DESCRIPTION OF A NEW SPECIES OF *ELAPHOGNATHIA* (ISOPODA: GNATHEIDAE) FROM JAPAN BASED ON MORPHOLOGICAL AND MOLECULAR TRAITS

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ABSTRACT

Elaphognathia cornigera is a sponge-dwelling species of isopod that occurs in the intertidal zone in the main island of Japan. Currently, another population of *Elaphognathia* has been found from Okinawa-jima Island (classified as *Elaphognathia* sp.). Although it is quite similar in morphology to *E. cornigera*, the habitat and seasonal traits are quite different between Okinawa-jima Is. and Izu Peninsula. In this paper, we examined *Elaphognathia* sp. and *E. cornigera* from several sampling sites using morphological analysis and ribosomal DNA ITS2 sequences. Morphological analysis revealed adult males of *Elaphognathia* sp. were distinguished from *E. cornigera* by the number of pylopod seta: *E. nunomurai*, 5.5 ± 1.1 (Haneji, $N = 18$) and 4.1 ± 1.4 (Odo, $N = 18$); *E. cornigera*, 11.6 ± 2.0 (Amakusa, $N = 18$) and 13.7 ± 1.4 (Miura, $N = 18$). *Elaphognathia* sp. were also distinguished from *E. cornigera* by means of rDNA ITS2 sequences, with differences between the *E. cornigera* group and *Elaphognathia* sp. being 5.3 and 5.9%, whereas the sequence differences between *E. cornigera* from Miura, Shimoda, and Amakusa were relatively low (0.5-1.4%). Thus, we describe a new species, *Elaphognathia nunomurai*.

INTRODUCTION

The isopod family Gnathiidae includes 12 genera and over 190 species (Hadfield et al., 2008; Kensley et al., 2009), and 6 genera and about 30 species have been recorded from Japan to date (Shimomura and Tanaka, 2008; Ota and Hirose, 2009). Gnathiids have morphological differences between larvae (known as praniza larvae) and sexually dimorphic adults (Monod, 1926). While non-feeding adults only reproduce in the benthic substrata such as sponge, dead coral, mud burrows, polychaete worm tubes, and similar structures, praniza larvae are temporal ectoparasites of fish and suck their blood. After feeding, they rest and molt into the next stage of larvae or alternatively metamorphose into adults (reviewed in Tanaka, 2007).

Gnathiids are distributed worldwide, occurring from the intertidal zone to abyssal waters up to 4000 m (Cohen and Poore, 1994; Smit and Davies, 2004). Specimens from the intertidal zone of *Elaphognathia cornigera* (Nunomura, 1992) have been found from demosponges in the main island of Japan; Amakusa, Kyushu (Nunomura, 1992), Shimonoseki, Chugoku (Ota, personal observation), the Izu Peninsula (Nunomura, 2006), the Boso Peninsula, and the Miura Peninsula (Tanaka, personal observation). Their ecological aspects have been studied at the Izu Peninsula, including the life cycle (Tanaka and Aoki, 1998), distribution pattern in sponge colonies (Tanaka and Aoki, 1999), female reproductive traits (Tanaka and Aoki, 2000), and population dynamics (Tanaka, 2003).

Ota et al. (2008) found a sponge-dwelling gnathiid of the genus *Elaphognathia* from Okinawa-jima Is. in southwestern Japan. The whole body and the mouthpart shapes in adult males of this *Elaphognathia* are very similar to those of *E. cornigera*, but there are a few differences between the two species. Ota et al. (2008) therefore classified this *Elaphognathia* as *Elaphognathia* aff. *cornigera* and demonstrated that this form is ecologically different from *E. cornigera* in the following points. First, the habitat of the gnathiids was quite different. *Elaphognathia* aff. *cornigera* (classified as *Elaphognathia* sp.) was found in muddy tidal flats in subtropical areas (Ota et al., 2008). On the other hand, *E. cornigera* was found in intertidal rocky shores in temperate areas (Nunomura, 1992; Tanaka and Aoki, 2000). In regard to the relationship to their environment, they inhabited different sponge species: our new *Elaphognathia* mainly in *Haliclona* sp. and *E. cornigera* in *Halichondria okadai* and *Haliclona permollis* (Nunomura, 1992; Tanaka and Aoki, 1998; Ota et al., 2008). Second, the seasonal fluctuations in populations were quite different between the new *Elaphognathia* from Okinawa-jima Is. and *E. cornigera* from the Izu Peninsula, central Japan. The abundance of our new *Elaphognathia* increased from winter to spring (Ota et al., 2008), whereas *E. cornigera* was most abundant in summer (Tanaka, 2003).

In this report, we compare the morphological characters of male adults of our *Elaphognathia* with those of *E. cornigera* from two sampling sites. We also observed the morphologies of female adults and the third praniza larvae of both species. Recently, Grutter et al. (2000) and Nagel et al. (2008) showed that the ribosomal DNA internal transcribed spacer 2 (ITS2) sequences are useful for distinguishing species of gnathiid isopods. We obtained ribosomal DNA ITS2 sequences from our *Elaphognathia* and *E. cornigera* at three sampling sites, and two additional
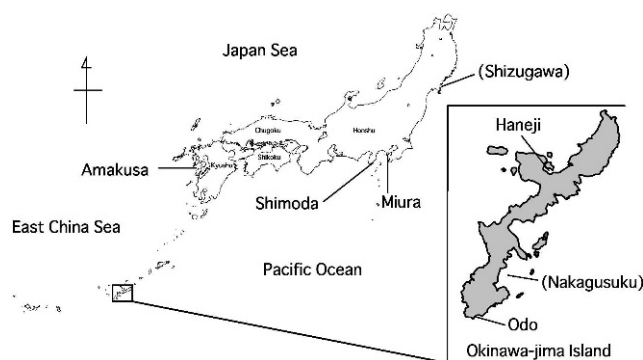
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Fig. 1. Sampling sites. The specimens of *Elaphognathia nunomurai* was collected from Haneji Island Sea and Odo Beach in Okinawa-jima Island. Material of *E. cornigera* was collected from Amakusa (Type locality), Shimoda from the Izu Peninsula, and Miura. Material of *E. discolor* was collected from Shizugawa Bay showed in parenthesis, and *E. rangifer* was obtained from Nakagusuku Bay.

species of *Elaphognathia*. We combined both morphological and molecular analyses as part of our analysis.

