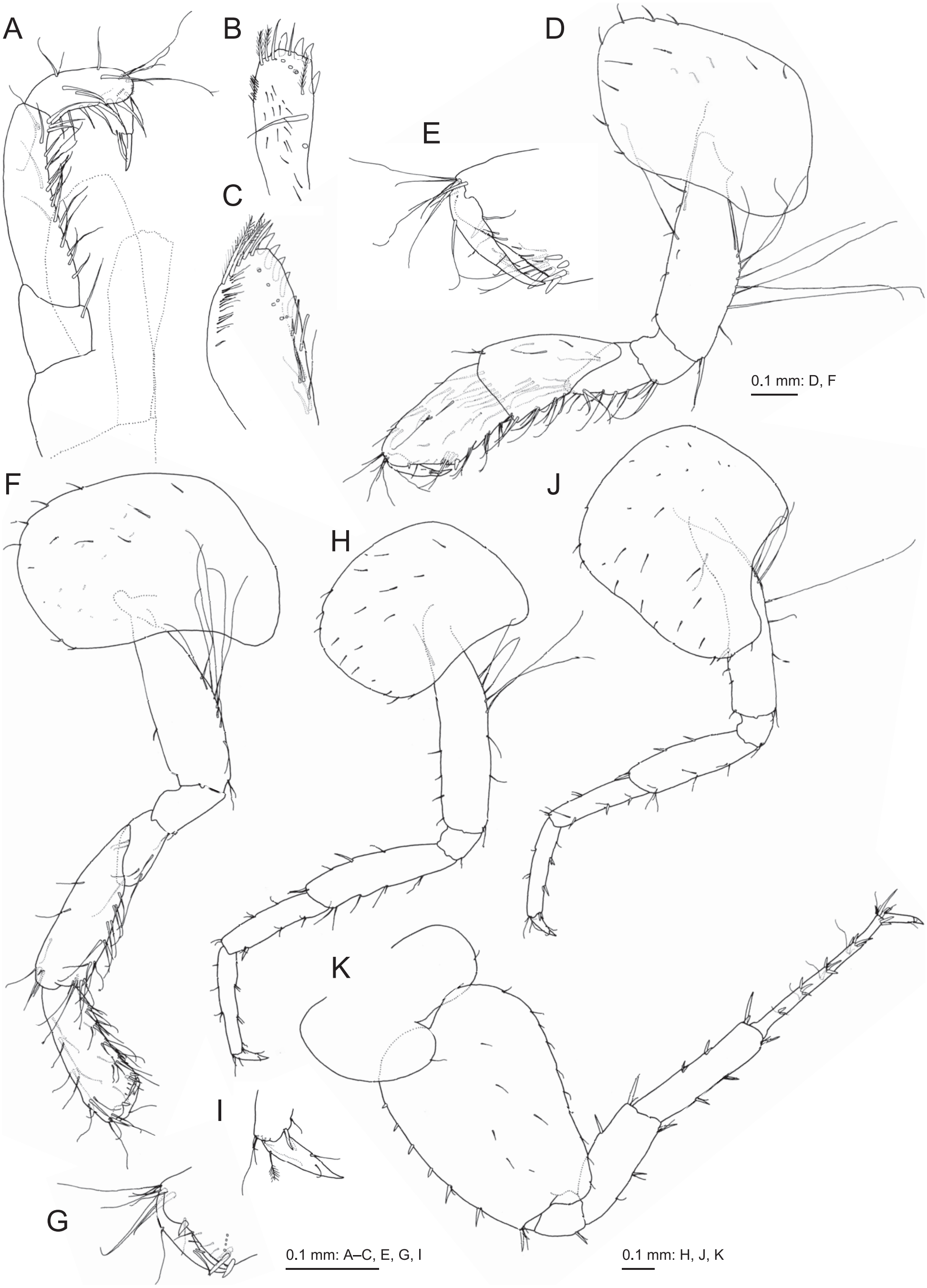

Figure 10. *Eoniphargus kojimai* Uéno, 1955, female (NSMT-Cr 30785) **A** maxilliped, dorsal view **B** inner plate of maxilliped, dorsal view **C** outer plate of maxilliped, dorsal view **D** gnathopod 1, lateral view **E** palmar margin and dactylus of gnathopod 1, medial view **F** gnathopod 2, lateral view **G** palmar margin and dactylus of gnathopod 2, medial view **H** pereopod 3, lateral view **I** dactylus of pereopod 3, lateral view **J** pereopod 4, lateral view **K** pereopod 5, lateral view.

