



A new species of *Bestiolina* (Copepoda, Calanoida, Paracalanidae) and complementary description of *B. similis* (Sewell, 1914) from the Nansei Islands, Japan

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Abstract

Two species of the calanoid copepod genus *Bestiolina* occurred in estuarine/coastal waters of the Nansei Islands, southernmost Japan. One of them is described herein as *Bestiolina okinawae* **sp. nov.**, which is distinguished from known congeners by a combination of the following characteristics: 1) fifth pedigerous somite bearing groups of tiny spinules on the posterior end, 2) female mandible bearing a rod-shaped first gnathal tooth, 3) male left leg 5 with short apical spine on long terminal segment, and 4) legs 2–3 with endopodal segment 2 lacking spinules. The new species was the dominant zooplankton in oligohaline waters. The other species is morphologically identifiable to *Bestiolina similis* (Sewell, 1914). Genetic analysis using COI revealed that the two species differed by 9.9–10.0%, in contrast to small intra-specific genetic variation (0.0–1.5%). The two species were spatially segregated in low-salinity (2–30) estuaries and high-salinity (25–36) coastal waters, respectively, but co-occurred in some samples. Their body length decreased from the winter (around 20°C) to the summer (around 30°C) by 9.5–18.1%, depending on the species and sex. In both species, adult males, which lack mandibular gnathobase, were larger than the adult females.

Key words: *Bestiolina*, calanoid copepod, brackish water, new species, taxonomy

Introduction

The planktonic copepod genus *Bestiolina* Andronov, 1991 (Calanoida, Paracalanidae) inhabits warm estuarine/coastal waters and sometimes becomes the dominant zooplankton in brackish waters (Oka et al. 1992; Vineetha et al. 2015; present study). The genus currently consists of nine species (Walter & Boxshall 2021). *Bestiolina inermis* (Sewell, 1912) and *B. similis* (Sewell, 1914) are widely distributed in the Indo-West Pacific region, while the other seven species have been confined to type localities or narrow ranges (Moon et al. 2010, Razouls et al. 2005–2021). Their geographical ranges have been considered to correspond largely to the Indomalayan realm (Moon et al. 2010). However, *B. mexicana* Suárez-Morales & Almeyda-Artigas, 2016 and *B. sarae* Dorado-Roncancio & Gaviria, 2019 were recently described from brackish waters outside the Indo-West Pacific region, i.e., the coast of Gulf of Mexico (Suárez-Morales & Almeyda-Artigas 2016) and the tropical Pacific coast of South America (Dorado-Roncancio et al. 2019). The species recorded from the Japan archipelago has only consisted of *B. similis* (Nishida 1985; Oka et al. 1992; Ueda 1997; Ueda & Hoshino 2003); *B. sinica* (Shen & Lee, 1966) was once described from the Nansei Islands, southernmost Japan, by Oka et al. (1992) but it was later assigned to *B. similis* (pers. comm. from S. Oka to HU on 14 February 2006).

Koike (1991) described two forms of *B. similis* from Okinawa Island, the largest island among the Nansei Islands. One is furnished with spinule rows on the fifth pediger and is smaller than the other (hereafter, small and large forms, respectively). These two forms are undoubtedly distinct species from each other, because the presence/absence of spinules on the fifth pediger has been considered a critical species-specific characteristic of *Bestiolina*

(Dorado-Roncancio et al. 2019, Moon et al. 2010, Suárez-Morales & Almeyda-Artigas 2016). In order to determine their taxonomic status, we re-examined Okinawan specimens and analyzed their mitochondrial gene cytochrome oxidase subunit I (COI). As a result, we identified the small form as an undescribed species and the large form as *B. similis*. Herein we describe the small form as a new species, and for comparative purposes, a short description of *B. similis* from the surveyed area, along with the results from genetic analyses.

Materials and methods

The present specimens were collected from six sampling sites from four locations in Okinawa and Ishigaki Islands of the Nansei Islands (Table 1). Sampling was performed from the shore or in shallow nearshore water using a small plankton net with a 0.1-mm mesh size. Samples were fixed and preserved in buffered formalin solution or 99% ethanol immediately after sampling. Surface water temperature and salinity were measured using a hand-held temperature-salinity meter (Sinar TS-meter NS-3P, Merbabu Corp., Japan) or a mercury thermometer.

TABLE 1. Summary of plankton samples for the present study with sampling location, date, environmental variables, and the sample-specific occurrence of *Bestiolina* (N=new species, S=*B. similis*).

sample	location	longitude, latitude	sampling date	temp. °C	salinity	species
#1	Hija River, Stn. 1	26.3625N, 127.7466E	21 Dec. 1989	21.3	28.5	N
#2			25 Jul. 1990	31.9	16.1	N
#3	Hija River, Stn. 2	26.3611N, 127.7453E	1 Sept. 2006	32.0	25.0	N,S
#4	Hija River Stn. 3	26.3673N, 127.7541E	25 Sept. 2010	n.d.	2	N
#5	Katabaru Beach	26.5096N, 127.9978E	1 Sept. 2006	32.0	25.0	N
#6	Aja Harbor	26.2386N, 127.6793E	28 Jul. 1986	28.0	36	S
#7			14 Jan. 1987	18.9	36	S
#8	Henoko Harbor	26.5191N, 128.0345E	1 Sept. 2006	30.3	34.3	S
#9	Naha Harbor	26.2100N, 127.6565E	21 Dec. 1979	n.d.	n.d.	S
#10	Tomari Harbor	26.2290N, 127.6809E	1 Sept. 2006	29.8	28.8	S
#11	Ishigaki Island	24.4564N, 124.2145E	7 Aug. 2007	33.1	33.1	N,S

Examination, measurement and drawing of specimens were done in 70% lactic acid or 50% glycerol under a microscope equipped with a drawing tube. For detailed examination, specimens were stained with a 0.1% chlorazol-black E solution and observed with a 100x long working distance objective lens (Nikon CF Plan Epi ELWD). In the case of a specimen with the urosome bent dorsalward, the body length was obtained by combining the prosome and urosome lengths. In addition to morphological observations, to assess potential seasonal variation we randomly measured the body and prosome lengths from 10 female and 10 male adult specimens from field samples #1 and #2 (Hija River, Stn 1) and #6 and #7 (Aja Harbor). The final figures were prepared by tracing drawings with computer software (Adobe Illustrator®).

The genomic DNA was extracted from specimens using NucleoSpin Tissue XS (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The COI sequences were analyzed for three female specimens of the new species from Hija River (sample #4), and two female *Bestiolina similis* specimens each from Henoko Harbor (sample #8) and Tomari Harbor (sample #10). The COI region was PCR-amplified using primers LCO1490 and HCO2198 (Folmer et al. 1994). The PCR was performed using TaKaRa Ex Taq (TaKaRa, Shiga, Japan) under

the following conditions: 30 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 30 s, and extension at 72°C for 1 min. The PCR products were cleaned using ExoSAP-IT Express (Affymetrix, Santa Clara, CA, USA) and directly sequenced with the 3730xl Genetic Analyzer DNA autosequencer (Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturers' instructions.