MATERIALS AND METHODS

Field Sampling

(Fig. 1)

We collected *Elaphognathia* from a colony of sponge *Haliclona* sp. at a tidal flat in Haneji Island Sea, Okinawa-jima Is., Ryukyu Archipelago, southwestern Japan (26°28′N, 128°0′E) on 3 March 2006 and 24 May 2008. We also collected other specimens of *Elaphognathia* from two types of unidentified demosponge in the tide pool (−50 cm depth) between sandy or rocky beach and coral reef lagoon, at Odo beach, on the south coast of Okinawa-jima Is. (26°33′N, 127°42′E) on 9 March 2009 and 5 December 2009. We collected the sponges, *Haliclona* sp., by hand and transferred them to a plastic bottle (1000 ml) filled with seawater. After the samples were taken to the laboratory, we dissected the colonies into smaller pieces with forceps and then sorted out the infaunal gnathiids. We wore latex gloves during the dissection because the sponge is venomous.

Because *E. cornigera* was originally described based on single specimen and type material was too damaged to examine, we collected a new *E. cornigera* from colonies of sponge *Halichondria japonica* in tidal pools on intertidal rocky shores of the type locality, Tûji-shima Is., Amakusa, Kyushu, west Japan (32°33′N, 130°6′E) on 10 July 2007 (called Amakusa). In accordance with the original description, we collected *E. cornigera* from *Haliclona permollis*, although we could not find the sponge at the original collection site. We collected other specimens of *E. cornigera* from colonies of the sponge *Halichondria okadai* at tidal pools on rocky shores of Cape Tsurugi-Zaki, Miura Peninsula, Honshu, central Japan (35°8′N, 139°40′E) on 15 October 2007 and Oura Bay, Shimoda, Izu Peninsula, central Japan (34°40′N, 138°57′E) on 3 October 2005. The sampling procedure was essentially the same as that described above for *Elaphognathia* spp.

We chose two species of *Elaphognathia* as out-groups in the molecular analysis. One was *Elaphognathia discolor* (Nunomura, 1988) reported by Tanaka and Nishi (2008) to inhabit terebellid polychaete tubes. Specimens of *E. discolor* were collected by scuba divers from the terebellid polychaete (*Nicolea gracilibranchis*) tubes in southern Shizugawa Bay, Miyagi Prefecture, northeastern Japan (38°38′N, 141°29′E) in 29 October 2008. Another species was found from unidentified sponges in coral reefs of Nakagusuku Bay (−2 to −4 m depth), Okinawa-jima Is. (26°17′N, 127°49′E). After comparison with the type specimen, the species appeared to be *E. rangifer* (Monod, 1926) originally recorded from Singapore. Specimens of *E. rangifer* for the present study were collected at Nakagusuku Bay in 23 October 2008 by scuba divers.

We fixed the gnathiid specimens in 70% ethanol for morphological analysis and 99% ethanol for molecular analysis.

Morphological Analysis

We morphologically examined 10 adult males specimens from both Haneji and Odo, as well as *E. cornigera* from Amakusa and Miura. We measured the body lengths with an ocular micrometer under a binocular stereomicroscope (SZX12, Olympus, Tokyo, Japan). After measurement, we extracted a pair of the mouthparts (pylopod and maxilliped) with sharpened tungsten needles and mounted them in a CMCP-10 High Viscosity Mountant (Polyscience, Warrington, PA, U.S.A.). We then counted the number of plumose setae on the inner margin of pylopod (article 1) and the outer margin of maxilliped under a light microscope equipped with phase-contrast and differential interference contrast optics. We also counted the number of setae on a pair of uropodal rami (exopod and endopod) under the microscope, although they were not mounted in CMCP-10. Even if the specimens lacked setae, they were counted if traces of plumose setae were clearly recognized. We did not include damaged appendages into the data. We applied the Tukey-Kramer test to compare the number of setae of mouthparts in *Elaphognathia* spp. from different localities. We performed these statistical analyses using InStat Ver. 3.0b software (GraphPad Software Inc., CA, U.S.A.).

We also described the shapes of body parts and appendages of adult females and the third praniza larvae in both our *Elaphognathia*, and *E. cornigera*. We separated the appendages from the body and mounted them in CMCP-10. We used a phase-contrast light microscope to record our observations.

All drawings were made with a camera lucida. We followed the gnathiid terminology described by Cohen and Poore (1994) and Smit and Davies (2004).

DNA Extraction and Amplification

We collected *Elaphognathia* from Haneji on 24 May 2008 and Odo on 5 December 2009, and we selected *E. cornigera* from Amakusa, Shimoda, and Miura for molecular analysis. We chose *E. rangifer* and *E. discolor* as the out-group taxa. We preserved tissue samples of gnathiids in ethanol at −30°C. Species of *Elaphognathia*, especially *E. cornigera*, are generally too small to obtain enough genomic DNA for sequencing from only one individual. Consequently, for this study, we suspended 1 to 5 specimens [four females each from Amakusa, Haneji and Odo, five males each from Shimoda and Miura, one female of *E. discolor*, and 2 females of *E. rangifer*] in 400 μl CTAB (hexadecyltrimethyl ammonium bromide) buffer [2% CTAB, 1.0 M NaCl, 75 mM EDTA (pH 8.0), 35 mM Tris-HCl (pH 8.0)] containing 0.1% sodium dodecyl sulfate (SDS) and 0.2% beta-mercaptoethanol, followed by incubated at 65°C for 1 h, according to the method of Hirose et al. (2009). We added proteinase K to the samples to a final concentration of 0.1 mg·ml^{-1} and incubated the samples overnight at 37°C. We extracted DNA with phenol-chloroform as described by Sambrook et al. (1989). We performed PCR amplification of the ribosomal DNA internal transcribed spacer 2 (ITS2) using EX *Taq* DNA polymerase (Takara) and a combination of *Gnathia* specific primers 3S (5′-GGTACC GGTGGATCACGTGGCTAGTG-3′) and ITS2.2 (5′ CCTGGTTAGTTT CTTTTCCTCCGC 3′) (Grutter et al., 2000). We performed PCR amplification under the following conditions: 5 min at 94°C for initial denaturation, followed by 35 cycles at 30 s at 94°C for denaturation, 30 s at 55°C for annealing, and 90 s at 72°C for extension, then a final extension at 72°C for 7 min. We then added 0.25 μl ExoSAP-IT (GE Healthcare) to each 10 μl PCR products and incubated at 37°C for 30 min to clean up the procedure. The sequencing reactions were performed using DTCS Quick Start Master Mix (Beckman Coulter), and the products were analyzed using a CEQ8800 (Beckman Coulter) automated DNA sequencing system. We obtained the DNA sequence of ITS2 region by direct sequencing using the primers mentioned above. However, the chromatographs of *E. cornigera* from Amakusa and *E. discolor* did not show distinct peaks when we use ITS2.2 primer for the sequencing reaction. Therefore, we cloned PCR-amplified DNA fragments of the 2 specimens using TOPO TA cloning kit for sequencing (Invitrogen). We randomly selected and sequenced at least 5 clones for each sample and sequenced. The sequences determined in this study can be retrieved from the GenBank/EMBL/DDBJ DNA databases under the accession numbers provided in Table 1.