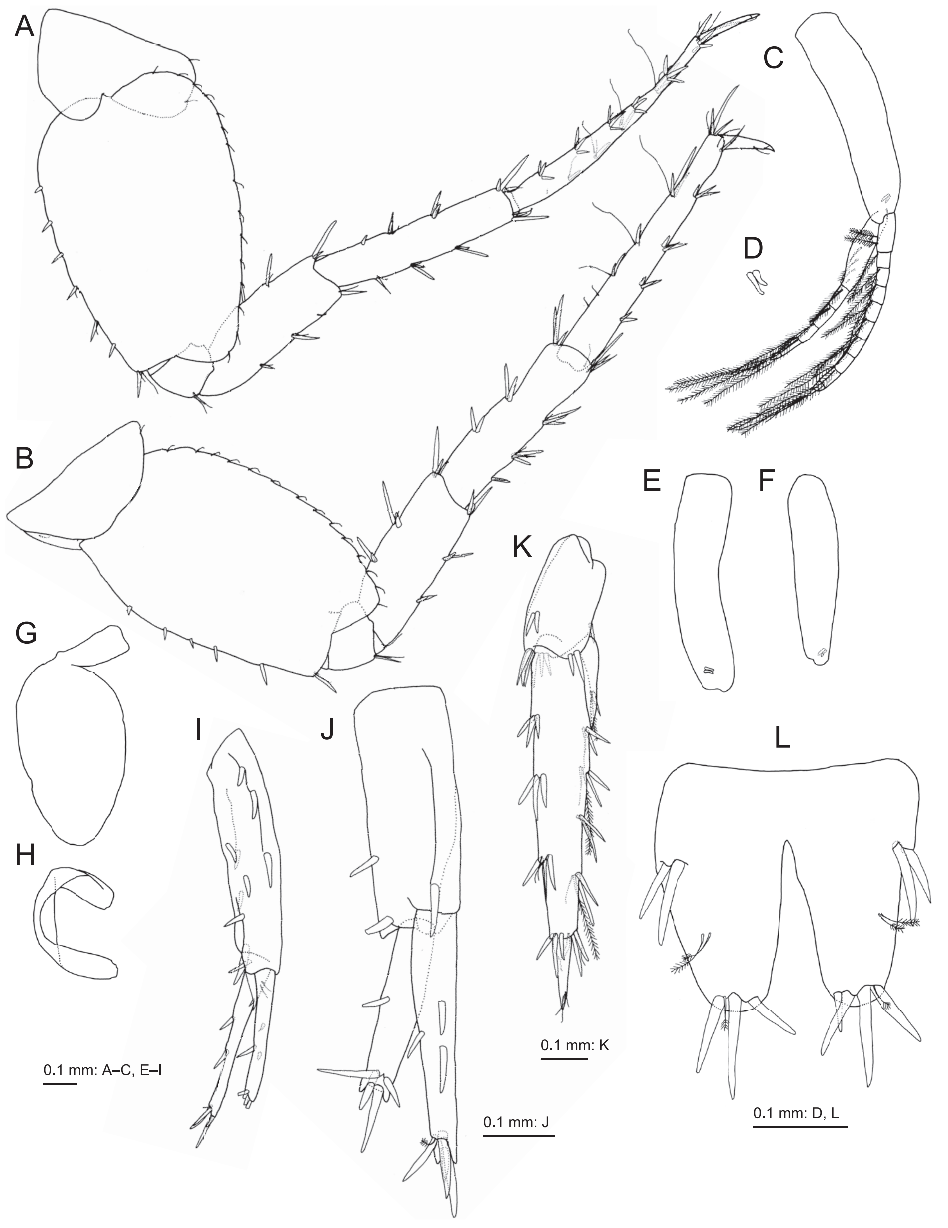

Figure 11. *Eoniphargus kojimai* Uéno, 1955, female (NSMT-Cr 30785) **A** pereopod 6, lateral view **B** pereopod 7, lateral views **C** pleopod 1, lateral view **D** retinacula of pleopod 1, lateral view **E** peduncle of pleopod 2, medial view **F** peduncle of pleopod 3 **G** coxal gill 4, lateral view **H** oostegite of pereopod 4, medial view **I** uropod 1, dorsal view **J** uropod 2, dorsal view **K** uropod 3, dorsal view **L** telson, dorsal view.

