

On the Brink of Extinction: A New Freshwater Amphipod *Jesogammarus Acalceolus* (Anisogammaridae) From Japan

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Research Article

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Abstract

Freshwater habitats, especially cold springs, are environments in which the extinction risk faced by organisms remains high due to human activities. The extinction risks faced by many species go unrecognized prior to their extinction. To conserve endangered species, it is important to describe and name them. Here, we describe a new, endangered freshwater anisogammarid amphipod species, *Jesogammarus (Jesogammarus) acalceolus* sp. nov., found in a spring in Aomori Prefecture, Japan, which is potentially the sole remaining habitat of this species. Both morphological and molecular phylogenetic results strongly support the nesting of the new species within *Jesogammarus*. *Jesogammarus (J.) acalceolus* sp. nov. is the first species of genus *Jesogammarus* that was found to lack a calceolus, a sensory organ located on male antenna 2. Thus, the diagnostic criteria for this genus required amendment. A reconstruction of ancestral calceoli, based on a molecular phylogenetic tree, revealed that the common ancestor of *Jesogammarus* possessed calceoli, which were secondarily lost in *J. (J.) acalceolus* sp. nov. Our results indicate that this new species, which is key to clarifying the evolution of the calceolus, is of high conservation significance.

Introduction

Freshwater is indispensable to human life. It is also an important habitat for many aquatic organisms. Freshwater accounts for about 2.5% of all water on Earth [1]. Approximately 9.5% of all known species live in fresh water [2]. Deterioration of freshwater environments due to human activities remains a worldwide issue [3, 4]. Species inhabiting freshwater habitats are reported to be at a greater risk of extinction compared to marine and terrestrial species [5–7].

Spring water is ground water that collects in soil due to rain and snow in mountainous areas. Recently, deterioration of spring water environments, leading to the depletion of spring water, caused by an inflow of domestic drainage and agricultural chemicals as well as excessive pumping of groundwater for drinking and agricultural purposes has become an issue of worldwide proportions. Therefore, of the species inhabiting freshwater habitats, those that depend on spring water are considered to be at an even higher risk of extinction [8]. However, currently available taxonomic data on invertebrates inhabiting spring water appear to be insufficient, with many species remaining undescribed [9]. Although the discovery rate of species appears to be increasing, many species go unrecognized before becoming extinct [10]. Thus, conducting taxonomic studies as well as naming and describing species is essential for conserving endangered species [11–15].

The order Amphipoda comprises peracarid crustaceans belonging to the class Malacostraca. Of the more than 10,000 amphipod species that have been described globally, approximately 20% occur in freshwater [16, 17]. Freshwater amphipods generally prefer cool environments [16], and cold spring water and flowing spring water are the best habitats for them. Springs in the Japanese archipelago reportedly harbour diverse endemic amphipods [18–20]. The anisogammarid genus, *Jesogammarus* Bousfield, 1979, is the most diverse group among Japanese freshwater amphipods. *Jesogammarus* was established by Bousfield [21], with *Anisogammarus jesoensis* Schellenberg, 1937 as the type species. In the same paper as that which described this type species, Bousfield established *Annanogammarus* Bousfield, 1979 and *Ramellogammarus* Bousfield, 1979 with *Gammarus annandalei* Tattersall, 1922 and *Gammarus ramellus* Weckel, 1907 as type species, respectively. *Annanogammarus* was later classified as a subgenus under *Jesogammarus* by Morino [22]. At present, *Jesogammarus* is known to contain 22 species from the Japanese Archipelago, the Korean Peninsula, and the Chinese Continent [23]. *Jesogammarus* is morphologically similar to *Ramellogammarus*, which is endemic to North American coastal fresh waters; these genera are considered to be closely related [21, 24]. The former is distinguished from the latter mainly by having an antennal sensory organ termed the calceolus [22, 25]. However, though molecular phylogenetic studies have been conducted previously on Anisogammaridae, the phylogenetic relationship between *Jesogammarus* and *Ramellogammarus* has not yet been fully clarified; [26, 27, 28].

Recently, a population of *Jesogammarus* species, sans a calceolus on male antenna 2, was found in a spring in the Aomori Prefecture of Japan, which is potentially the sole remaining habitat of this species (Fig. 1). We describe this species as *J.*

(*J. acalceolus* sp. nov.). Describing and naming this species, as have been done here, can be considered important first steps leading to its conservation. In addition, we investigated the evolution of calceoli in *Jesogammarus* species with molecular phylogenetic analyses and ancestral state reconstruction.

Methods

Sample collection

Specimens of *J. (J.) acalceolus* sp. nov. were collected from Haguro Shrine Spring, Hirosaki, Aomori Prefecture, Japan (40.6153°N, 140.3854°E). Amphipods were collected by a fine-mesh hand net from fallen leaves and mosses. Specimens were fixed in 99% ethanol on the site.

Morphological observation

Appendages of the examined amphipods were dissected using needles under a stereomicroscope (Olympus SZX7) and mounted in gum-chloral medium on glass slides. Prepared specimens were examined by a light microscope (Nikon Eclipse Ni) and illustrated using the aid of a camera lucida attached to the light microscope. The body length was measured from the tip of the rostrum to the base of the telson along the dorsal curvature to the nearest 0.1 mm following Tomikawa et al. [23]. The specimens have been deposited in the Tsukuba Collection Center of the National Museum of Nature and Science, Tokyo (NSMT).

PCR and DNA sequencing

Genomic DNA was extracted from the pleopod muscle of the specimens following procedures detailed by Tomikawa et al. [42]. The primer sets for PCR and cycle sequencing reactions used in this study were as follows: for 28S rRNA (28S), 28SF and 28SR [43]; for cytochrome *c* oxidase subunit I (COI), Am-COI-H and Am-COI-T [37]; and for 16S rRNA (16S), 16STf [26] and 16Sbr [44]. PCR and DNA sequencing were performed using the method detailed by Tomikawa [37]. The newly obtained DNA sequence has been deposited in the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan (DDBJ) (Table 1).