Phylogenetic Analyses

We initially aligned the sequences using MUSCLE (Edgar, 2000) and then eliminated poorly aligned positions and divergent regions by Gblocks (Castresana, 2000) for phylogenetic analysis. We estimated the rDNA-

Table 1. List of *Elaphognathia* ribosomal DNA ITS2 sequences for the phylogenetic tree reconstruction.

Species	Abbreviation	Collection site	Acc. no.
E. cornigera	*E. cornigera*_Miura	Miura, Kanagawa	AB519154
E. cornigera	*E. cornigera*_Shimoda	Shimoda, Shizuoka	AB519155
E. cornigera	*E. corrnigera*_Amakusa	Amakusa, Kumamoto	AB519156
E. nunomurai	*E. nunomurai*_Haneji	Haneji, Okinawa	AB519157
E. nunomurai	*E. nunomurai*_Odo	Odo, Okinawa	AB547450
E. rangifer	*E. rangifer*	Nakagusuku, Okinawa	AB519158
E. discolor	*E. discolor*	Shizugawa, Miyagi	AB519159

ITS2 region in reference to previously reported ITS2 sequences of *Gnathia* (Grutter et al., 2000; Nagel et al., 2008). The alignment data set of ITS2 (7 taxa/496 sites) is available on request from the corresponding author. We performed the following analyses on the aligned DNA sequences of the ITS2 region: maximum likelihood (ML) using TREEFINDER January 2008 version (Jobb, 2008), phylogenetic analysis based on Baysian inference (BI) using MrBayes 3.12 (Ronquist and Huelsenbeck, 1993), and maximum parsimony (MP) and neighbor-joining (NJ) using PAUP* 4.0 beta10 (Swofford, 2003). To select an appropriate nucleotide substitution model, we used modeltest ver. 3.7 (Posada and Crandall, 1998) with PAUP. On the basis of the Akaike information criterion, we selected the HKY+I model (Hasegawa, Kishino, and Yano model with invariant site) as the best model. We searched the unweighted MP trees using a heuristics approach with 100 random initial trees. Statistical support for the ML, MP, and NJ trees was evaluated using a non-parametric bootstrap test with 1000 re-sampling events. For the BI analysis, a Markov chain Monte Carlo (MCMC) analysis was run for 1,000,000 generations, and trees were built in 100-generations intervals (burn in = 2500).

SYSTEMATICS

Gnathiidae Leach, 1814
Elaphognathia Monod, 1926
Elaphognathia nunomurai n. sp.
(Figs. 2-5)

Material Examined.—Adult male holotype (2.40 mm), deposited in the National Science Museum, Tokyo, Japan (NSMT-Cr 21127), from a colony of sponge *Haliclona* sp. from muddy tidal flats in Haneji Island Sea, Okinawa-jima Is., (26°28′N, 128°0′E) collected by Y. Ota in 3 March 2006. Paratypes; 11 males, 24 females and 27 larvae (NSMT-Cr 21128). The sampling data is the same as the holotype.

Description.—Male (Figs. 2, 3).

Body length 1.89-2.67 mm (mean ± SD; 2.36 ± 0.2; *n* = 11). Brown pigments scattered over entire body of live specimens, except for dorsal buccal part and inner area of pleonites. Cephalosome (Fig. 2A) rectangular, approximately one-fourth of body length. Frontal border (Fig. 2B) with pair of seta and triangular mediofrontal process. Eyes well developed, approximately one-seventh-length of cephalosome.

Pereionites 2-7 approximately one-half of body length. Pereionite 1 fused with cephalosome. Pereionites 2 and 4 slightly wider and longer than pereionite 3. Pereionites 5 and 6 together subequal in length to pereionites 2-4 combined. No areae laterales and lobi laterals. Each article of pleonite (Fig. 2A) subequal in length. Pleonites and pleotelson approximately two-thirds of body length. Pleonal epimera not pronounced. Pleotelson (Fig. 2C) slightly concave on lateral margins. Two pairs of small setae on distal margins.

Antenna 1 (Fig. 2D) composed of three peduncular and four flagellar articles. One to five simple setae on distal margin of each article. One aesthetasc on articles 2 and 4 each. Antenna 2 (Fig. 2E) composed of four peduncular and seven flagellar articles. Two and four feather-like setae on distal margins of peduncles 3 and 4, respectively. A few setae on distal margins of flagellar articles 1-7.

Mandibles (Fig. 2B) as long as cephalosome, curved internally. Apex bifit and fork dentate. Erisma prominent. Basal neck absent. Pylopod (Fig. 3A) composed of three articles, fringed by fine setae on lateral margins of articles 1 and 2. Article 1 with three areolae, large and elliptical. Six plumose setae on internal margin. Article 2 oval-shaped. Article 3 min.

Maxilliped (Fig. 2F) with fine setae on external margin, composed of basis and four-articled palp. Endite not prominent. Palp articles 1-4 of holotype bearing three, four, six, and six plumose setae on external margin, respectively. Article 4 terminates in two simple setae.

Pereiopod 2 (Fig. 2G). Basis and ischium rectangular. Merus, rectangular, approximately two-third lengths of ischium; bears five setae, five projections and one denticulate composed of spine on distal margin. Carpus, elliptical and as long as merus; bearing five tooth-like tubercles on inner margin, pectinate scales on lateral margin, and four setae distally. Propodus about 1.2 times longer than carpus; bearing two denticulate composed of spines on inner-mid and inner-distal margins. Dactylus terminating in unguis. All pereiopods similar in shape and size.

Penes (Fig. 2H) composed of two small contiguous papillae and not prominent.

Pleopodal peduncle of pleopod 2 (Fig. 2I) with one seta on outer distal corner and coupling hook on inner margin. Both pleopodal rami oval-shaped and equal in length. Exopod and endopod bearing nine plumose setae and six plumose setae, respectively. Appendix masculina lacking on pleopod 2.