Coxal gills (Fig. 11G) ovate with stalks on gnathopod 2 and pereopods 3–6.

Pleopods 1–3 (Fig. 11C–F) with peduncles longer than rami, bearing distal seta on pleopod 1; retinacula paired (Fig. 11D); mediobasal margin of inner ramus with bifid plumose setae; rami well developed.

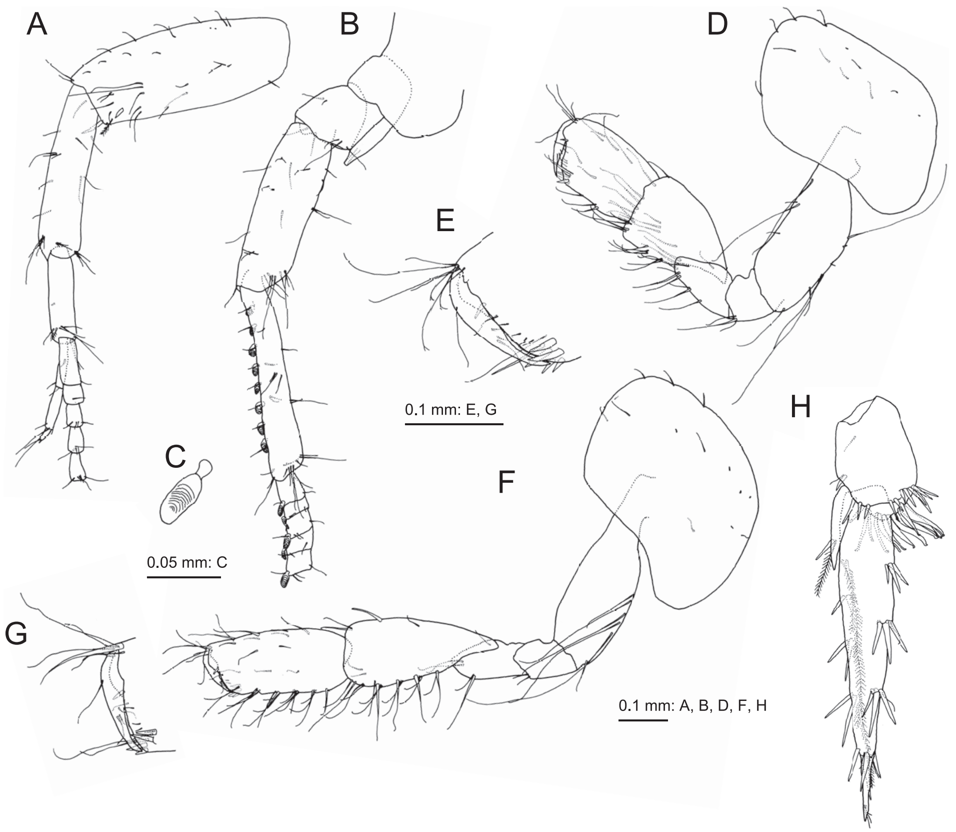

Figure 12. *Eoniphargus kojimai* Uéno, 1955, male (NSMT-Cr 30786) **A** antenna 1 (some flagellar articles are omitted), lateral view **B** antenna 2 (some flagellar articles are omitted), lateral view **C** calceolus on flagellar article of antenna 2, medial view **D** gnathopod 1, lateral view **E** palmar margin and dactylus of gnathopod 1, medial view **F** gnathopod 2, lateral view **G** palmar margin and dactylus of gnathopod 2, medial view **H** uropod 3, dorsal view.

Uropod 1 (Fig. 11I) with peduncle bearing three basofacial robust setae and dorsal robust setae; inner ramus length 0.6 times as long as peduncle, with two medial robust setae and two ventroproximal setae; outer ramus length 0.9 times as long as inner ramus, with two medial and one lateral robust setae. Uropod 2 (Fig. 11J) with peduncle bearing dorsal robust setae; inner ramus as long as peduncle, bearing two medial and one lateral robust setae; outer ramus length 0.7 times as long as inner ramus, with lateral robust seta. Uropod 3 (Fig. 11K) with peduncle length 0.3 times as long as outer ramus, bearing robust setae on distal edge; inner ramus length 0.3 times as long as outer ramus, with seta on medial margin; outer ramus 2-articulate, proximal article with plumose setae on medial margin and robust setae on medial and lateral margins, terminal article length 0.2 times as long as proximal article with subapical setae.