The COI sequences were aligned using MAFFT (Katoh & Standley 2013). The COI pairwise p-distances were calculated using MEGA X. The maximum likelihood (ML) phylogenetic trees and the corresponding bootstrap support values (1000 replicates) were calculated using MEGA X software (Kumar et al. 2018) for the resulting datasets. Initial trees for a heuristic search were constructed automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with the superior log likelihood. For these datasets, Bayesian analyses were performed using MrBayes v3.2.7a (Ronquist et al. 2012). Six parallel metropolis-coupled Markov chain Monte Carlo (MCMCMC) runs, each consisting of three heated chains and one cold chain with default chain temperatures, were run for 1,000,000 generations. Log-likelihood scores and trees with branch lengths were sampled every 1000 generations. The first 250,000 generations were excluded as burn-in, and the remaining trees were summarized to obtain Bayesian posterior probabilities. Convergence of parallel MCMCMC runs was judged by the average standard deviation of split frequencies. For both ML and Bayesian analyses, the most appropriate model (the general time reversible model with gamma-distribution) was selected with the "Find Best DNA/Protein models (ML)" setting of MEGA X. The nucleotide sequences determined in the present study have been deposited in the GenBank databases under accession nos. LC621162–LC621164 for the new species (named *Bestiolina okinawae* sp. nov.), and LC621165 and LC621166 for the large form as *B. similis*.

Specimens of the two species in ethanol with glycerol were deposited into the National Museum of Nature and Science, Tokyo (NSMT). The morphological terminology herein follows Huys & Boxshall (1991) and Boxshall & Halsey (2004), except for the following abbreviations on the rami of legs: exp1–exp3 and enp1–enp3 for the first–third exopodal segments and the first–third endopodal segments, respectively.

Results

Taxonomy

Order Calanoida G.O. Sars, 1903

Family Paracalanidae Giesbrecht, 1893

Genus *Bestiolina* Andronov, 1991

Bestiolina okinawae sp. nov.

(Figs. 1–4)

urn:lsid:zoobank.org:pub:4D1BCA86-9A37-4362-A9D4-CFA2A53CFB2B

Bestiola similis small form: Koike (1991), 16, figs. 17–19, 20a,b.

Materials examined. Female holotype (NSMT-Cr 28994), male allotype (NSMT-Cr 28995) collected from Hija River Stn. 1 on 21 December 1989. Paratypes: 17 females and 14 males from Hija River Stn. 1 on 21 December 1989 and 11 female and 10 males from the same site on 26 July 1990; 1 female from Hija River Stn. 2 on 1 September 2006; 3 females and 2 males from Katabaru Beach on 1 September 2006; 1 female and 1 male from Ishigaki Island on 7 August 2007. The other 10 females (NSMT-Cr 28996) and 10 males (NSMT-Cr 28997) from Hija River Stn. 1 on 21 December 1989 were deposited.

Descriptions. *Female.* Body (Fig. 1A, B) length 0.60–0.73 mm (n=11, holotype 0.68 mm) prosome length 0.48–0.52 mm (n=4, holotype 0.51 mm), widest at posterior margin of cephalothorax. Fifth pedigerous somite small, separated from fourth pedigerous somite by complete suture, bearing groups of tiny spinules on posterior end (Fig. 1C, D). Genital double somite (Fig. 1D) strongly produced ventrally; seminal receptacle rounded, located

within ventral projection in lateral view. Anal somite length about 0.8 times genital double somite and as long as preceding 2 urosomites. Caudal rami (Fig. 1E) slightly longer than twice its width, armed with 4 long terminal and 1 short subterminal medial setae; medialmost terminal seta (arrow in Fig. 1E) almost as long as adjacent lateral seta.

Antennule (Fig. 1F) 25 segmented; ancestral segments I–III and XXVII–XXVIII fused, with incomplete suture between I and II; segmentation and setation (ae = aesthetasc, sp = spine): 1(I–III)=6+ae, 2(IV)=1+ae, 3(V)–4(VI)=2+ae, 5(VII)=1, 6(VIII)=1+ae, 7(IX)=sp+ae, 8(X)–9(XI)=1+ae, 10(XII)–11(XIII)=1, 12(XIV)=0, 13(XV)=1+ae, 14(XVI)–20(XXII)=1, 21(XXIII)–23(XXV)=2, 24(XXVI)=1, 25(XXVII–XXVIII)=3. Ancestral segments I–VII with row of tiny spinules near distal margin.

Antenna (Fig. 1G) with coxa and basis armed with 1 and 2 medial setae, respectively. Exopod 7-segmented, with setal formula 2, 2, 1, 1, 1, 1, 4. Endopod 2-segmented; segment 1 with 2 setae at midlength of medial margin and fine setules on distolateral margin; segment 2 with 9 setae medially, 7 terminal setae and fine setules along distal half of lateral margin.

Mandible (Fig. 1H, I) coxal gnathobase with cutting blade medially, seta at postmedial corner and row of tiny spinules on dorsal surface. Cutting edge of blade consisting of rod-shaped first tooth (arrow in Fig. 1H), 4 sclerotized molar-like teeth, 4 pointed teeth posteriorly, and hyaline teeth on molar-like teeth; length of rod-shaped first tooth about 3 times width at base, with hyaline tip. Palp basis with 2 short and 2 long setae medially, 5-segmented exopod armed with 1, 1, 1, 1, 2 setae, and 2-segmented endopod with 4, 11 setae.

Maxillule (Fig. 2A) praecoxal arthrite with 10 long spines, 4 setae, and 2 rows of tiny spinules; coxal epipodite with 7 long and 3 short setae; coxal endite with 3 setae; basal endites with 3, 4 setae; exopod with 11 setae; endopod with 7 setae medially and 7 distally.

Maxilla (Fig. 2B) praecoxa and coxa fused with 4 endites bearing 5, 3, 3, 3 setae; basal endite with 4 setae, one of them setiform; endopod 3-segmented with 2, 3, 3 setae.

Maxilliped (Fig. 2C) syncoxal with 4 endites each bearing 1, 2, 3, 4 setae; basis with 3 setae; endopod 6-segmented with 2, 3, 3, 4, 3+1, 4 setae.

Leg 1 (Fig. 1D) endopod 2-segmented; exp3 length 2 times width. Spine and setal formula of legs 1–4 (Fig. 1D–G) as follows.

	Coxa	Basis	Exopodal segment	Endopodal segment
Leg 1	0-0	0-1	0-1; 0-1; 2,1,4	0-1; 1,1,3
Leg 2	0-1	0-0	I-1; I-1; II,I,5	0-1; 0-2; 1,2,3
Leg 3	0-1	0-0	I-1; I-1; II,I,5	0-1; 0-2; 1,2,3
Leg 4	0-1	0-0	I-1; I-1; II,I,5	0-1; 0-2; 1,2,3

Legs 1–4 rami with tiny spinules as follows (spinule number on anterior/posterior surfaces): leg 1, exp1=0/3; leg 2, exp1=4/0, exp2=3/0, exp3=0/3; leg 3, exp2=3–4/0; leg 4, exp2=3/0, exp3=4/0.

Leg 5 (Fig. 2J) reduced into pair of 1-segmented small lobes; right leg slightly larger than left leg; right leg of one specimen from Hija River (sample #1) abnormally with additional segments (Fig. 2I).