Molecular phylogenetic analyses

The alignment of COI was trivial, as no indels were observed. The sequences of 28S and 16S were aligned using the Muscle algorithm implemented in MEGA X [45]. 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 and Bayesian Information Criterion (BIC) for BI using MEGA X [45]. ML phylogenies were conducted using MEGA X [45] under the substitution model GTR+G+I, and 1,000 bootstrap replications [46] were performed to estimate statistical support for branching patterns. BI analyses were estimated using MrBayes v3.2.6 [47] under the substitution model GTR+G+I, with Markov chains of 10 million generations. Parameter estimates and convergence were checked using Tracer v1.7.1 [48], and the first 1 million trees were discarded as burn-in. Two gammarid species, *Gammarus mukudai* Tomikawa, Soh, Kobayashi, and Yamaguchi, 2014 and *G. nipponensis* Uéno, 1966, were included in the analyses as outgroup taxa.

Ancestral state reconstruction

The ancestral states of the calceolus on male antenna 2 were reconstructed on the tree (Fig. 1) via the likelihood model using Mesquite v3.61 [49]. The Markov K-state 1 parameter model was used for likelihood reconstruction at each ancestral node with equal probability for all particular character state changes.

Results

Molecular phylogenetic analyses

The monophyly of *Jesogammarus* was inferred with maximum (100% bootstrap support [BS]) and relatively low (0.85 posterior probability [PP]) support values in the maximum likelihood (ML) and Bayesian inference tree (BI) trees, respectively (Fig. 2). Although, *Jesogammarus* formed a sister group with *Barrowgammarus* Bousfield, 1979 (87% BS), their relationship was not supported by BI analyses. The new species collected in this study, *J. (J.) acalceolus*, was nested within *Jesogammarus* and clustered with *J. (J.) hinumensis* Morino, 1993 and *J. (J.) ikiensis* Tomikawa, 2015. In this study, the phylogenetic position of *J. (A.) koreanus* was also clarified for the first time: this species formed a sister group with *J. (A.) debilis*, with high support values (98% BS, 1.0 PP). Of the 22 species of *Jesogammarus*, 20, excluding *Jesogammarus (Jesogammarus) fontanus* Hou & Li, 2004 and *Jesogammarus (Jesogammarus) ilhoii* Lee & Seo, 1992, were included in the molecular phylogenetic analyses of this study.

Ancestral state reconstruction

The likelihood reconstruction (Fig. 2) demonstrated that the calceolus on male antenna 2 was an ancestral character state of the most recent common ancestor (MRCA) of the *Jesogammarus* species, with 0.96 proportional likelihood (PL). The character state of the MRCA of *J. (J.) acalceolus* sp. nov. + *J. (J.) hinumensis* and *J. (J.) acalceolus* sp. nov. + *J. (J.) hinumensis* + *J. (J.) ikiensis* was the presence of calceolus, with 0.92 and 0.99 PL, respectively. The character state of the MRCA of *Barrowgammarus* + *Eogammarus* + *Jesogammarus* + *Ramellogammarus* was the presence of calceolus, with 0.52 PL.

Taxonomic account

Family Anisogammaridae Bousfield, 1977

Genus *Jesogammarus* Bousfield, 1979

Type species. *Anisogammarus jesoensis* Schellenberg, 1937

Diagnosis. Pleonites not carinate dorsally, with slender and robust setae (robust setae often lacking). Dorsal margins of urosomites with 4 (3), 4 (2), 2 (4) clusters or single robust setae; urosomite 2 without prominent median tooth. Antenna 1 longer than antenna 2; article 1 of peduncle subequal to or slightly longer than article 2. Male antenna 2, flagellum with or without calceoli. Maxilla 1, palp article 1 without setae. Female gnathopods 1 and 2 strongly dissimilar. Coxal gills on gnathopod 2 and pereopods 3–7, gills 2–5 each with 2 accessory lobes, gills 6 and 7 each with 1 accessory lobe. Uropods 1 and 2, rami extending beyond peduncle of uropod 3. Uropod 3, inner ramus not longer than 0.4 times of that of outer ramus; terminal article distinct. Brood plate 2 of female broadly expanded anteroproximally.

Remarks. The presence of a calceolus on the flagellum of male antenna 2 is a major diagnostic feature of *Jesogammarus*, which distinguishes it from *Ramellogammarus* [21,22]. However, the discovery of the new species, *J. acalceolus*, which lacks a calceolus, indicated that the calceolus was not critical for diagnosis. The genus *Jesogammarus* is distinguishable from *Ramellogammarus* by the dissimilar female gnathopods 1 and 2 and the expanded brood plates of the female. The genus *Jesogammarus* shares a similar coxal gill type with marine *Locustogammarus* Bousfield, 1979 and *Spasskogammarus* Bousfield, 1979 but differs from these two genera in terms of the following features (features of *Locustogammarus* and *Spasskogammarus* in parentheses): from *Locustogammarus*, in terms of longer antenna 1 than antenna 2 (subequal), dissimilar female gnathopods 1 and 2 (similar), uropods 1 and 2 with rami extending beyond the peduncle of uropod 3 (not extending), and a distinct terminal article of uropod 3 (very small); from *Spasskogammarus*, in terms of dorsal margins of pleonites with slender setae (lacking), longer antenna 1 than antenna 2 (subequal), and slender pereopods 5–7 (short).

Jesogammarus (J.) acalceolus sp. nov.

[New Japanese name: Shitsuko-yokoebi]

Figures 1B, 3–5

Material examined. Holotype: male (7.4 mm, NSMT-Cr 29003), Haguro Shrine Spring, Hirosaki, Aomori Prefecture, Japan (40.6153°N, 140.3854°E), collected by A. Ohtaka, N. Kimura, and K. Tomikawa on 10 December 2020. Paratypes: two females (7.3 mm, NSMT-Cr 29004; 6.7 mm, NSMT-Cr 29005 [G1845]), two male (7.7 mm, NSMT-Cr 29006; 7.5 mm, NSMT-Cr 29007 [G1844]), data same as for the holotype; male (6.8 mm, NSMT-Cr 29008 [G1625]), same locality of the holotype, collected by A. Ohtaka on 23 December 2018; 3 males (7.3–7.6 mm, NSMT-Cr 29009) and three females (6.4–7.3 mm, NSMT-Cr 29009), same locality of the holotype, collected by A. Ohtaka on 17 June 2018; 3 males (5.8–8.0 mm, NSMT-Cr 29009) and three females (5.3–6.4 mm, NSMT-Cr 29009), same locality of the holotype, collected by N. Kimura on 23 December 2018; seven males (7.6–8.8 mm, NSMT-Cr 29009) and three females (5.6–6.6 mm, NSMT-Cr 29009), same locality of the holotype, collected by N. Kimura on 10 December 2020; 10 males (6.9–9.9 mm, NSMT-Cr 29009) and 11 females (5.9–8.3 mm, NSMT-Cr 29009), same locality of the holotype, collected by A. Ohtaka on N. Kimura on 12 December 2020.