Uropodal rami (Fig. 2C) subequal in length, extending beyond apex of pleotelson. Exopod bearing five simple setae and four plumose setae on external and internal margins, respectively. Endopod bearing one simple seta and six plumose setae on external and internal margins, respectively. Four feather-like setae on dorsal surface.

Female (Fig. 4).

Body length 1.95-2.43 mm (2.18 ± 0.2 mm, *n* = 24). White pigmentation on whole body of live specimens.

Cephalosome (Figs. 4A, B) pentagonal; length approximately two-thirds of width. Frontal margin convex. Eyes approximately one-third length of the cephalosome. Pereion (Fig. 4A). Pereionite 1 fused with cephalosome. Lateral shields of pereiopods 2-6 visible dorsally. Pleonites 1-5 subequal in length (Fig. 4A). Pleotelson (Fig. 4C) slightly concave on lateral margin. Two pairs of setae on lateral margin and apex.

Article numbers of antennae same as those of males (Figs. 4D, E). Simple and plumose setae on peduncle article 4, fewer than those of males.
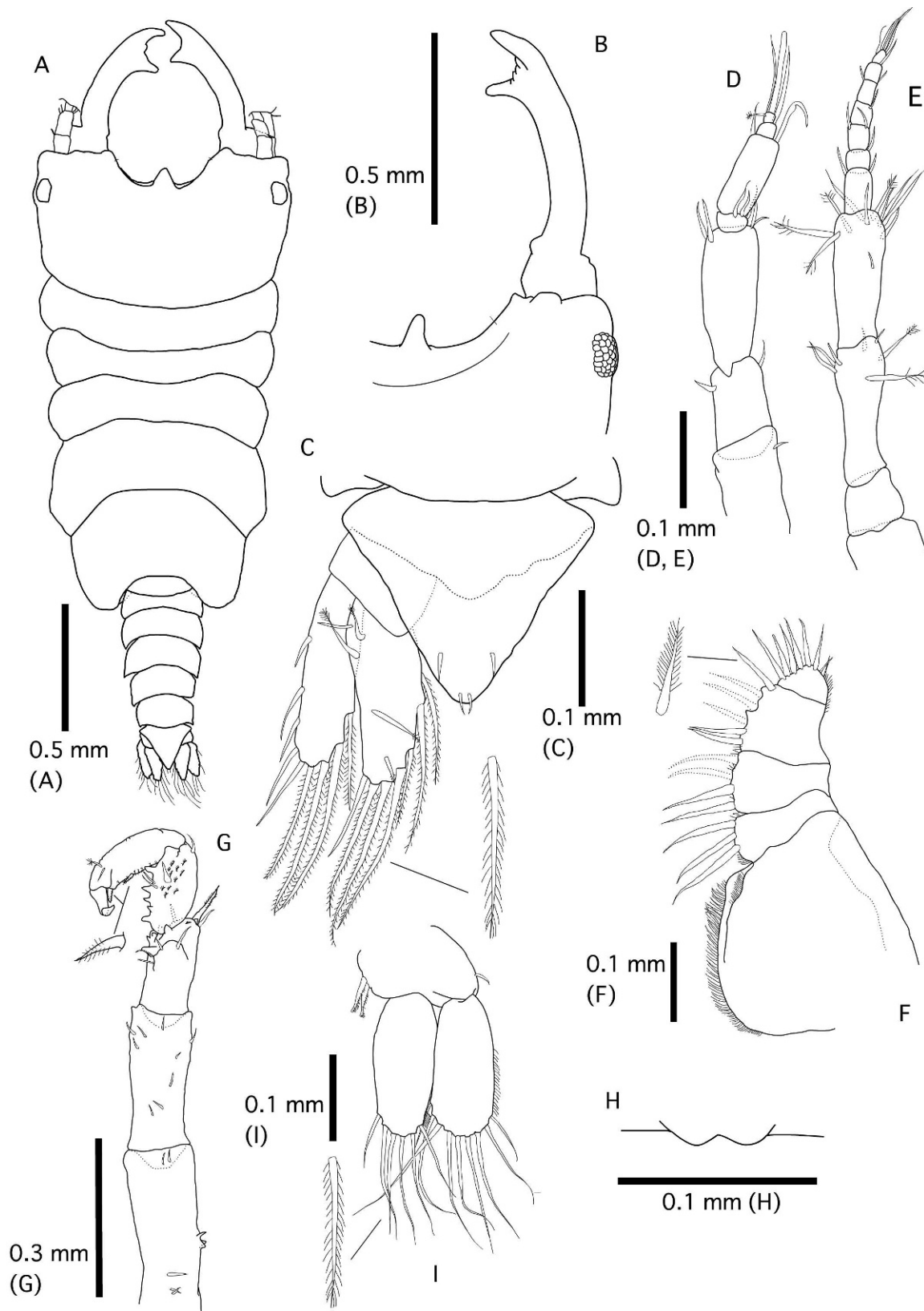
Fig. 2. *Elaphognathia nunomurai* n. sp., male holotype, 2.4 mm (NSMT-Cr 21127). A, Dorsal view of body; B, Mandible and frontal border; C, Pleotelson; D, Antenna 1; E, Antenna 2; F, Maxilliped; G, Pereiopod 2; H, Penes; I, Pleopod 2 (paratype, 2.3 mm, NSMT-Cr 21128).
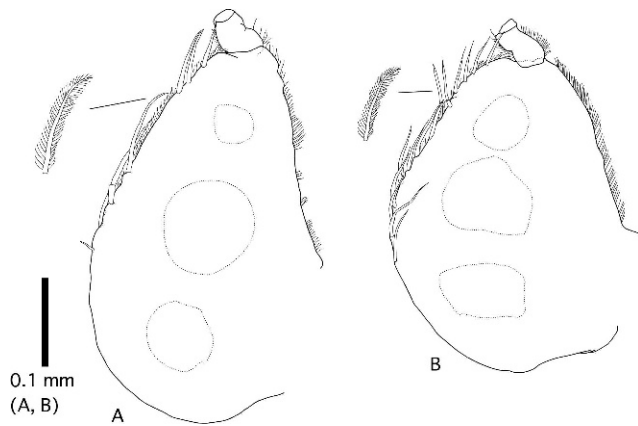
Fig. 3. Pylopod of A, *Elaphognathia nunomurai* n. sp. (holotype); B, *E. cornigera* from Amakusa.