Male. Body (Fig. 3A, B) length 0.62–0.87 mm (n=7, allotype 0.81 mm); prosome length 0.45–0.59 mm (n=6, allotype 0.57 mm), widest at 1/3 length of prosome, with dorsal hump at 1/4 length of cephalothorax. Urosome usually bent dorsalward. Spinule rows on fifth pedigerous somite (Fig. 3C), caudal rami and setae (Fig. 3D) as in female. Caudal rami of some specimens strongly divergent.

Antennule (Fig. 3E) 19-segmented; considering ancestral segments I–VIII, IX–X, and XXVII–XXVIII fused; articulate segments 1–3 with conspicuous aesthetascs; armature on each segment as follows: 1(I–VIII) = 6+11ae, 2(IX–X)=3+sp+2ae, 3(XI)=1+3ae, 4(XII)–5(XIII)=1, 6(XIV)=ae, 7(XV)=0, 8(XVI)=1+ae, 9(XVII)–13(XXI)=1, 14(XXII)=ae, 15(XXIV)=1, 16(XXV)–18(XXVI)=2, 19(XXVII–XXVIII)=3+ae. Antennular segments with no spinules; aesthetascs on segments 1 and 2 turned ventralward.

Antenna (Fig. 4A) basis with single seta on distomedial corner. Exopod 4-segmented, armed with 1, 1, 2, 1 setae. Endopod 2-segmented; segment 1 unarmed; segment 2 with 13 setae arranged in 2 groups, 6 of them medially and 7 terminally.

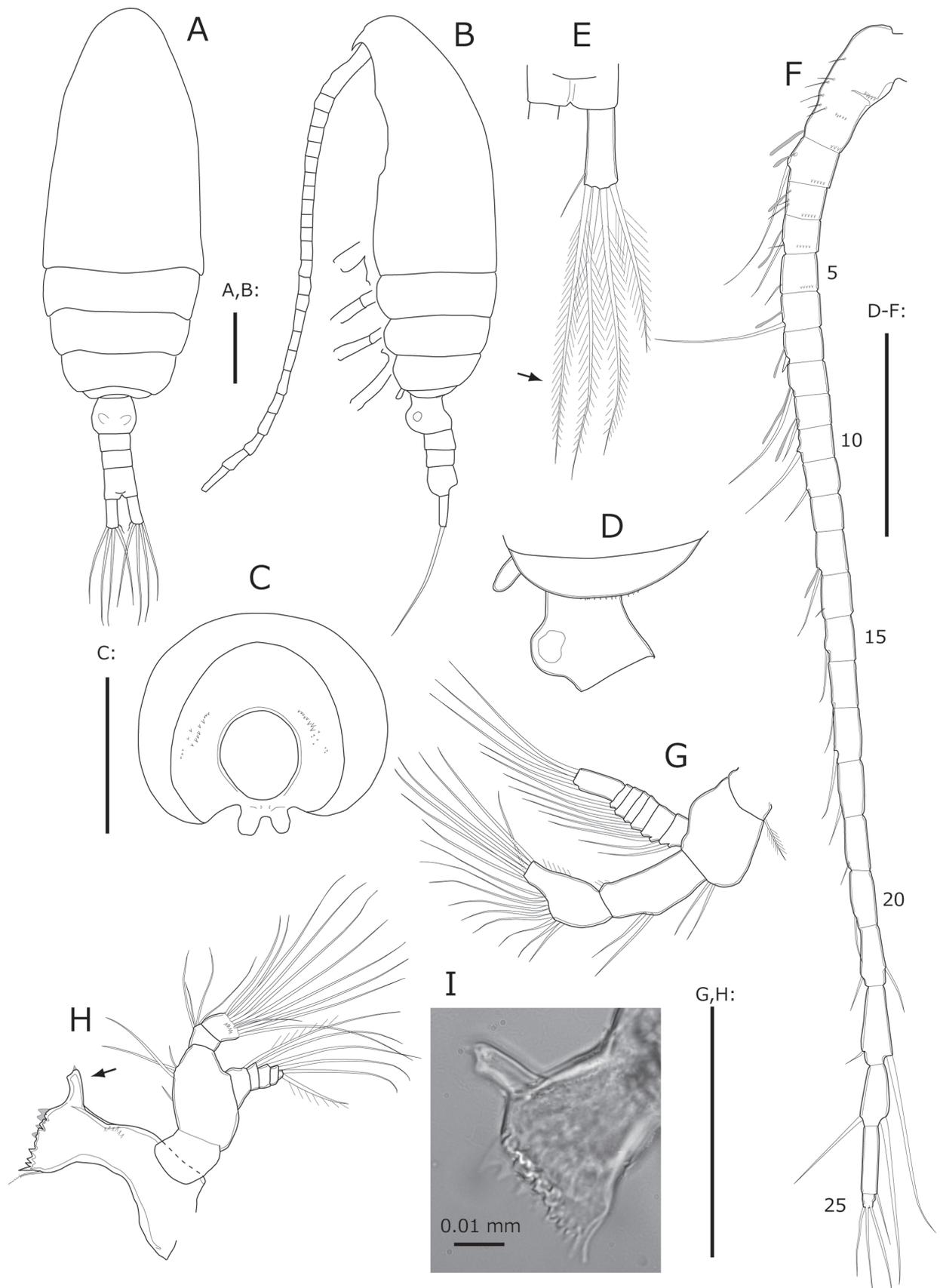


FIGURE 1. *Bestiolina okinawae* sp. nov. female (A, B, holotype; C–I, from sample #1). A, habitus, dorsal; B, habitus, lateral; C, fourth and fifth pedigerous somites with leg 5, posterior; D, fifth pedigerous somite with leg 5 and genital double somite; E, right caudal ramus, dorsal; F, antennule; G, antenna; H, mandible; I, mandible gnathal blade (same specimen as H). Scale bars (except for I), 0.1 mm.