Description. Male [7.4 mm, NSMT-Cr 29003]. Head (Fig. 3), rostrum short; lateral cephalic lobe with ventral margin weakly concave; antennal sinus rounded; eyes small, subreniform, major axis $0.3 \times$ head height. Pereonites, dorsal surfaces smooth (Fig. 3). Pleonites 1–3 (Fig. 3B–D), dorsal margins each with 3, 2, and 2 setae. Epimeral plate 1 with rounded posterior margin bearing seta, seta on posteroventral corner (Fig. 3A); epimeral plate 2 with posterior margin almost straight bearing 3 setae, seta on weakly produced posteroventral corner, 2 and 1 robust setae on ventral margin and submargin, respectively (Fig. 3A); epimeral plate 3 with posterior margin almost straight bearing 3 setae, seta on quadrate posteroventral corner, 3 robust setae on ventral margin (Fig. 3A). Urosomite 1 (Fig. 3E) with dorsal margin bearing a pair of lateral robust setae and a middle cluster of robust setae; urosomite 2 (Fig. 3F) with dorsal margin bearing a pair of lateral robust setae and clusters of robust setae; urosomite 3 (Fig. 3G) with dorsal margin bearing a pair of robust setae.

Antenna 1 (Fig. 3H) $0.6 \times$ length of body; length ratio of peduncular articles 1–3 $1.0 : 0.9 : 0.6$; peduncular article 1 with posterodistal corner lacking robust seta, posterior margin with 3 pairs of setae and single seta; peduncular article 2 with posterior margin bearing 6 clusters of setae; peduncular article 3 with posterior margin bearing 4 clusters of setae; accessory flagellum comprising 4 articles; primary flagellum comprising 20 articulate, aesthetasc on each article. Antenna 2 (Fig. 3J) $0.7 \times$ length of antenna 1; article 4 of peduncular $1.1 \times$ article 5; peduncular articles 4 and 5 with posterior margins each bearing 5 setal clusters; flagellum comprising 12 articles, calceoli absent.

Upper lip (Fig. 3K) with fine seta on rounded distal margin. Mandibles (Fig. 3L–N), left and right incisors comprising 5 and 4 teeth, respectively, left lacinia mobilis comprising 4 teeth, right lacinia mobilis bifid with many denticles; molar process triturative with plumose seta; left and right mandibles with 7 and 5 blade-like setae on accessory setal rows, respectively; palp comprising 3 articles with length ratio of $1.0 : 3.3 : 2.8$; article 1 of palp without setae; article 2 with 25 setae; article 3 bearing pair of setae on inner surface, 3 clusters of setae and single seta on outer surface. Lower lip (Fig. 3O), outer lobes broad, inner lobes indistinct. Maxilla 1 (Fig. 3P) with medial margin of inner plate bearing 20 plumose setae; 11 serrate robust setae on outer plate apically (Fig. 3Q); palp comprising 2 articles, article 1 marginally bare, apical margin of article 2 with 5 robust setae and 2 slender seta. Maxilla 2 (Fig. 4A) with inner plate bearing oblique inner row of 17 plumose setae. Maxilliped (Fig. 4B) with inner plate bearing 3 apical and 2 inner marginal robust setae; outer plate, apical margin with plumose setae and inner margin with robust setae; palp comprising 4 articles, inner margin and submargin of article 2 with rows of setae, article 3 bearing facial setae, slightly curved article 4 with slender nail.

Gnathopod 1 (Fig. 4C, D) with coxa bearing marginal setae ventrally; basis with long setae on anterior and posterior margins; length of carpus $1.4 \times$ width, with seta on anterior margin; length of propodus $1.3 \times$ carpus and $1.4 \times$ width, bearing 2 clusters of setae on anterior margin, propodus with oblique and weakly convex palmar margin bearing 6 medial and 10 lateral peg-like robust setae; dactylus weakly curved, as long as palmar margin. Gnathopod 2 (Fig. 4E, F) with coxa

bearing marginal setae ventrally; masis with anterior and posterior margins bearing long setae; length of carpus $1.8 \times$ width, bearing setae on anterior margin; length of propodus $1.1 \times$ carpus and $1.6 \times$ width, respectively, with 2 clusters of setae on anterior margin, propodus with oblique and weakly convex anterior margin bearing 8 medial and 5 lateral peg-like robust setae; dactylus weakly curved, as long as palmar margin.

Pereopods 3 and 4 (Fig. 3A) similar, coxa of pereopod 3 subrectangular with ventral setae; coxa of pereopod 4 expanded with posterior concavity, anterodistal corner and ventral margin with setae. Pereopod 5 (Fig. 3A, 4G) with bilobed coxa bearing apical seta on anterior lobe, 2 robust setae on ventral margin of posterior lobe, posterodistal corner of posterior lobe rounded with robust seta; basis with weakly expanded posterior margin bearing setae, posterodistal corner not lobate. Pereopod 6 (Fig. 3A, 4H) with bilobed coxa bearing anteroproximal setae and apical seta on anterior lobe, 2 robust setae on ventral margin of posterior lobe, posterodistal corner of posterior lobe quadrate with robust seta; basis with weakly expanded posterior margin bearing setae, posterodistal corner not lobate. Pereopod 7 (Fig. 3A, 4I) with weakly concave coxa in ventral margin bearing setae; basis with weakly expanded posterior margin bearing setae, posterodistal corner not lobate with robust and slender setae.

