



A new *Pseudodiaptomus* (Copepoda, Calanoida) from Korea supported by molecular data

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Abstract

A new species, *Pseudodiaptomus koreanus* **sp. nov.** (Calanoida, Pseudodiaptomidae), which has so far been identified as *P. inopinus* Burckhardt, 1913, is described from Korean estuaries. This new species differs from *P. inopinus* in the broad and short posterior process of the female genital flap, rounded posterioventral projection between the gonopores, presence of a posterodorsal spinule on the fifth pediger of the male, and a row of fine ventral spinules on the male second urosomite. The new species is also distinguishable from *P. nansei* Sakaguchi and Ueda, 2010 by the presence of a row of fine spinules along posterior margins of the second and third pedigers in both sexes, rounded posteromedial projection between the gonopores, separation of ancestral segment XXI and compound segments XXII–XXIII of the male right antennules, and a row of fine ventral spinules on the male second urosomite.

Their ribosomal internal transcribed spacer 1 (ITS 1) and mitochondrial gene cytochrome oxidase subunit I (mtCOI) sequences supported the conclusion based on morphological data, with considerable divergence values between *P. koreanus* and *P. inopinus*: 12–14% for ITS 1 and 14–22% for mtCOI. The ITS 1 and mtCOI sequences of specimens of two morphs of *P. koreanus* with different male fifth legs differed by <1.2% for ITS1 and <3.5% for mtCOI, suggesting that these morphological differences represent interspecific variability.

Key words: Pseudodiaptomidae, copepods, taxonomy, new species, ITS 1, mtCOI, estuary

Introduction

Pseudodiaptomus inopinus Burckhardt, 1913 has been known as one of the major components of the copepod assemblages in the fresh and brackish waters of northeastern Asia (Suh et al. 1991; Oka et al. 1991; Shen & Song 1979). However, Sakaguchi and Ueda (2010) verified that *P. inopinus* from the Nansei Islands, southernmost Japan was a new species, *P. nansei* Sakaguchi and Ueda, 2011. They also suggested that *P. inopinus* in western Japan may comprise two morphological groups. On the other hand, *P. inopinus* has two morphs with a finger-shape or paddle-shape terminally on the distal exopodal segment of the left fifth leg of male (Burckhardt 1913; Shen & Song 1979; Chang & Kim 1986; Chang 2009). In calanoid copepods the morphological differences of the male fifth legs play a very important role in the classification and identity of species. In spite of these facts two morphs of *P. inopinus* have still been considered as a single species, even though Shen and Tai (1962) established the latter of two morphs as a separate subspecies *P. inopinus saccupodus* Shen and Tai, 1962, because they can be found to live sympatrically.

Recently, many studies show that genetic characters can be used to provide unambiguous taxonomic discrimination when morphological characters are not practical for routine identification at the species level (Bucklin et al. 1996; Bucklin & Wiebe 1998; Lindeque et al. 1999). The internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA and the mitochondrial gene cytochrome oxidase subunit I (mtCOI) are appropriate

markers for species-level studies because they contain sufficient diversity to address intra- and interspecific phylogenetic relationships in invertebrates (Vogler & DeSalle 1994; Schlötterer et al. 1994; Odorico & Miller 1997; Schizas et al. 1999; Ueda & Bucklin 2006; Eyun et al. 2007; Sakaguchi & Ueda 2010).

In this study we used ITS 1 and mtCOI genes to address the genetic differentiation between *Pseudodiaptomus inopinus* and *P. koreanus* **sp.nov.** from estuaries on the southern and eastern coast of Korea. As well as aiming to verify that the latter species is a distinct, we also wanted to investigate the status of the two morphs with different male fifth legs.

Material and methods

Zooplankton samples were collected from the Hyungsan River estuary on 9 September 1997, and from the Mankyung River and the Seomjin River estuaries from January 2000 to December 2000 monthly, using a NORPAC net (0.2 mm mesh) (Fig. 1). All samples were preserved in 6%-neutralized formalin/seawater immediately after capture. Zooplankton for molecular analysis were also obtained by net tows: from Beolgyo Stream on 15 May 2008, from Hyungsan River on 9 September 1997, from Mankyung River on 14 January 2000, and from Seomjin River on 16 October 1999. These samples were preserved in 99.9% ethyl alcohol. All pseudodiaptomids were sorted from the samples. Pseudodiaptomids for morphological taxonomy were dissected and mounted in polyvinyl lactophenol. The bodies and appendages were observed with a differential interference contrast microscope (Nikon Optiphot) equipped with a drawing tube. The female genital structures of *Pseudodiaptomus* species were examined with a scanning electron microscope (SEM) Hitachi S-4700. The general morphological terminology follows Huys and Boxshall (1991) and terminology of the female genitalia follows Barthélémy et al. (1998) and Walter et al. (2002). The type specimens were deposited in the collections of the National Institute of Biological Resources, Korea (NIBR).