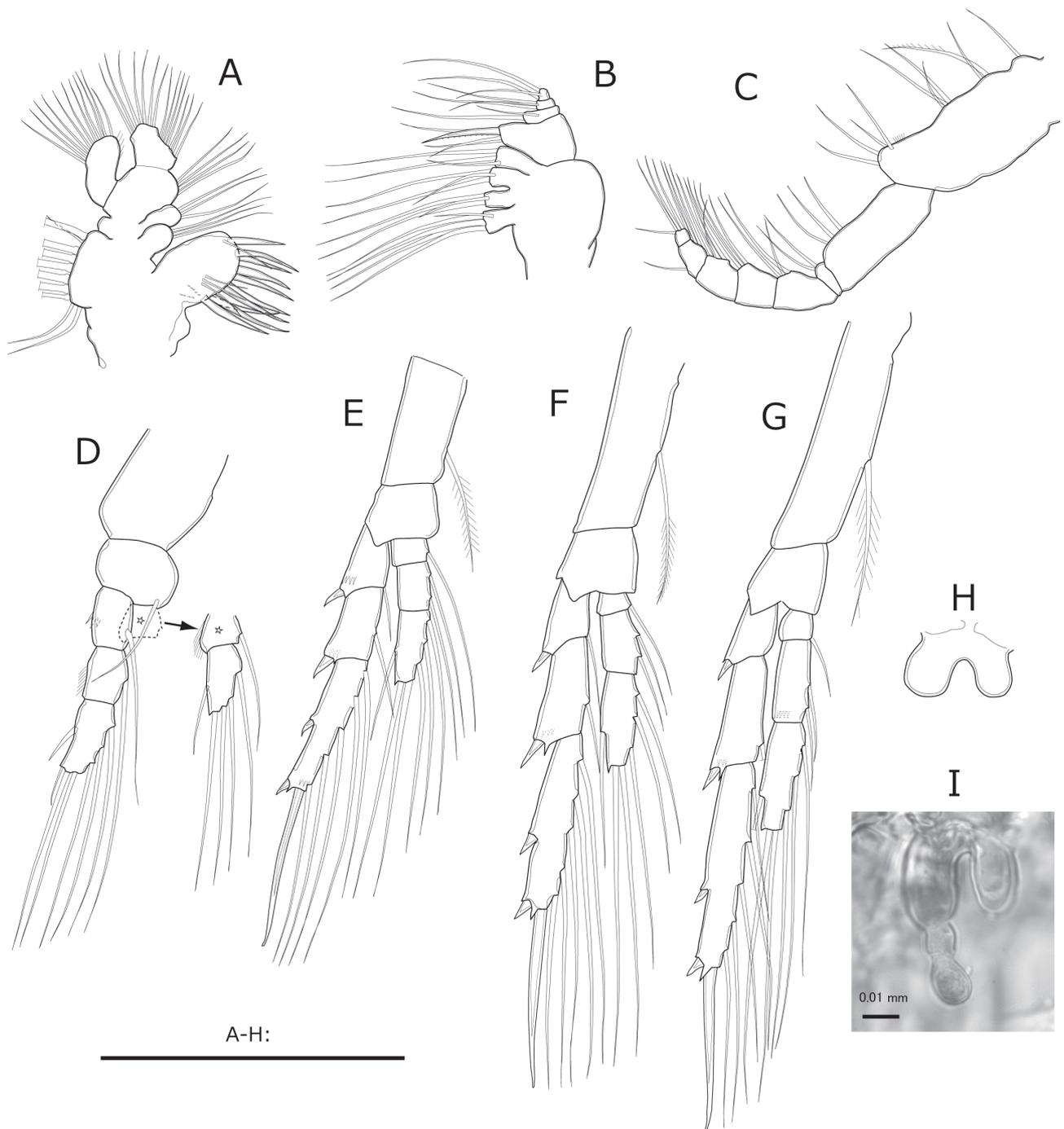


FIGURE 2. *Bestiolina okinawae* sp. nov. female (A, D–H, from sample #5; B, C, I, from sample #1). A, maxillule; B, maxilla; C, maxilliped; D, leg 1, anterior; E–H, legs 2–4, posterior; I, anomaly of leg 5. Scale bars (except for I), 0.1 mm.

Mandible (Fig. 4B) coxal gnathobase absent. Basis with seta as long as seta on enp1. Exopod 4-segmented, with 0, 1, 1, 4 setae; lateralmost seta of segment 4 as long as half of next seta.

Maxilliped (Fig. 4C) reduced, comprising syncoxa, basis, and 2-segmented endopod. Syncoxa naked; basis with unipinnate medial seta. Endopod segment 1 with 7 seta, one of them spiniform and located on lateral margin, remaining 6 on medial margin and distal 3 curved distalward; segment 2 with 3 setae, lateral and terminal ones of them spiniform; 2 spiniform lateral setae directed proximalward. Maxillule and maxilla rudimentary.

Leg 1 (Fig. 4D) basis with unipinnate seta and strongly curved lateralward; exp3 length 3 times width, with spine and setal formula 1+I, I, 4, distolateral spine small and shorter than segment width. Legs 1–4 devoid of spinules.

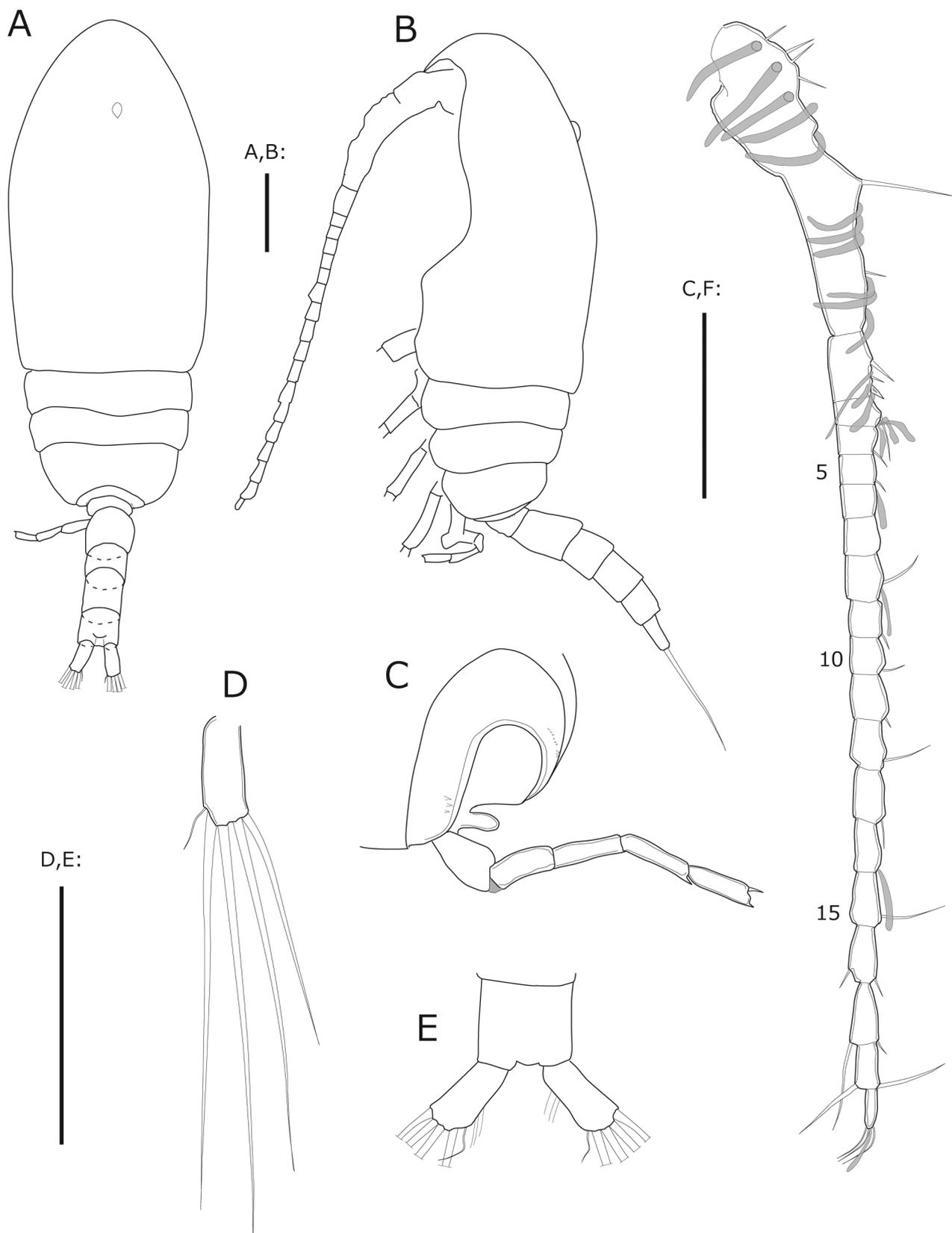


FIGURE 3. *Bestiolina okinawae* sp. nov. male (A, B, allotype; C–E, from sample #1). A, habitus, dorsal; B, habitus, lateral; C, fifth pedigerous somite with leg 5, dorsolateral; D, right caudal ramus; E, antennule. Scale bars, 0.1 mm.