Coxal gills (Figs. 4J–O, 5H, I) with 2 accessory lobes on gills 2–5, posterior lobes longer than or equal to anterior ones, 1 accessory lobe on gills 6 and 7.

Pleopods 1–3 (Fig. 4P) with peduncle bearing paired retinacula (Fig. 4Q) on inner margin; inner ramus with inner basal margin bearing bifid plumose setae.

Uropod 1 (Fig. 4R) with peduncle bearing basofacial robust seta, 2 robust setae on inner and outer margins, 1 and 2 robust setae on inner and outer distal corners, respectively; length of inner ramus $0.8 \times$ that of peduncle, inner margin of inner ramus with 2 robust setae; length of outer ramus $0.9 \times$ that of inner ramus, inner margin of outer ramus with robust seta. Uropod 2 (Fig. 4S) with peduncle bearing 2 robust setae on inner and outer margins, respectively, and robust seta on inner and outer distal corners; length of inner ramus $0.9 \times$ that of peduncle, inner margin of inner ramus with 2 robust seta; length of outer ramus $0.8 \times$ that of inner ramus, inner margin of outer ramus with robust seta. Uropod 3 (Fig. 4T, U) with peduncle length $0.3 \times$ that of outer ramus; length of inner ramus $0.3 \times$ that of outer ramus, inner ramus with slender setae on inner margin and setae apically; outer ramus comprising 2 articles, proximal article with 2 clusters of setae on inner and outer margins, some of which robust, lacking plumose setae, length of terminal article $0.2 \times$ that of proximal article, apical part of terminal article with simple setae.

Telson (Fig. 4V) 0.8 times as long as wide, cleft for 67% of length, with robust seta and slender setae on each lobe.

Female [7.3 mm, NSMT-Cr 29004]. Antenna 1 (Fig. 5A), length ratio of peduncular articles 1–3 $1.0 : 0.8 : 0.6$; peduncular article 1 with pair of setae and single seta on posterior margin; peduncular article 2 with 5 clusters of setae on posterior margin; accessory flagellum comprising 3 articles; primary flagellum comprising 17 articles. Antenna 2 (Fig. 5B) with peduncular article 4 bearing 6 clusters or single setae on posterior margin; peduncular article 5 with 5 clusters or single setae on posterior margin; flagellum comprising 11 articles, lacking calceoli.

Gnathopod 1 (Fig. 5C, D) with carpus bearing cluster of setae on anterior margin; length of propodus $1.2 \times$ that of carpus and $1.5 \times$ width; propodus with 8 medial and 2 lateral robust setae on palmar margin. Gnathopod 2 (Fig. 5E, F) with carpus bearing cluster of setae on anterior margin; propodus and carpus about the same length, propodus with 3 medial and 2 lateral robust setae and 1 medial and 1 lateral pectinate robust setae on palmar margin.

Pereopods 5–7 with more expanded posterior margin of bases than those of male (Fig. 5G–I).

Brood plates (= oostegites) (Fig. 5J) wide, with numerous setae on its margins.

Uropod 3 (Fig. 5K), length of peduncle $0.4 \times$ that of outer ramus; length of inner ramus $0.2 \times$ that of outer ramus.

Variation. Although almost all specimens have a pleonite 1 with a pair of setae on the dorsal margin, a few specimens have three setae. Some specimens have a urosomite 1 with a pair of lateral robust setae and a pair of clusters of robust setae on its dorsal margin. The numbers of setal clusters on the posterior margins of the peduncular articles 1–3 ranged from two to four, six to seven, and two to four, respectively. The number of setal clusters on the posterior margins of the peduncular articles 4 and 5 ranged from five to six and four to five, respectively. Some specimens have robust setae on the outer margin of the outer ramus of uropod 1 and lack robust setae on the inner margin of the outer ramus of uropod 2. Some specimens have a telson with two robust setae on each lobe. The number of eggs number is up to nine.

Etymology. The new specific name derived from the absence of calceolus.

Remarks. *Jesogammarus acalceolus* (*J.*) sp. nov. differs from its congeners by lacking a calceolus on the flagellum of antenna 2 in male. This new species is similar to *J. (J.) bousfieldi* Tomikawa, Hanzawa and Nakano, 2017 and *J. (J.) paucisetulosus* Morino, 1984 in having the following features: eyes are small; antenna 1 lacks robust setae on the posterodistal corner of the peduncular article 1; antennae 1 and 2 have many long setae on the posterior margins of the peduncular articles; the maxilla 1 lacks setae on the outer margin of the palp article 2; and gnathopods 1 and 2 have few setae on the ventral margins of the coxae in female. In addition to the absence of a calceolus, *J. (J.) acalceolus* sp. nov. is distinguished from *J. (J.) bousfieldi* by the pleonites 1–3 each with less than three setae on the dorsal margins (vs. more than 4 setae in *J. (J.) bousfieldi*).

Discussion

Among freshwater habitats, springs have an especially high risk of extinction of species [8]. The highly diverse genus *Jesogammarus*, which is found in spring water habitats of the Japanese Archipelago, has a sensory organ termed the calceolus on male antenna 2. We described a new endangered freshwater amphipod species, *Jesogammarus (Jesogammarus) acalceolus* sp. nov., found in a spring in Aomori Prefecture, Japan, which is potentially the sole remaining habitat of this species.

The calceolus is a typically club- or paddle-shaped structure found on the antennae of amphipods [29]. Although structures similar to the calceolus are also found in the antennal articles of Anaspidacea and Mysida, these are not considered to be homologous to amphipod calceoli [30]. Calceoli are used mainly as a taxonomic character in the higher taxa of amphipods [30,31–33]. In Anisogammaridae, the presence or absence of calceoli is used as a genus-level taxonomic feature [21]. However, the molecular phylogenetic tree generated in this study confirmed that the non-calceolate species, *J. (J.) acalceolus* sp. nov., is nested in *Jesogammarus*, and not in *Anisogammarus*, *Ramellogammarus* or *Spasskogammarus*, the other Anisogammaridae with non-calceolate species (Fig. 2). These results indicated that the calceolus should no longer be used as a diagnostic feature of *Jesogammarus* and the genus needs to be redefined. Therefore, in this study, we have amended the diagnosis of *Jesogammarus*.