Telson (Fig. 11L) length slightly shorter than width, each lobe with two lateral and three apical robust setae, and two dorsolateral and 1 subapical penicillate setae, cleft for 69%.

Male (NSMT-Cr 30786). Antenna 1 (Fig. 12A) length 0.24 times as long as body length; accessory flagellum 3-articulate; primary flagellum 25-articulate. Antenna 2 (Fig. 12B) length 0.6 times as long as antenna 1; peduncular article 4 length 0.8 times as long as peduncular article 5; peduncular article 5 and flagellum with calceoli (Fig. 12C); flagellum 16-articulate. Gnathopod 1 (Fig. 12D, E) with carpus length 1.2 times as long as length of propodus and 1.6 times width of carpus; propodus length 1.5 times width. Gnathopod 2 (Fig. 12F, G) with carpus length 1.1 times as long as length of propodus and 2.3 times width of carpus; propodus length 2.2 times width, palmar margin with two medial and two lateral robust setae. Uropod 3 (Fig. 12H) with peduncle length 0.4 times as long as outer ramus, peduncle bearing bent robust setae on distal edge.

Distribution and environment. This species has been found in interstitial waters in Tokyo: the sand-filter bed of the Komae Purification Plant near the Tama River (Uéno 1955); two springs — Mamashita Spring, Kunitachi and Hinochūōtoshokan Spring, Hino (this study).

Remarks. The present specimens conform with the original description of *E. kojimai* by Uéno (1955). However, our specimens differed from the original description in the left mandible with 4-dentate lacinia mobilis and maxilla 1 with 11 serrate robust setae on the outer plate, which was 6-dentate incisor and 5-dentate lacinia mobilis of the left mandible, and 10 serrate robust setae in Uéno's (1955) description. The exact number of these setae is difficult to ascertain because they are minute and overlap. This suggests that he may have misstated the number of setae. Unfortunately, the type specimen of this species is believed to be lost (Tomikawa et al. 2007), so the character could not be verified. Examination of the present material from Kunitachi and Hino, Tokyo revealed some features that were not mentioned in the original description: maxilla 2 with inner plate bearing nine plumose setae in oblique row, uropod 1 peduncle with facial robust setae, and maxilliped inner plate with three subapical robust setae and medial robust seta.

Key to species of *Eoniphargus*

1	Antennal sinus shallow; antenna 1 peduncular article 1 with slender seta on posterodistal corner; antenna 2 peduncular article 2 with elongate gland cone exceeding peduncular article 3; uropod 1 peduncle without basofacial robust setae; uropod 3 with uniarticulate outer ramus .... ***E. glandulatus* Stock & Jo, 1990**
–	Antennal sinus deep; antenna 1 peduncular article 1 with robust seta on posterodistal corner; antenna 2 peduncular article 2 with gland cone reaching distal end of peduncular article 3; uropod 1 peduncle with basofacial robust setae; uropod 3 with 2-articulate outer ramus **2**
2	Right mandible with lacinia mobilis bearing 3 or 4 teeth; maxilla 1 with 6 medial setae on inner plate..***E. toriii* sp. nov.**
–	Right mandible with bifid lacinia mobilis bearing many teeth; maxilla 1 with 8 medial setae on inner plate..**3**

3 Urosomite 3 with robust setae on dorsal margin; maxilla 2 with inner plate bearing 9 setae in oblique row; uropod 2 with robust seta on lateral margin of outer ramus .... ***E. kojimai*** **(Uéno, 1955)**

– Urosomite 3 without robust setae on dorsal margin; maxilla 2 with inner plate bearing 7 setae in oblique row; uropod 2 without robust seta on lateral margin of outer ramus .... ***E. iwataorum*** **sp. nov.**