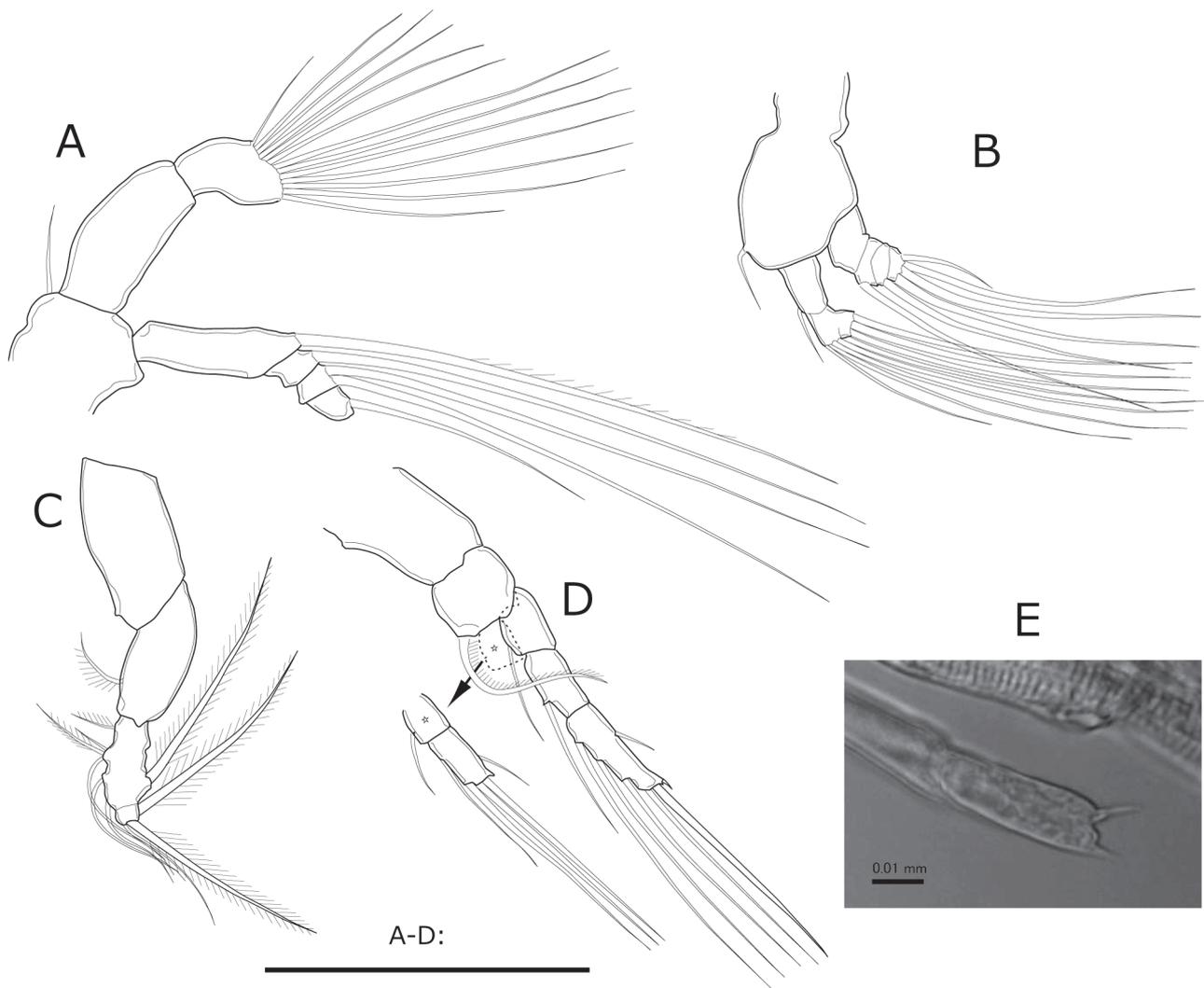


FIGURE 4. *Bestiolina okinawae* sp. nov. male from sample #1. A, antenna; B, mandibular palp; C, maxilliped; D, leg 1, anterior; E, leg 5 terminal segment. Scale bar (except for E), 0.1 mm.

Leg 5 (Figs. 3C, 4E) right leg reduced to rudimentary lobe as in female; left leg 5-segmented, uniramous; geniculate between swollen segment 1 and normal segment 2, and generally turned to left (Fig. 3A); segment 5 slightly shorter than segment 4; apical spine length 1/5–1/4 times segment 5.

Etymology. The name *okinawae* makes reference to the island of Okinawa, the type locality.

TABLE 2. Length ratios of the apical spine (AS) to segment 5 (S5) and of S5 to segment 4 (S4) of the male left leg 5 of *Bestiolina* spp. measured from the original descriptions of the species. Segment length did not include distal attenuation.

	<i>amoyensis</i> ¹	<i>arabica</i> ²	<i>coreana</i> ³	<i>inermis</i> ⁴	<i>mexicana</i> ⁵	<i>sarae</i> ⁶	<i>similis</i> ⁷	<i>sinica</i> ⁸	<i>zeylonica</i> ⁹	<i>okinawae</i> sp. nov.
AS/S5	0.7	0.8	1.0	1.6	0.7	0.9	0.9	0.8	0.5	0.2
S5/S4	0.4	0.5	0.5	0.5	0.6	0.5	0.4	0.4	0.7	0.9

Original descriptions: 1, Li & Huang (1984); 2, Ali et al (2007); 3, Moon et al. (2010); 4, Sewell (1912); 5, Suárez-Morales & Almeyda-Artigas (2016); 6, Dorado-Roncancio et al. (2019); 7, Sewell (1914); 8, Shen & Lee (1966); 9, Andronov (1972).

Remarks. The most characteristic feature of *Bestiolina okinawae* **sp. nov.** is the short apical spine on the long terminal segment 5 of the male left leg 5. The length ratios of the apical spine to the segment 5 and of the segment 5 to the segment 4 are 0.2 and 0.9, respectively, whereas these ratios in the original descriptions of the nine congeners were 0.5–1.6 and 0.4–0.7, respectively (Table 2).

Sewell (1914) noted that the absence of spinules on posterior surfaces of enp2 of legs 2–4 of *Bestiolina similis* female was a distinct character from *B. inermis*. In the female of *B. okinawae* **sp. nov.**, there are no spinules on enp2 of legs 2 and 3, which is unique in the genus because spinules are present on the anterior and/or posterior surfaces of these segments in all known congeners (Dorado-Roncancio et al. 2019).