The phylogenetic relationship between *Jesogammarus* and *Ramellogammarus* could not be clarified in this study. This may be due to the lack of information available for *Ramellogammarus* because only the 28S rRNA gene was available for molecular phylogenetic analyses. Future molecular phylogenetic research using adequate sequence data are therefore needed to reveal whether the two genera are closely related.

Although the calceolus is thought to be a sensory organ, its function and evolution are not well understood [31,34–36]. Therefore, the discovery of *J. (J.) acalceolus* sp. nov., which lacks calceoli, provides important clues regarding the function and evolution of calceoli. Bousfield & Shih [30] contended that, while the calceolus represents a plesiomorphic state, its absence signifies an apomorphic state. However, molecular phylogenetic analyses conducted in this study indicated that the non-calceolate *Anisogammarus* and *Spasskogammarus* are located at the base of the phylogenetic tree, suggesting that calceoli likely represent an apomorphic state in the Anisogammaridae. An ancestral reconstruction of calceoli via the molecular phylogenetic tree generated during this study revealed that the common ancestor of

Jesogammarus possessed calceoli, which were secondarily lost in *J. (J.) acalceolus* sp. nov. (Fig. 2). Since *Jesogammarus* carried calceoli only on the flagellum of antenna 2 of males, it is considered that calceoli have a reproductive function [30]. Females of amphipods lay eggs just after moulting when the exoskeleton is soft. Therefore, some species display a reproductive behaviour termed "precopula", in which a male holds and guards a female for a couple of days till the female's moulting and subsequent laying eggs. Dunn [36] reported that calceoli can be used to evaluate the molting interval of females to find suitable females for mate guarding. In this study, *J. (J.) acalceolus* sp. nov., the males of which lack calceoli, was also found to practice precopulatory guarding, which suggested that calceoli are not always necessary for precopulatory guarding in *J. (J.) acalceolus* sp. nov.. Comparison of *J. (J.) acalceolus* sp. nov. that have lost calceoli, which were acquired during the course of evolution via calceolate congeners, may shed light on the adaptive significance of calceoli in *Jesogammarus*.

Jesogammarus (J.) acalceolus sp. nov. was found in a spring located 120 m above sea level, on the slope of the volcanic Mt. Iwaki, Aomori Prefecture, Japan. Although we conducted an intensive survey of inland waters at more than 400 sites in the Aomori Prefecture, this new species was present only in this one spring described above and not found in any others (unpublished data). In most of the freshwater habitats that were investigated, *J. (J.) jesoensis* Schellenberg, 1937, which is distributed in Hokkaido and northern Honshu, was present. Because *J. (J.) acalceolus* sp. nov. and *J. (J.) jesoensis* are not closely related (Fig. 2), it is expected that the current distributions of both species are a result of different evolutionary processes. Freshwater amphipods have low dispersal ability, and there thus exists a high tendency for endemic species to be distributed throughout each region [20]. In addition, our taxonomic studies revealed a considerable presence of *Jesogammarus* fauna in the Japanese Archipelago [18,19,23,37]. For these reasons, it is unlikely that *J. (J.) acalceolus* sp. nov. will be found outside type localities, thereby limiting the current habitat of this species to a great extent. In the past, there have been many cold springs in Hirosaki with the type locality of this new species. However, recent, rapid urbanization has led to a depletion of such springs [38]. Besides, the habitat of this species may have been lost due to the disappearance of springs and/or environmental pollution caused by the use of agrochemicals, both of which were associated with apple plantations that flourished in this region. As a positive aspect, the type locality of *J. (J.) acalceolus* sp. nov. is in the precincts of the Iwaki Haguro Shrine, built in AD 807, as a result of which this type locality has been treated with care by locals for more than 1,000 years [38]. Therefore, the environment of this spring has been preserved in good condition, allowing the present *J. (J.) acalceolus* sp. nov. population to survive. At present, this spring has an abundance of water (60 m³/day) [39], and its environment is stable. However, amphipods are known to be highly sensitive to chemicals, such as pesticides [40,41]. This species inhabits only a few meters of a spring brooklet surrounded by apple plantations. Therefore, the deterioration of its habitat due to an inflow of agricultural chemicals into spring water may lead to its extinction. Thus, to conserve what is possibly the only remaining population of *J. (J.) acalceolus* sp. nov., it will be necessary to conduct further investigations into risk factors and develop a conservation plan with the cooperation of local communities and policymakers. In conclusion, our results indicate that this new species, which is key to clarifying the evolution of the calceolus, is of high conservation significance.

Declarations

Data availability statement

Type materials have been deposited in the Tsukuba Collection Center of the National Museum of Nature and Science, Tokyo (NSMT). The DNA sequence has been deposited with the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan (DDBJ).

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Author contributions statement

KT and NK collected samples and described them. KT conducted the molecular phylogenetic analyses and wrote the main text. All authors reviewed the manuscript.

Additional Information

Competing interest

There authors declare that there are no competing interests.