Molecular phylogenetic analyses

The obtained ML tree showed a topology identical to that of the BI tree (Fig. 13). The monophyly of Mesogammaridae was shown with the highest support (100% bootstrap support [BS] and 1.0 Bayesian posterior probability [BPP] in the ML and BI trees, respectively). Mesogammaridae formed a monophyletic group with two gammarids genera, *Barnardiorum* Iwan & Löbl, 2007 and *Rhipidogammarus* Stock, 1971 (BS = 99%, BPP = 1.0). Within Mesogammaridae, the first split formed *Mesogammarus* and (*Eoniphargus* + *Octopupilla*) (BS = 69%, BPP = 1.0). *Eoniphargus kojimai* is sister to *E. iwataorum* sp. nov. (BS = 76%, BPP = 1.0). The uncorrected pairwise distances among the three *Eoniphargus* species are 16.9–19.8% for COI and 1.3% for 28S.

Discussion

Although there are many taxonomic studies on the amphipods that occur in East Asian groundwaters (Akatsuka and Komai 1922; Derzhavin 1927; Uéno 1927, 1934), their species diversity is not fully understood. To date, studies on the amphipods in groundwater have been based on specimens from small streams in caves that are relatively easily accessible and incidentally collected from dug wells (Tomikawa et al 2008; Tomikawa and Nakano 2018). Recently, a variety of groundwater amphipods have been reported to occur in the interstitial waters of rivers and springs (e.g. Tomikawa et al. 2007), but little is known about their species diversity and evolutionary history.

In this study, we conducted a molecular phylogenetic analysis of Mesogammaridae, including one marine genus, *Mesogammarus*, and two subterranean genera, *Eoniphargus* and *Octopupilla*, and showed that they form a monophyletic group (Fig. 13). Our phylogenetic trees show that *Mesogammarus* was the first to diverge within Mesogammaridae, followed by *Octopupilla* and *Eoniphargus*. *Mesogammarus* occurs under coastal cobbles, *Octopupilla* in brackish and freshwater interstitial environments, and *Eoniphargus* only in freshwater groundwater (Tzvetkova 1965; Tomikawa et al. 2007). This suggests that species diversification in Mesogammaridae likely occurred through the adaptive dispersal of epigean marine ancestor into brackish and freshwater groundwater environments.

Mesogammaridae formed a monophyletic group with gammarid genera *Barnardiorum* and *Rhipidogammarus*. *Barnardiorum* occurs in epigean freshwaters in Tajikistan and Afghanistan, and *Rhipidogammarus* in brackish and freshwater groundwaters (interstitial waters) in the Mediterranean belt (Barnard and Barnard 1983;