Maxilliped (Fig. 4F) composed of basis and 4-articled palp. Endite with two setae, extending beyond half-length of palp article 1. Four, two, five, six, and five plumose setae on basis and articles 1, 2, 3, and 4, respectively. Inner margins of all palps fringed with fine setae. Pylopod (Fig. 4G) composed of three articles. Article 1 with suture, bearing several setae. Article 2 oval-shaped, bearing few setae. Article 3, minute.

Pereiopod 2 (Fig. 4H) with one feather-like seta on outer margin of basis. Three projections on merus and carpus. Pectinate scales on carpus and propodus. Pereiopods 4-6 larger than pereiopods 2 and 3. Pleopod biramous (Fig. 4I). Exopod fan-shaped, with nine plumose setae. Endopod oval, with seven plumose setae. Uropod (Fig. 4C). Uropodal rami extended beyond apex of pleotelson. Exopod bearing seven setae and four plumose setae on external and internal margins, respectively. Endopod bearing seven setae and four plumose setae on external and internal margins, respectively.

Third praniza larva (Fig. 5)

Body length, 2.43 mm. Cephalosome circular (Figs. 5A, B). Length same as width. Labrum, pentagonal; frontal margin, concave. Eyes occupying two-third length of cephalosome. Pereionite 1 (Fig. 5B) short. Pereionite 2 and 3 with concave anterior margin. Pereionite 3 slightly wider than pereionite 2. Lateral shields of pereiopods 4-6 elliptical, visible in dorsal view. Pleon about half length of pereionites 4-6 (Fig. 5A). Pleonites 1-5 bearing one seta on lateral margins. Pleotelson (Fig. 5C) similar to that of females.

Article numbers of antennae same as those of adults (Figs. 5D, E). Peduncle articles of antennae shorter than those of adults. Mandible (Fig. 5F) with eight teeth. Paragnath (Fig. 5G) slightly curved. Maxillule (Fig. 5H) with two slender articles, seven teeth on apex. Maxilliped (Fig. 5I) composed of basis and 2-articled palp. Basis with one coupling hook on inner margin and four teeth on outer margin. One seta on palp 1. Apex of palp 2 divided into two parts; one seta and two teeth on inner apex, three setae on outer part. Gnathopod (Fig. 5J) pereiopodal shape with reduced carpus. Several ridges on inner margins of ischium, merus, and carpus.

Pereiopod 2 (Fig. 5K) lacks projection on merus and carpus, same shape as that of adults. Pleopod 4 (Fig. 5L). Exopod fan-shaped with nine plumose setae. Endopod oval-shaped with seven plumose setae. Uropod (Fig. 5C) extending beyond apex of pleotelson. Exopod bearing six setae and four plumose setae on external and internal margins, respectively. Endopod bearing one seta and five plumose setae on external and internal margins, respectively.

Etymology.—The species was named in honor of Noboru Nunomura, the former director of Toyama Science Museum, for his great contribution to taxonomy of Japanese gnathiids, including *E. cornigera*.

Remarks.—Currently, 19 species of *Elaphognathia* are known worldwide, of which 5 have been recorded from Japan and adjacent waters; an additional species *E. rangifer* is also found at Okinawa-jima Is., in southwestern Japan. The male of *E. nunomurai* is morphologically quite similar to that of *E. cornigera* but distinguishable by a fewer number of pylopod setae (Fig. 3). There is also a difference in the morphology of gnathopod of third praniza larvae (presence or absent of ridges) between *E. nunomurai* and *E. cornigera* (Fig. 5).

Among Japanese species of *Elaphognathia*, *E. rangifer* from Okinawa-jima Is. is relatively similar to *E. nunomurai*. However, in *E. rangifer*, the internal lobe is present on the mandible with a deeply excavated frontal border extending beyond the eyes (Monod, 1926). The internal lobe is absent and frontal border is not beyond the eyes in *E. nunomurai*.

Table 2 shows the result of the morphological analysis in *E. nunomurai* from Haneji and Odo, and *E. cornigera* from Amakusa and Miura. Body length of *E. nunomurai* from Odo was significantly smaller (mean $\pm$ SD = 1.74 $\pm$ 0.21) than the other three populations (2.36 $\pm$ 0.24; Haneji, 2.17 $\pm$ 0.14; Amakusa; 2.47 $\pm$ 0.27) ($P < 0.05$). The numbers of maxilliped setae varied in each article between individuals; 2-4 (palp 1), 3-6 (palp 2), 3-8 (palp 3), 2-7 (palp 4), and 11-25 (all palps combined) (Table 3). The number of plumose setae on palp 1, 2, and 3 were not significantly different between populations. However, the number of palp 4 setae was significantly different between *E. nunomurai* from Odo and *E. cornigera* from Miura ($P < 0.001$), and between *E. nunomurai* from Haneji and Odo ($P < 0.05$), and *E. cornigera* from Amakusa and Miura ($P < 0.05$). The total number of plumose setae on maxilliped was not significantly different among each population except for populations from Odo and Miura ($P < 0.01$; Table 2). In *E. nunomurai*, the number of pylopodal setae was significantly lower (mean $\pm$ SD = 5.5 $\pm$ 1.1; Haneji, 4.1 $\pm$ 1.4; Odo) than that of *E. cornigera* (11.6 $\pm$ 2.0; Amakusa, 13.7 $\pm$ 1.4; Miura). The number of setae of uropodal exopods and endopods were not different within and between species.