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Tables

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 | 16S rRNA |
| <i>Anisogammarus pugettensis</i> | G1500 | Akkeshi Bay, Hokkaido, Japan | LC624749* | LC624757* | LC624742* |
| <i>Barrowgammarus macginitiei</i> | G37 | Akkeshi Bay, Hokkaido, Japan | LC624750* | LC624758* | LC624743* |
| <i>Eogammarus kygi</i> | G1 | Naibetsu River, Hokkaido, Japan | LC214759 | LC052229 | LC052250 |
| <i>E. possjeticus</i> | G3 | Lake Akkeshi, Hokkaido, Japan | LC214760* | LC052230 | LC052251 |
| <i>Jesogammarus (Annanogammarus) annandalei</i> | G1162 | Lake Biwa, Shiga, Japan | LC214786 | LC052248 | LC052269 |
| <i>J. (A.) debilis</i> | IZCAS-I-A0325 | Fangshan, Beijing, China | EF582997 | | EF582846 |
| <i>J. (A.) fluvialis</i> | G83 | Samegai, Shiga, Japan | LC214766 | LC052236 | LC052257 |
| <i>J. (A.) koreaensis</i> | G1376 | Deoksin-ri, Onsan-eup, Ulju-gun, Ulsan, Korea | LC624751* | LC624759* | |
| <i>J. (A.) naritai</i> | G1167 | Lake Biwa, Shiga, Japan | LC214787 | LC052249 | LC052270 |
| <i>J. (A.) suwaensis</i> | G88 | Lake Suwa, Nagano, Japan | LC214767 | LC052237 | LC052258 |
| <i>Jesogammarus (Jesogammarus) acalceolus</i> sp. nov. | NSMT-Cr 29008 (G1625) | Haguro Shrine Spring, Aomori, Japan | LC624752* | LC624760* | LC624744* |
| <i>J. (J.) acalceolus</i> sp. nov. | NSMT-Cr 29005 (G1845) | Haguro Shrine Spring, Aomori, Japan | LC624753* | LC624761* | LC624745* |
| <i>J. (J.) bousfieldi</i> | KUZ Z1799 | Aburato, Tsuruoka, Yamagata, Japan | LC214778 | LC214538 | LC214795 |
| <i>J. (J.) fujinoi</i> | G17 | Yamagata, | LC214762 | LC052232 | LC052253 |

| | | Japan | | | |
|------------------------------------|---------------|--|-----------|-----------|-----------|
| <i>J. (J.) hebeiensis</i> | IZCAS-I-A0294 | Yanqing, Beijing, China | EF582998 | | EF582847 |
| <i>J. (J.) hinumensis</i> | G52 | Lake Hinuma, Ibaraki, Japan | LC214765 | LC052235 | LC052256 |
| <i>J. (J.) hokurikuensis</i> | G1838 | Shimizucho, Fukui, Japan | LC624754* | LC624762* | LC624746* |
| <i>J. (J.) ikiensis</i> | G515 | Iki, Nagasaki, Japan | LC214772 | LC052242 | LC052263 |
| <i>J. (J.) jesoensis</i> | G164 | Sapporo, Hokkaido, Japan | LC214769 | LC052239 | LC052260 |
| <i>J. (J.) mikadoi</i> | G13 | Rokugo, Akita, Japan | LC214761 | LC052231 | LC052252 |
| <i>J. (J.) paucistulosus</i> | G1037 | Mito, Ibaraki, Japan | LC214780 | LC052247 | LC052268 |
| <i>J. (J.) shonaiensis</i> | G192 | Sakata, Yamagata, Japan | LC214770 | LC052240 | LC052261 |
| <i>J. (J.) spinopalpus</i> | G32 | Onjuku, Chiba Prefecture, Japan | LC214763 | LC052233 | LC052254 |
| <i>J. (J.) uchiyamaryui</i> | KUZ Z1803 | Tanie River, Iki, Nagasaki, Japan | LC214773 | LC214533 | LC214790 |
| <i>Ramellogammarus oregonensis</i> | G1537 | Willamette River, Corvallis, Oregon, USA | LC624755* | | |
| <i>R. similimanus</i> | G1540 | Alice Springs, Portland, Oregon, USA | LC624756* | | |
| <i>Spasskogammarus spasskii</i> | G35 | Akkeshi Bay, Hokkaido, Japan | LC214764* | LC052234 | LC052255 |
| <i>Gammarus mukudai</i> | G858 | Iki, Nagasaki, Japan | AB893234 | LC624763* | LC624747* |
| <i>G. nipponensis</i> | G797 | Kiyotaki, Kyoto, Japan | AB893232 | LC624764* | LC624748* |

Figures



Figure 1

Habitat and live specimens of *Jesogammarus (Jesogammarus) acalceolus* sp. nov. (A) The type locality, Haguro Shrine Spring, Hirosaki, Aomori Prefecture, Japan. (B) Mate guarding pair, male is upper and female is lower, photographed by Ryu Uchiyama.