A rod-shaped anterior tooth of the mandibular gnathobase of the female is probably a characteristic feature of *Bestiolina okinawae* **sp. nov.** According to previous illustrations of the gnathal blade of congeners, the corresponding tooth is considerably larger in *B. sarae* (Dorado-Roncancio et al. 2019) and *B. mexicana* (Suárez-Morales & Almeyda-Artigas 2016), or much broader at the base in *B. inermis* (Sewell 1912), *B. arabica* Ali, Al-Yamani & Prusova, 2007 (Ali et al. 2007) and *B. coreana* Moon, Lee & Soh, 2010 (Moon et al. 2010). In addition, the medi-almost terminal caudal seta, which is slightly shorter than the longest next seta in *B. okinawae* **sp. nov.**, is possibly a characteristic. Although this seta has never been mentioned in previous descriptions of *Bestiolina*, illustrations of *B. coreana* (Moon et al. 2010) and *B. mexicana* (Suarez-Morales & Almeyda-Artigas 2016) show they are shorter than a half of the next seta.

Bestiolina okinawae **sp. nov.** occurred at salinities from 2 to 30 and was highly abundant from oligohaline waters (Table 1). In sample #4 (Hija River Stn. 3, salinity 2), copepods were the dominant zooplankton with 75% consisting of *B. okinawae* **sp. nov.**, followed by the small form of *Oithona dissimilis* Lindberg, 1940 (Ueda et al. 2011) (25%).

Bestiolina okinawae **sp. nov.** occurred year-round in Okinawa Island, and showed clear seasonal variation in body size. Measurements of 10 specimens from winter (23.1°C) and summer (31.9°C) samples revealed that the body length decreased from winter to summer by 18.1% in both sexes (Table 3). The mean body length of males was significantly larger than females in both seasons ($p < .001$, *t*-test).

TABLE 3. Mean and range (in parentheses) of body length (in mm) of *Bestiolina okinawae* **sp. nov.** (Bo) and *B. similis* (Bs) from Okinawa Island. Each value is based on 10 specimens randomly selected from sample #1 (Bo, winter), #2 (Bo, summer), #6 (Bs, summer), and #7 (Bs, winter). See Table 1 for sample data.

		winter	summer
<i>B. okinawae</i> sp. nov.	female	0.72 (0.66–0.77)	0.59 (0.57–0.61)
	male	0.83 (0.79–0.84)	0.68 (0.64–0.71)
<i>B. similis</i>	female	0.74 (0.72–0.76)	0.67 (0.64–0.71)
	male	0.80 (0.73–0.85)	0.70 (0.61–0.74)

Bestiolina similis (Sewell, 1914)

(Figs. 5, 6)

Acrocalanus similis Sewell, 1914: Sewell (1914), p. 211–213, pl. 17, figs. 3–5; Wellershaus (1967), p. 252–254, figs. 10–20;

Bestiola similis: Greenwood (1976), p. 20, fig. 7a–j.

Bestiola similis large form: Koike (1991), p. 14–15, fig. 15, 16, 20c,d.

Bestiola sinicus: Oka et al. (1992), p. 83–85, fig. 2.

Materials examined. 13 females and 14 males from Aja Harbor on 28 July 1986, and 13 females from the same site on 21 December 1979; 1 female and 1 male from Ishigaki Island on 7 August 2007; 2 females and 4 males from Naha Harbor on 28 July 1986; 3 females and 1 male from Ishigaki Island on 7 August 2007. The other 10 females (NSMT-Cr 28998) and 10 males (NSMT-Cr 28999) from Aja Harbor on 28 July 1986 were deposited.

Descriptions. *Female.* Body (Fig. 5A, B) length 0.70–0.80 mm (n=6); prosome length 0.54–0.60 mm (n=6); no spinules on posterior corner of prosome.

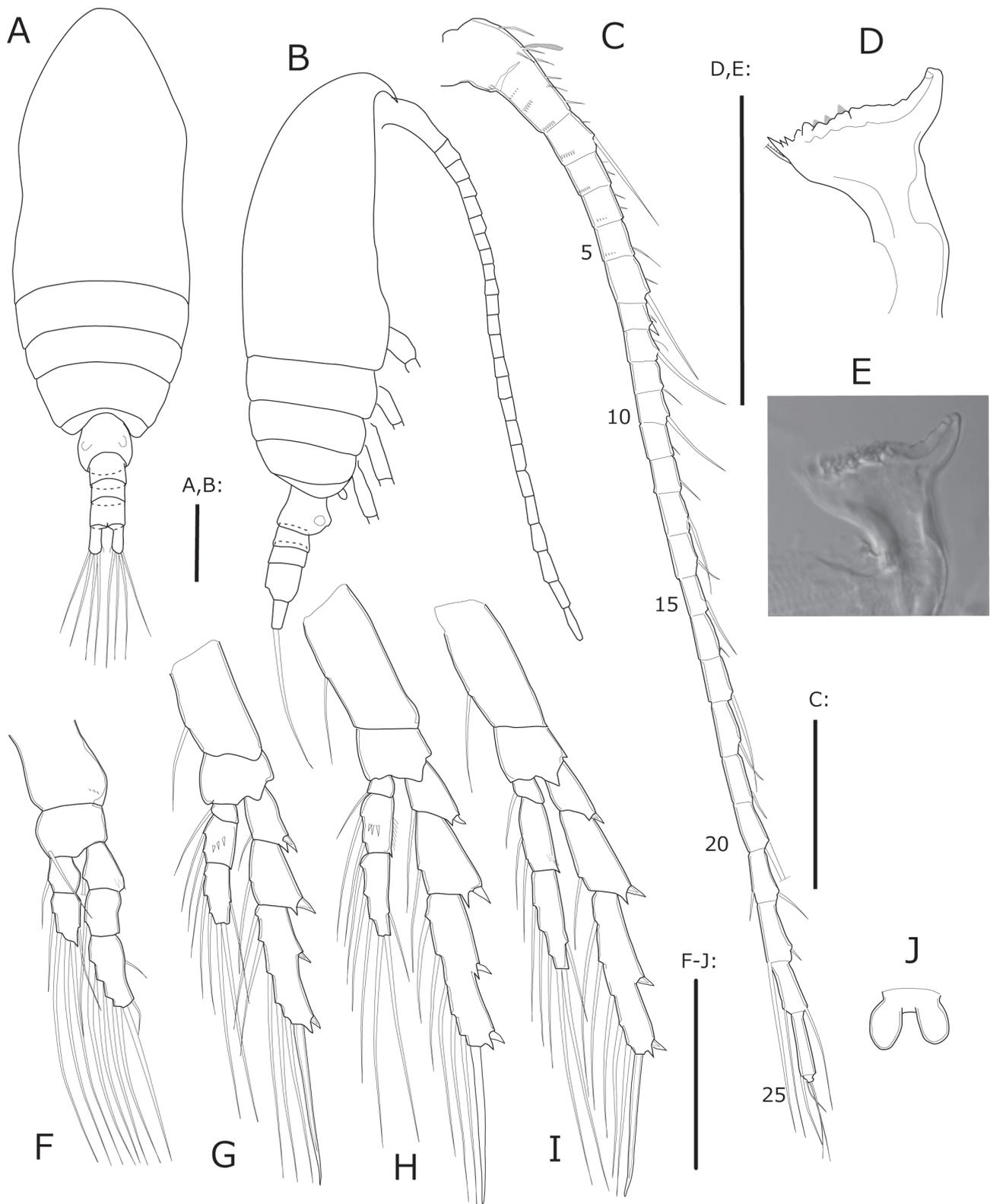


FIGURE 5. *Bestiolina similis* female from Okinawa Island (A, B, D, E. from sample #7; C, F–I, from sample #9). A, habitus, dorsal; B, habitus, lateral; C, antennule; D, E, mandible gnathal blade; F, leg 1, anterior; G–I, legs 2–4, posterior; J, leg 5, anterior. Scale bars, 0.1 mm.