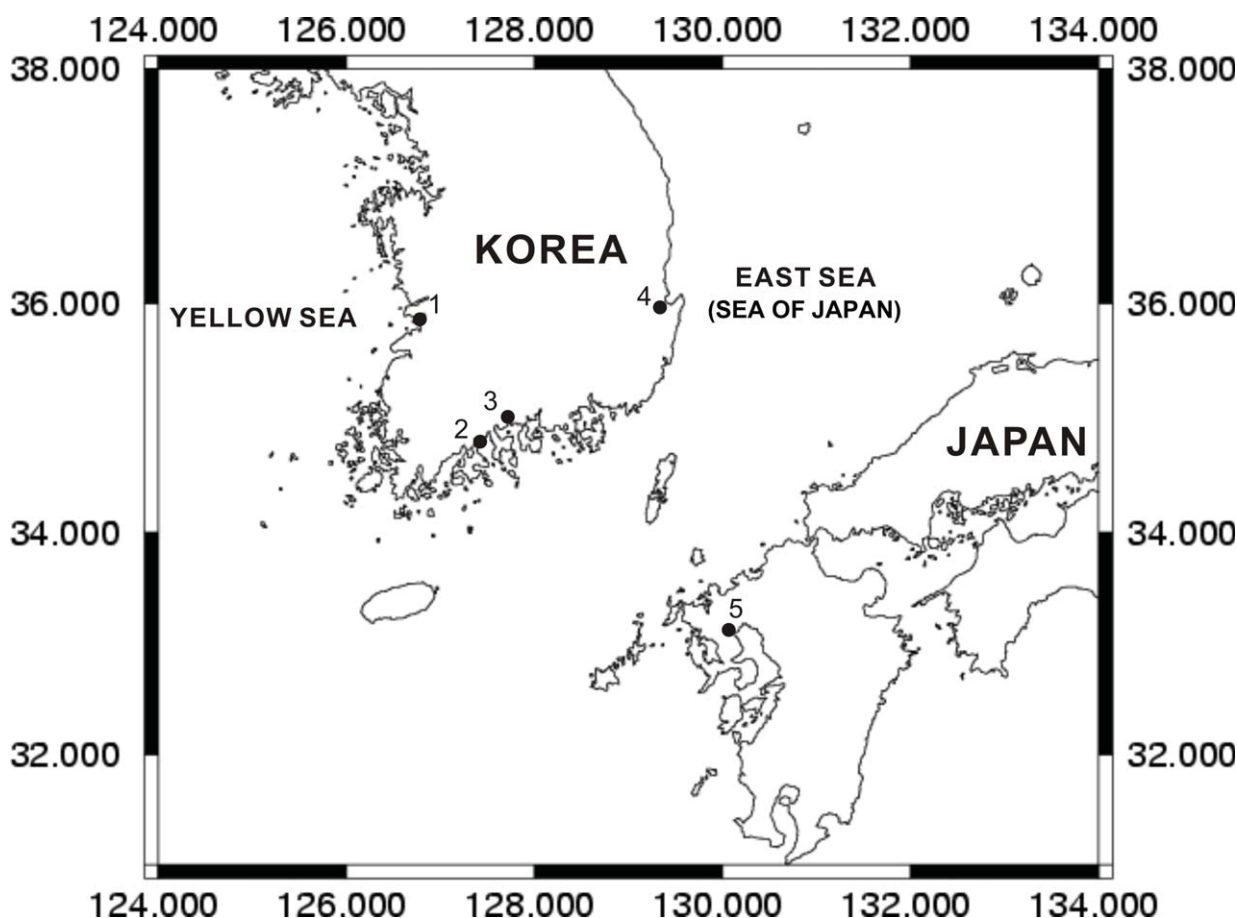


FIGURE 1. Map showing locations of Korean and Japanese estuaries sampled for this study: 1, Mankyung River estuary (MRE); 2, Beolgyo Stream estuary (BS); 3, Seomjin River estuary (SRE); 4, Hyungsan River estuary (HRE); 5, Rokkaku River (RR).