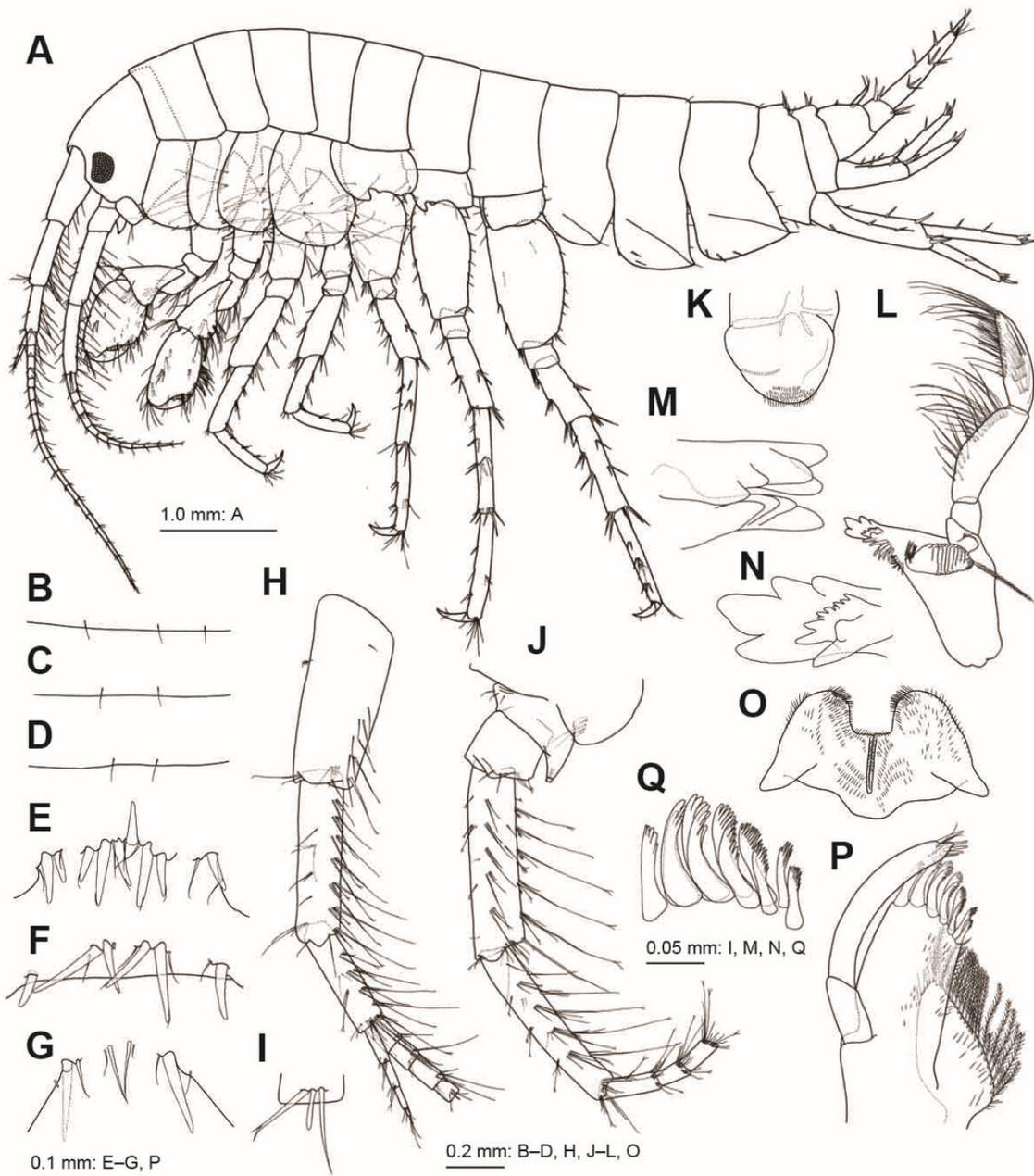


Figure 3

Jesogammarus (Jesogammarus) acalceolus sp. nov., male (7.4 mm), NSMT-Cr 29003. (A) Habitus, lateral view. (B)–(D) Dorsal margins of pleonites 1–3, respectively, dorsal views. (E)–(G) Dorsal margins of urosomites 1–3, respectively, dorsal views. (H) Peduncular articles 1–3, accessory flagellum, and flagellar articles 1–4 of antenna 1, medial view. (I) Aesthetasc and associate setae on the flagellum of antenna 1, medial view. (J) Peduncular articles 1–5 and flagellar articles 1–3 of antenna 2, medial view. (K) Upper lip, posterior view. (L) Right mandible, medial view. (M)–(N) Incisor and lacinia mobilis of left and right mandibles, medial views. (O) Lower lip, ventral view. (P) Maxilla 1, medial view. (Q) Serrate robust setae on outer plate of maxilla 1, medial view.