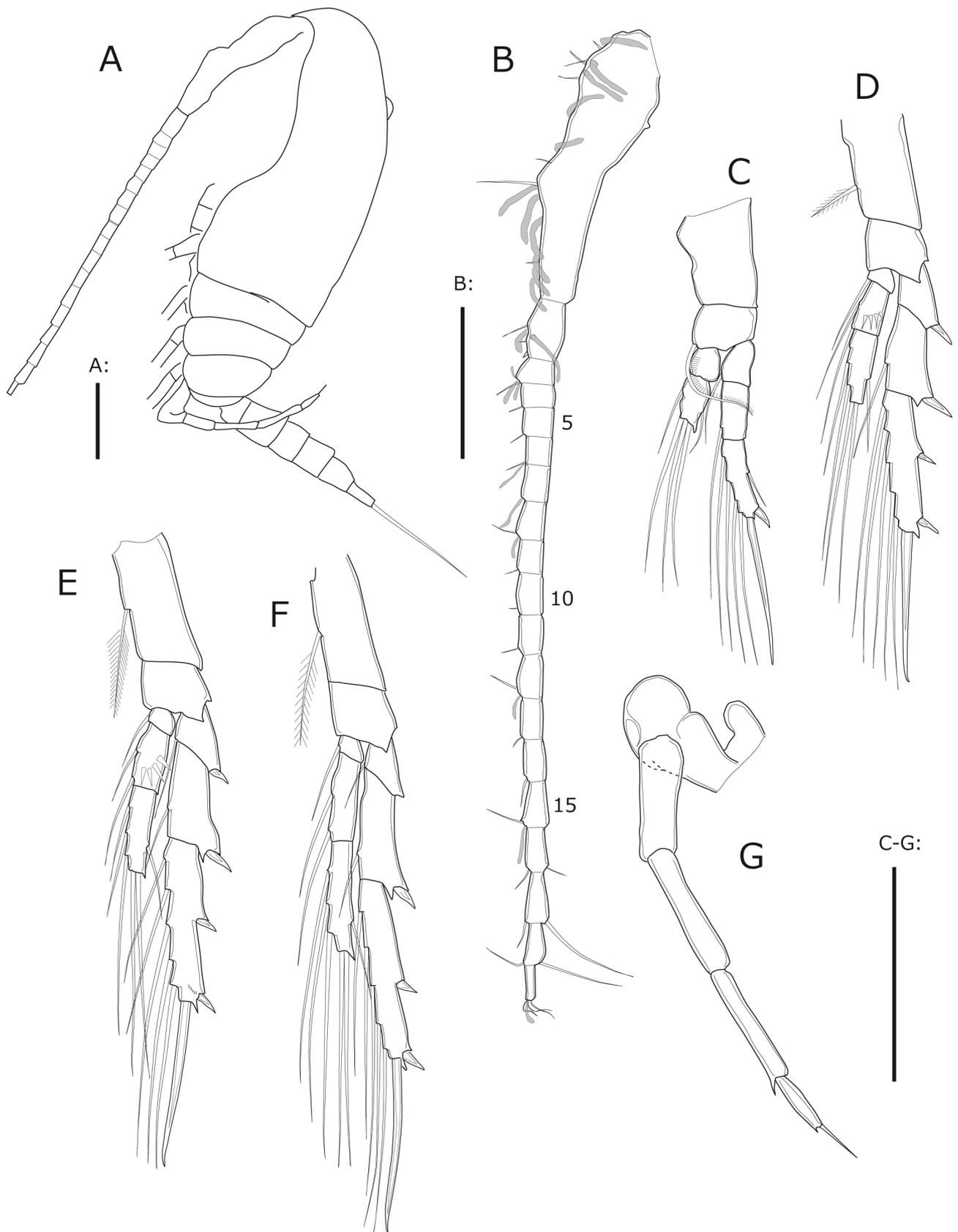


FIGURE 6. *Bestiolina similis* male from sample #7. A, habitus, lateral; B, antennule; C, leg 1, anterior; D–F, legs 2–4, posterior; G, leg 5. Scale bars, 0.1 mm.

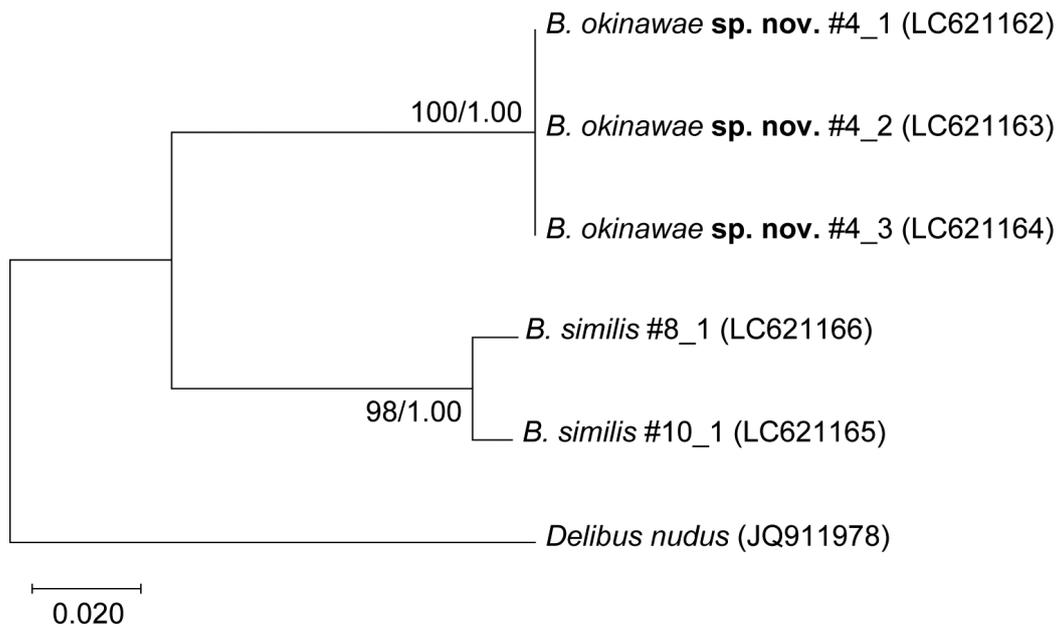


FIGURE 7. Maximum likelihood (ML) phylogeny of COI gene of *Bestiolina okinawae* **sp. nov.** from the Hija River (#4), *B. similis* from Henoko (#6) and Tomari (#10) Harbors in Okinawa, Japan. *Delibus nudus* was used as an outgroup to root the tree. ML bootstrap probabilities and Bayesian posterior probabilities for bipartitions are shown. Accession numbers in GenBank are shown in parentheses.