Phylogenetic Analysis

We obtained total six unique sequences from *E. nunomurai*, *E. cornigera*, *E. rangifer*, and *E. discolor*. We confined our
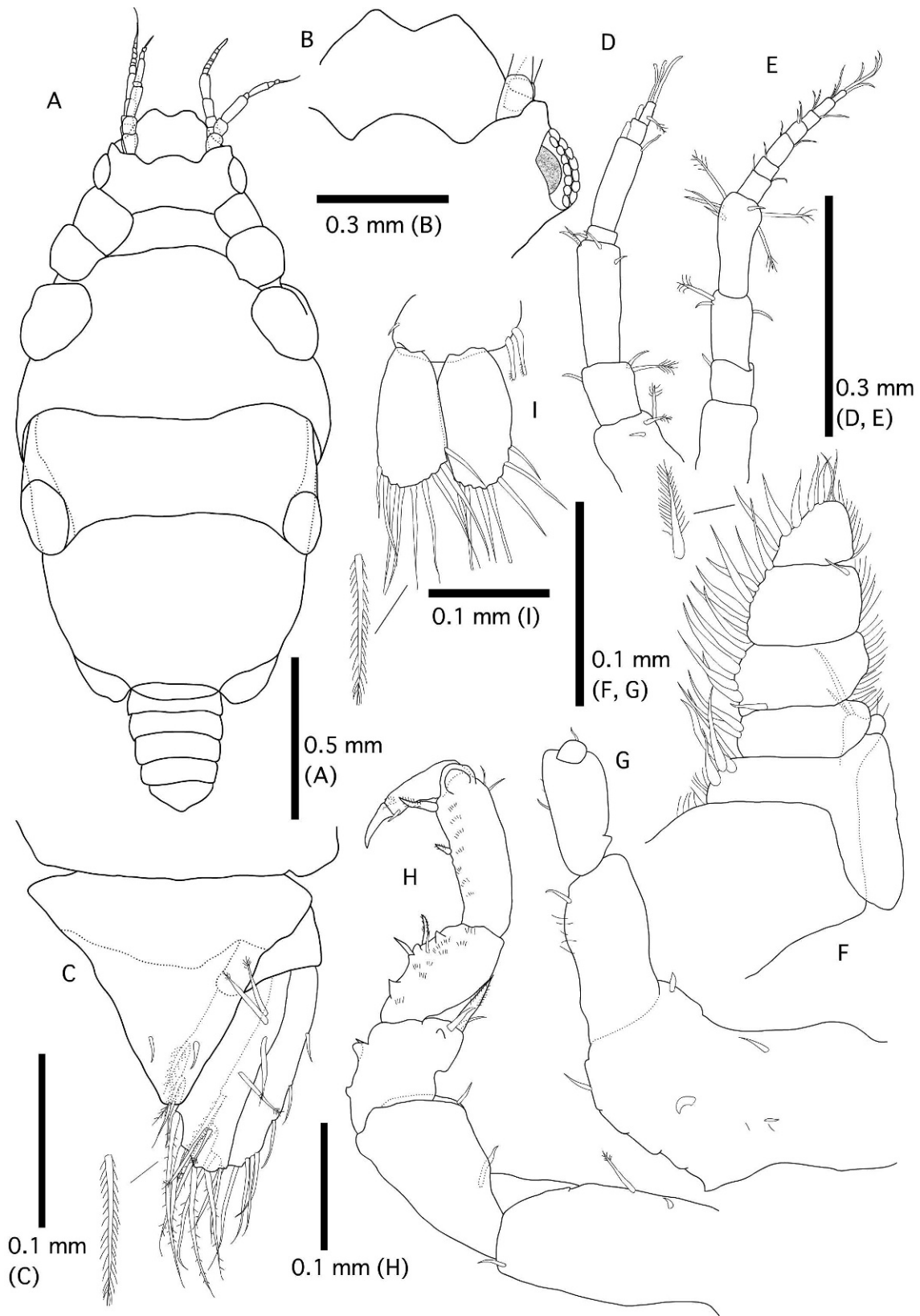
Fig. 4. A-E, Female of *Elaphognathia nunomurai* n. sp. H and I, from paratype (NSMT-Cr 21128); F and G, are from paratype (NSMT-Cr 21128). A, Dorsal view of body, body length is 2.1 mm; B, Right eye and frontal border; C, Pleotelson; D, Antenna 1; E, Antenna 2; F, Maxilliped; G, Pylopod; H, Pereiopod 2; I, Pleopod 4.
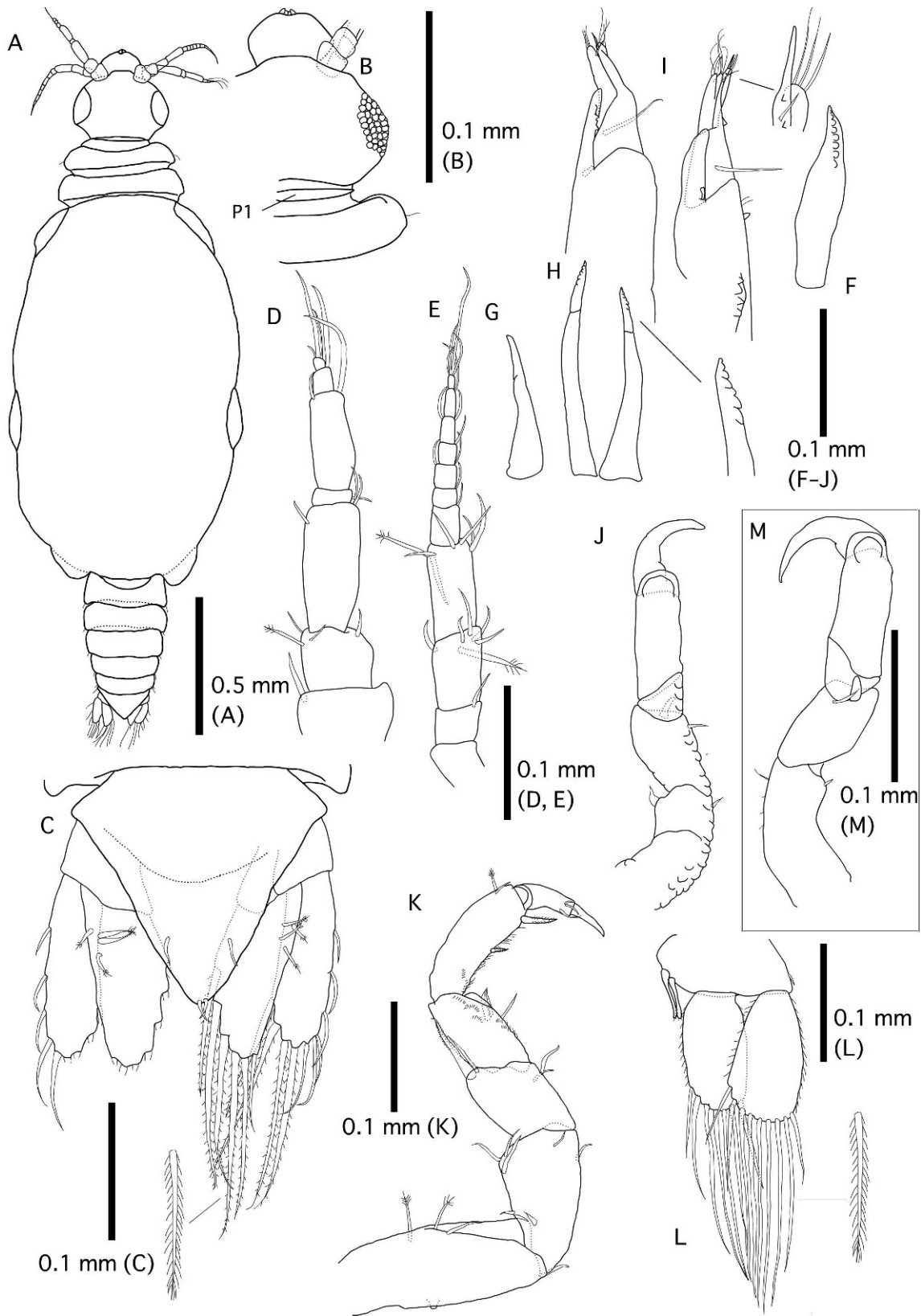
Fig. 5. *Elaphognathia nunomurai* n. sp. larva paratype. A, C-L, 2.4 mm (NSMT-Cr 21128); B, another paratype, body length is unknown (NSMT-Cr 21128). A, Dorsal view of body; B, Cephalosome with pereionite 1 (P1); C, Pleotelson; D, Antenna 1; E, Antenna 2; F, Mandible; G, Paragnath; H, A pair of maxillules; I, Maxilliped; J, Gnathopod; K, Pereiopod 2; L, Pleopod 4; M, in square, Gnathopod of *E. cornigera* from Amakusa.