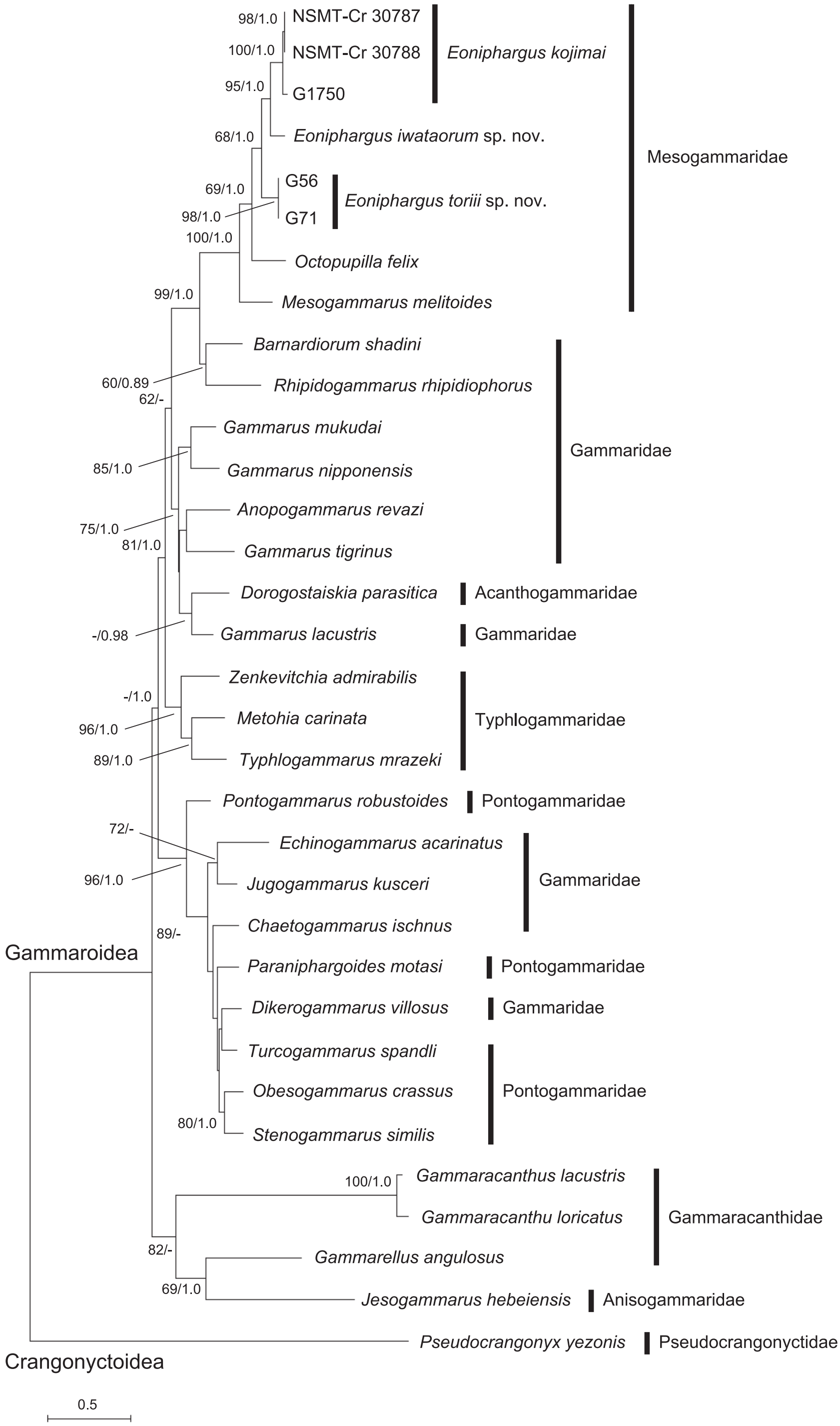

Figure 13. Maximum likelihood tree for 1550 bp of nuclear 28S rRNA and mitochondrial cytochrome *c* oxidase subunit I markers. Numbers at nodes represent maximum likelihood bootstrap values and Bayesian posterior probabilities (Values below 60% for the former and 0.8 for the latter are omitted).

Özbek and Sket 2020). Hou et al. (2014) showed the Tethys Sea origin of Gammaridae and that geohistorical and environmental changes in the Tethys Sea facilitated the diversification of this group. Although the details of the evolutionary history of Mesogammaridae and related groups are not clear in this study, judging from the fact that their distribution extends from the North Pacific coast to Central Asia and the Mediterranean Sea, ancestral species that inhabited the coastal surface waters of the Tethys Sea may have diversified by adapting to different salinity and subterranean environments. *Barnardiorum* and *Rhipidogammarus* are presently treated as members of Gammaridae. Our results challenge their family affiliation, but the low statistical support for the results of the phylogenetic analyses in this study precluded further discussion of the monophyly of Gammaridae.

Prior to this study, two species of *Eoniphargus*, *E. kojimai* and *E. glandulatus*, were found in the interstitial waters in Japan and cave pools in the Korean Peninsula, respectively. In this study, we described two new species, *E. iwataorum* and *E. toriii*, found in the Kanto region of Japan, indicating that the species diversity of amphipods in interstitial water is higher than previously thought. Interestingly, *Eoniphargus* mainly occurs in interstitial waters and is rarely reported from the groundwater in caves. In contrast, the groundwater amphipod genus *Pseudocrangonyx* Akatsuka & Komai, 1922 is widely distributed in East Asian groundwater systems, and species of this genus often occur in cave groundwater (Lee and Min 2020). Speciation in subterranean environments is commonly a consequence of geographic isolation, as subterranean environments are always dark, oligotrophic, and ecological niches are narrow (Mammola and Isaia 2016; Mammola et al. 2018). Recently, however, the potential for diversification through niche differentiation has been demonstrated in *Niphargus* amphipods and *Troglohyphantes* spiders, even in energy-poor environments such as underground habitats (Fišer et al. 2013; Mammola et al. 2018). In *Eoniphargus* and *Pseudocrangonyx*, the choice between different groundwater environments, relatively open cave groundwater, and interstitial water may have played a role in the differentiation of the two genera.

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