Antennule (Fig. 1F) 25 segmented; segmentation and setation as follows: 1(I–III)=7+ae, 2(IV)–4(VI)=2, 5(VII)=1, 6(VIII)=2, 7(IX)=1+sp, 8(X)=2, 9(XI)–20(XXII)=1, 21(XXIII)–23(XXV)=2, 24(XXVI)=1, 25(XXVII–XXVIII)=3. Ancestral segments I–VIII with row of tiny spinules near distal margin.

Mandible (Fig. 5D, E) gnathal blade with wide-based, gently curved strong first tooth.

Legs 1–4 (Fig. 5F–G) rami with spinules on anterior/posterior surfaces as follows: leg 1, exp1=0/2; leg 2, exp2=0/3; leg 3, exp2=0/3–4; leg 4, exp2=3/0. Leg 5 (Fig. 2H) as in *Bestiolina okinawae* **sp. nov.**

Male. Body (Fig. 6A, B) length 0.73–0.81 mm (n=4); prosome length 0.52–0.56 mm (n=4).

Antennule (Fig. 6B) 19-segmented; armature on each segment as follows: 1(I–VIII) = 7+11ae, 2(IX–X)=2+sp+2ae, 3(XI)=1+3ae, 4(XII)–5(XIII)=1, 6(XIV)=1+ae, 7(XV)=ae, 8(XVI)=1+ae, 9(XVII)–11(XIX)=1, 12(XX)=1+ae, 13(XXI)–14(XXII)=1, 15(XXIV)=1+ae, 16(XXV)–18(XXVI)=2, 19(XXVII–XXVIII)=3+ae.

Leg 1 (Fig. 6C) exp3 length 4 times width; distolateral spine thick and as long as segment width. Legs 1–4 (Fig. 6C–F) rami with spinules as follows: leg 2, exp2=0/4, leg 3, exp3=0/5, exp2=0/4; spinules on leg 3 exp3 very minute, spinules on legs 2, 3 exp2 larger than those of female.

Left leg of leg 5 (Fig. 6G), segment 5 length 0.5 times segment 4; apical spine length 0.8 times segment 5.

Remarks. The present specimens are identifiable to *Bestiolina similis* by the following characteristics: 1) Spinules on the posterior corner of the prosome, which are absent in *B. inermis*, *B. similis* and *B. arabica* among the currently known nine species of the genus; 2) The posterior surfaces of the legs 1–4 exp2 are devoid of spinules as in *B. similis*, which Sewell (1914) remarked as a critical difference between *B. similis* and *B. inermis*; 3) The male leg 1 exp3 is 4 times longer than wide and apparently longer than that of *B. arabica*, of which the corresponding segment is about 2.5 times longer than wide according to the original figure of the species (Ali et al. 2007, fig. 4g). The present specimens agree well to the original description of *B. similis* by Sewell (1914), but there are two points of difference between them. First, transverse spinule rows on proximal segments of the female antennule are present in the present specimens but absent in the original description. This was used for the discrimination of *B. similis* from *B. inermis* by Sewell (1914). Second, the number of spinules on leg 3 exp2 is 3–4 in the present specimens whereas 5 are in the original description (Sewell 1914, plate 17, fig. 3). However, it is unknown whether these differences are inter- or intra-specific variations, because an intra-specific variation of the spinule number and spinulation pattern is not rare in calanoid copepods (Moon et al. 2010, Ueda & Sakaguchi 2019, present study).

Bestiolina similis occurred in waters where salinities ranged from 25 to 36, and co-occurred with *B. okinawae* **sp. nov.** at salinities of 25 (sample #3) and 30 (sample #11) (Table 1). Of 18 adult copepods (12 females and 6 males)

of *Bestiolina* randomly sorted from sample #11, five were *B. okinawae* **sp. nov.** females and the remaining 13 were seven females and six males of *B. similis*. The body length of *B. similis* in summer (28.0°C) decreased from winter (18.9°C) by 9.5% in females and 12.5% in male (Table 3). Males were significantly larger than females in both seasons ($p < .001$, t -test).

Genetic diversity

The DNA sequences of the 618-bp fragment of COI from individuals were determined. The COI sequence differed by 9.9–10.0% between *Bestiolina okinawae* **sp. nov.** and *B. similis*, in contrast to small variations (0.0–1.5%) within each population. The genetic differences of these species from *Delibus nudus* (Sewell, 1929) used as the outgroup species was 12.8–13.1%. The phylogenetic tree demonstrated a clear separation between the present two species (Fig. 7).

Discussion

Sewell (1914) proposed the presence of spinules on the posterior surfaces of legs 2–4 exp2 as a distinctive characteristics of *Bestiolina similis* with respect to *B. inermis*. Shen & Lee (1966) and Andronov (1972) also used spinules on posterior surfaces of all exopodal segments in their descriptions of *B. sinica* and *B. zeylonica* (Andronov, 1972), respectively. Ali et al. (2007) at last presented a list of the number of spinules on each segment on both the posterior and anterior surfaces of exp1–exp3 and the anterior surface of enp2 of legs 2–4 in their description of *Bestiolina arabica*. This list has been used in subsequent new species descriptions of *Bestiolina* (Moon et al. 2010, Dorado-Roncancio et al. 2019). However, the identification of *Bestiolina* to species should not be performed solely by the spinule number on leg rami, because the number of these spinules has been known to vary, as noted above.

Body lengths of the present two species decreased by 9.5 to 18.1% from winter to summer, between which temperatures varied by around 10°C. Although body length is generally an important species-specific characteristic, such the seasonal variation needs to be considered even in subtropical species. In adult calanoid copepods, the males are generally smaller than the females (Mauchline 1998), whereas in the present two species the adult males were significantly larger than the females. This is possibly related to the characteristic feeding habits of the genus. That is, it is thought that adult males are incapable of feeding because of lack of mandibular gnathobase and therefore they store necessary nutrients in the body by the last molt and grow larger than females in post-molt.

This is the first record for the genus in that two *Bestiolina* species co-occurred in the same sample. Oka et al. (1992) described *B. similis* (as *Bestiola sinicus*) from river estuaries of the three main islands of the Nansei Islands, i.e., Amami-oshima, Okinawa, and Iriomote islands. The species they described is most certainly not *B. okinawae* **sp. nov.** but rather *B. similis*, because the morphological characteristics of *B. similis* were illustrated in their figures. However, it is uncertain from which island(s) *B. similis* occurred, because they did not specify from which island(s) was used for the illustrated specimens. It is also uncertain whether *B. okinawae* **sp. nov.** co-occurred in their samples. Accordingly, the current reliable range of *B. okinawae* **sp. nov.** is from Okinawa to Ishigaki Island of the Nansei Islands.

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