Table 2. Body proportions and number of setae in male *Elaphognathia nunomurai* from Haneji Island Sea and Odo beach, and *E. cornigera* from Amakusa and Miura. Body length (BL) is expressed as mean ± SD. The setae of pylopod (PY) and maxilliped (MP) were counted using only plumose setae. The number of maxilliped seta is shown as the total and mean number of each palp article (Total No; palp 1-2-3-4). Exopod and endopod of uropod shows both simple seta (s) and plumose seta (p). The results of the Tukey-Kramer Multiple Comparison Test are shown on the lower lines. [a] The total number of seta in all palp articles were compared in the statistical analysis. The lower lines show the statistical significance: – not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	BL ($N = 10$) (mm)	PY seta ($N = 18$)	MP seta [a] ($N = 20$)	Exopod seta ($N = 20$)	Endopod seta ($N = 20$)
E. nunomurai Haneji	2.36 ± 0.24	5.5 ± 1.1	18.4; 2.9-4.0-6.5-5.0	5.9s, 4.0p	1.0s, 6.0p
E. nunomurai Odo	1.74 ± 0.21	4.1 ± 1.4 ($N = 20$)	17.3; 2.9-4.0-6.4-4.0	6.0s, 4.0p ($N = 18$)	1.0s, 6.0p ($N = 18$)
E. cornigera Amakusa	2.17 ± 0.14	11.6 ± 2.0	18.6; 3.0-4.3-6.7-4.6	6.0s, 4.0p	1.0s, 6.0p
E. cornigera Miura	2.47 ± 0.27	13.7 ± 1.4	1.99; 3.2-4.3-6.8-5.6 ($N = 19$)	6.0s, 4.0p	1.0s, 5.9p
Haneji vs. Odo	***	–	–	–	–
Haneji vs. Amakusa	–	***	–	–	–
Haneji vs. Miura	–	***	–	–	–
Odo vs. Amakusa	***	***	–	–	–
Odo vs. Miura	***	***	**	–	–
Amakusa vs. Miura	*	*	–	–	–

analysis to a specific subset of 424-441 base pairs of the rDNA ITS2 sequences. The average base-pair proportions were: A = 0.252, C = 0.191, G = 0.238, and T = 0.319. The sequences of rDNA ITS2 region were identical between the specimens of *E. nunomurai* from the two sites of Okinawa-jima Island. We found three haplotypes among the three sampling sites (Amakusa, Miura, and Shimoda) of *E. cornigera*. The aligned sequences from Miura and Shimoda had no gaps and one variable site over 424 bp. The aligned sequence from Amakusa had one site of insertion/deletion that consisted of three base pairs compared with sequences from Miura and Shimoda, and three and four variable sites between Miura and Shimoda, respectively. The aligned sequences from Haneji and Odo (*E. nunomurai*) had three unique sites of insertion/deletion that consisted of two, three and seven base pairs each compared with three haplotypes of *E. cornigera*, and the haplotype had 15 unique variable sites among three haplotypes of *E. cornigera*. The alignment data set of rDNA ITS (496 positions) were eliminated to 356 positions as poorly aligned and divergent regions by Gblocks. Following analyses were performed with this no gaps dataset. The percentage sequence-divergence among the six haplotypes was 0.5-73.9% (Table 4). The sequence differences between *E. cornigera* from Miura, Shimoda and Amakusa were 0.5-1.5%. The sequence differences between the *E. cornigera* group and *E. nunomurai* were 5.3-5.9%. The sequence differences between *E. cornigera*, *E. rangifer* and *E. discolor* were 32.0-60.5%.

Four topologies from the ML, BI, NJ, and MP analyses were nearly identical. The BI consensus tree using HKY+I substitution model is presented in Figure 6. Monophyly of *E. nunomurai* + *E. cornigera* was supported by high PI (Bayes posterior probability) value (1.0) and high bootstrap values [ML = 99%. MP = 100%; NJ = 100%]. However, monophyly of *E. cornigera* had low PI (0.63) and moderate bootstrap supports (ML = 54%; MP = 70%; NJ = 82%).

DISCUSSION

The habitat of *E. nunomurai* was originally found from the muddy tidal flat at Haneji Island Sea, located in north area of Okinawa-jima Island (Ota et al., 2008). In this study, we found *E. nunomurai* from different environments: the tide pools between sandy or rocky beaches, and the coral-reef lagoon at Odo Beach, south coast of Okinawa-jima Is., and also from Toguchi Beach on the west coast of central Okinawa-jima Is. Furthermore, the sponge species in which *E. nunomurai* lived were different between Haneji and Odo. Thus, *E. nunomurai* may adapt to their environmental circumstances. Although this species has been found form Okinawa-jima Is. only, we believe specimens will also occur in the other islands of the Ryukyus.

Body lengths of male adults from Odo were significantly smaller than those from Haneji. The reason of the difference is not clear. However, their body lengths may be related with the different environment. Generally, gnathiid larvae attach on their host fish to feed their body fluids. On the other hand, the adults never feed nor molt (Monod, 1926; Mouchet, 1928; Stoll, 1962), and they survive only on the reserves provided by their larvae. This indicates that body size of the adult depends on the quality and/or quantity of fish body

Table 3. The numbers of plumose setae on pylopod (PY) ($N = 20$) and maxilliped (MP) ($N = 20$). All data is based on 10 adult males in species of *Elaphognathia*.