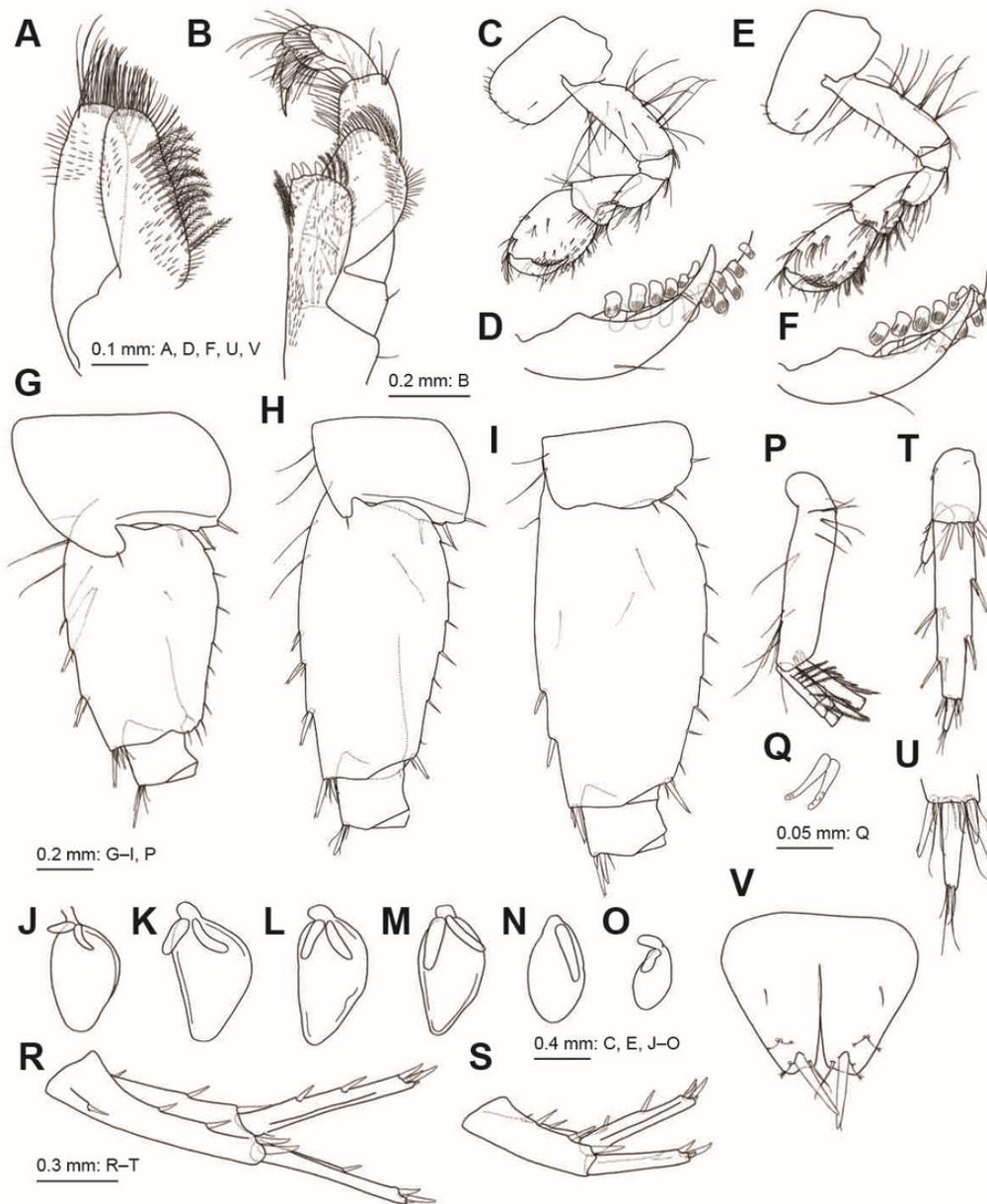


Figure 4

Jesogammarus (Jesogammarus) acalceolus sp. nov., male (7.4 mm), NSMT-Cr 29003. (A) Maxilla 2, medial view. (B) Maxilliped, dorsal view. (C) Gnathopod 1, medial view. (D) Palmar margin of propodus and dactylus of gnathopod 1, medial view, some setae omitted. (E) Gnathopod 2, medial view. (F) Palmar margin of propodus and dactylus of gnathopod 2, medial view, some setae omitted. (G)–(I) Coxa–ischium of pereopods 5–7, respectively, lateral views. (J)–(O) Coxal gills on gnathopod 2–pereopod 7, respectively, lateral views. (P) Pleopod 1, lateral view, distal parts of rami omitted. (Q) Retinacula on peduncle of pleopod 1, lateral view. (R)–(S) Uropods 1–2, respectively, dorsal views. (T) Uropod 3, ventral view. (U) Distal part of proximal article and terminal article of outer ramus of uropod 3, ventral view. (V) Telson, dorsal view.

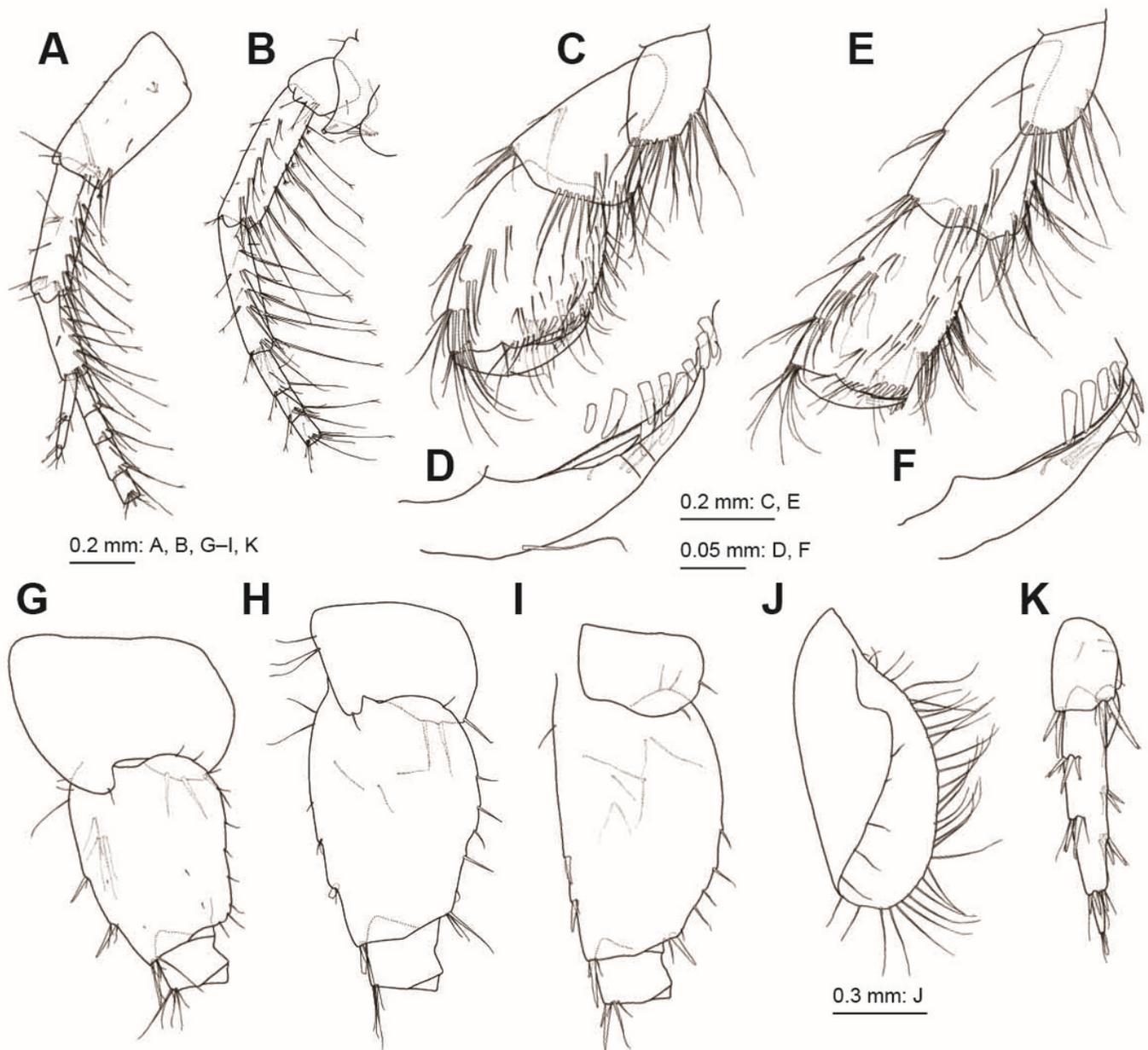


Figure 5

Jesogammarus (Jesogammarus) acalceolus sp. nov., female (7.3 mm), NSMT-Cr 29004. (A) Peduncular articles 1–3, accessory flagellum, and flagellar articles 1–4 of antenna 1, medial view. (B) Peduncular articles 1–5 and flagellar articles 1–3 of antenna 2, medial view. (C) Ischium–dactylus of gnathopod 1, medial view. (D) Palmar margin of propodus and dactylus of gnathopod 1, medial view, some setae omitted. (E) Ischium–dactylus of gnathopod 2, medial view. (F) Palmar margin of propodus and dactylus of gnathopod 2, medial view, some setae omitted. (G)–(I) Coxa–ischium of pereopods 5–7, respectively, lateral views. (J) Brood plate on gnathopod 2, lateral view. (K) Uropod 3, ventral view.