	PY seta	MP seta				
		Palp 1	Palp 2	Palp 3	Palp 4	Total
E. nunomurai Haneji	4-8 ($N = 18$)	2-3	3-5	4-8	3-6	15-21
E. nunomurai Odo	2-7	2-3	3-5	3-7	2-5	11-20
E. cornigera Amakusa	7, 10-14 ($N = 18$)	3	4-5	6-8	3-6	17-20
E. cornigera Miura	12-16 ($N = 18$)	3-4 ($N = 19$)	3-6 ($N = 19$)	5-8 ($N = 19$)	4-7 ($N = 19$)	17-25 ($N = 19$)

Table 4. Sequence divergence (using HKY+I model) for DNA ITS2 sequences in the *Elaphognathia* ribosome.

	E. cornigera			*E. nuno-murai*		*E. rangifer*	*E. discolor*
	Miura	Shimoda	Amakusa	Haneji	Odo		
E. cornigera Miura		0.5	1.4	5.9	5.9	32.6	59.4
Shimoda			1.5	5.9	5.9	32.7	59.9
Amakusa				5.3	5.3	32.0	60.5
E. nunomurai Haneji					0	35.8	73.9
Odo						35.8	73.9
E. rangifer							46.4

fluids, and fish species. Unfortunately, host fish of *E. nunomurai* at Haneji and Odo have not been found. However, their larvae may feed on the different fish species between the two different environments.

We compared the number of plumose setae on mouthparts (pylopod and maxilliped) and both rami of uropod in *E. nunomurai* and *E. cornigera*. The latter was stable within and between species. In contrast, the former was variable within each population. Smit and Davies (2004) observed that the number of plumose seta on maxilliped articles were constant within species, but different between species in *Gnathia*. On the other hand, the present study is based on two species of *Elaphognathia*. Our results showed that the number of setae on the maxilliped varied within the same population and the range of number of pylopod seta may be important for dividing the species.

DNA sequences have potential as useful name tags for species, if the sequences have species-specific variation. Recently, the ''Folmer region'' at the 5′end of the cytochrome oxidase subunit I mitochondrial region is emerging as the standard barcode region (Ratnasingham and Herbert, 2007), but the DNA fragments were not amplified with the universal primer (Folmer et al., 1994) in some crustacean studies, such as *Metanephrops* (Decapoda: Nephropidae) (Chan et al., 2009) and Munnopsidae (Isopoda: Asellota) (Osborn, 2009). In the present study, we could not obtain reliable sequences of the standard barcode region from the *Elaphognathia* spp. Grutter et al. (2000) developed the specific primers to amplify *Gnathia* rDNA ITS2 region, and they showed that ITS2 sequences are useful for distinguishing species of gnathiid isopods. On the other hand, many studies revealed that the presence of intragenomic sequence variations within the rDNA ITS region, e.g., in sponges (Wörheide et al., 2004; Alvarez et al., 2007), cnidarians (Márquez et al., 2003), nematodes (Conole et al., 1999), crustaceans (Harris and Crandall, 2000), insects (Leo and Barker, 2002; Keller et al., 2006; Li and Wilkerson, 2007; Sword et al., 2007; Fisher and Smith, 2008), or salmonid fishes (Reed et al., 2000). Thonhill et al. (2007) showed that the sequences obtained by direct sequencing of PCR products and via bacterial cloning were identical to the most commonly cloned sequence and the one that occupied the centre of a phylogenetic polytomy in eukaryotic unicellular alga (*Symbiodinium*). The stability
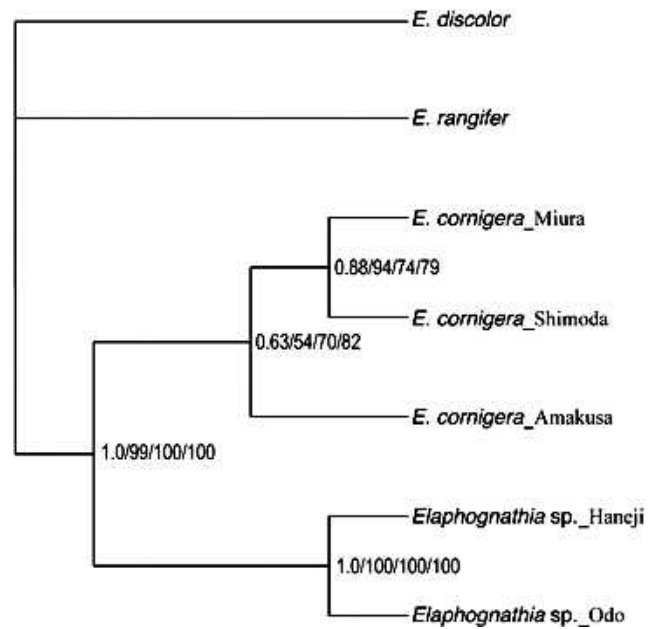

Fig. 6. BI consensus tree of *Elaphognathia* based on internal transcribed spacer 2 of ribosomal DNA (rDNA-ITS2) sequences. The HKY+I substitution model was used for the analysis. The bootstrap probabilities of ML analysis, MP analysis and NJ analysis lager than 50%, and the Bayesian posterior probability were noted (the order of there values is BI/ML/MP/NL). The length if each branch is not proportional to the estimated substitution number for each branch.

and eventual replacement of a dominant rDNA sequence operates though concerted evolution, a process that requires the turnover of numerous generations on evolutionary time scales (Dover, 1986). Therefore, putative dominant sequences may be useful 'markers' for identification among closely related species. In this study, the sequences of ITS2 region were obtained by direct sequencing in two samples. Following sub-cloning, we completed the sequences as the dominant sequences among five or more clones for each of the two samples.

Grutter et al. (2000) reported divergence in ribosomal DNA ITS2 sequences between gnathiid isopod species ranging from 38.5% to 43% as compared to within-species divergence levels of < 1%. However, they did not examine the morphological characters of each species. In this study, we obtained six haplotypes from seven specimens of four species of *Elaphognathia*. The divergence of the ribosomal DNA ITS2 sequences was 0-1.5% within species and 32.0-73.9% between species. These data are not in conflict with those of Grutter et al. (2000). The present study confirms that ribosomal DNA ITS2 sequences are useful for distinguishing species of *Elaphognathia* that are closely similar in morphology.

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