DNA sequences were determined for portions of the internal transcribed spacer (ITS) of the nuclear ribosomal gene and the mitochondrial gene cytochrome oxidase subunit I (mtCOI). DNA for ITS was isolated by the method of Bucklin et al. (1996) without extracting the DNA. The amplification primers for ITS used were ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-R (5'-TCCTCCGCTTATTGATATGC-3') based on the nucleotide sequence of fungal ribosomal RNA genes (White et al. 1990). Each PCR mixture (50 µl) contained 0.25 µl of 20 µM solutions of each primers, 4 µl 2.5mM deoxynucleotide triphosphates, and 0.5 µl 2.5 unit Taq polymerase. The reaction mix was covered with 1 drop of mineral oil. The DNA thermal cycler (Perkin-Elmer Co.) used for amplification was programmed as follows: an initial extensive denaturation step consisting of 95 °C for 4 min; 40 cycles, with each cycle consisting of 95°C for 1 min, 46°C for 1 min, and 72°C for 2 min; and a final extension step consisting of 72°C for 8 min. The PCR solutions for ITS were electrophoresed on 1% agarose gels and then the ITS regions were purified with a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). Purified ITS regions were ligated into pUC119 vectors. Ligated plasmids were then transformed into *Escherichia coli* DH5aF' cells and transformants were selected by the blue-white screening procedure (Sambrook et al. 1989). Plasmids containing the ITS fragments were isolated by using a QIAquick plasmid minikit (Qiagen GmbH). Purified plasmids were manually sequenced using both the T7 sequencing kit (Pharmacia Biotech, Inc., Piscataway, N.J.) and the fmol DNA sequencing system (Promega Co.) according to the suppliers' instructions. The sequencing primers were used Sp6 promotor (5'- AT TTA GGT GAC ACT ATA G – 3') and T7 promotor (5'- TA ATA CGA CTC ACT ATA GGG – 3').

Each individual for mtCOI was placed in 0.2 ml microcentrifuge tube with a cap and 20 µl dHOH was added, and microwaved for several minutes. The 18 µl template DNA and each 1.0 µl 10 µM primer solutions were placed in 0.2 ml microcentrifuge tube including AccuPower™ HotStart PCR Premix (Bioneer co., Korea) and well mixed. PCR primers for mtCOI were LCO1490 and HCO2198 (Folmer et al. 1994). The reaction condition was 94°C (1 min); 47°C (2 min); 72°C (3 min); repeated for 40 cycles. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) and sequenced directly using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing products were purified using the QIAGEN DyeEx 20.0 spin Kit (QIAGEN, Germany).

DNA sequencing was carried out in an Automated DNA Sequencer, Model 377 (Applied Biosystems Inc, USA). The machine-read sequences were compiled using the Sequencing Analysis (Ver. 3.3, ABI prism), and manually checked for accuracy. Multiple-sequence alignments were done using CLUSTAL X version 2.0 (Larkin et al. 2007). Pairwise distance measures and phylogenetic analyses were conducted using MEGA 4 (Tamura et al. 2007). Ambiguous sites were eliminated from the dataset. The Neighbor-Joining (Saito & Nei 1987) tree with the p-distance model was used for likelihood optimization. Significance of the tree topology for NJ was done by the bootstrap test (Felsenstein 1985) with 1000 replicates. The mtCOI DNA sequences for *Pseudodiaptomus poplesia* (Shen), collected from the Mankyung River estuary, western Korea, and available from GenBank (AF536521), were used as outgroups for our phylogenetic analysis.

Results

Order Calanoida G. O. Sars, 1903

Family Pseudodiaptomidae G. O.Sars, 1902

Genus *Pseudodiaptomus* Herrick, 1884

Pseudodiaptomus koreanus sp. nov.

(Figs. 2, 3A, 4–8)

Type material. Holotype female dissected and mounted on seven glass slides using CMC-10, aqueous mounting medium (Masters Company, Inc., Wood Dale, IL) (NIBR0000245889); paratypes: one female and two males dissected and mounted on 18 glass slides (NIBR0000245890) and undissected six females and seven males in alcohol (NIBR0000245891); all collected from the Hyungsan River estuary (36°54'43.21"N 129°22'22.48"E), eastern Korea by H.Y. Soh on 7 September 1997. Additional paratypes: undissected 20 females and 20 males in alcohol (NIBR0000245892) from the Seomjin River estuary (35°04'45.45"N 127°43'22.08"E), southern Korea, collected by H.Y. Soh on 16 October 1999.

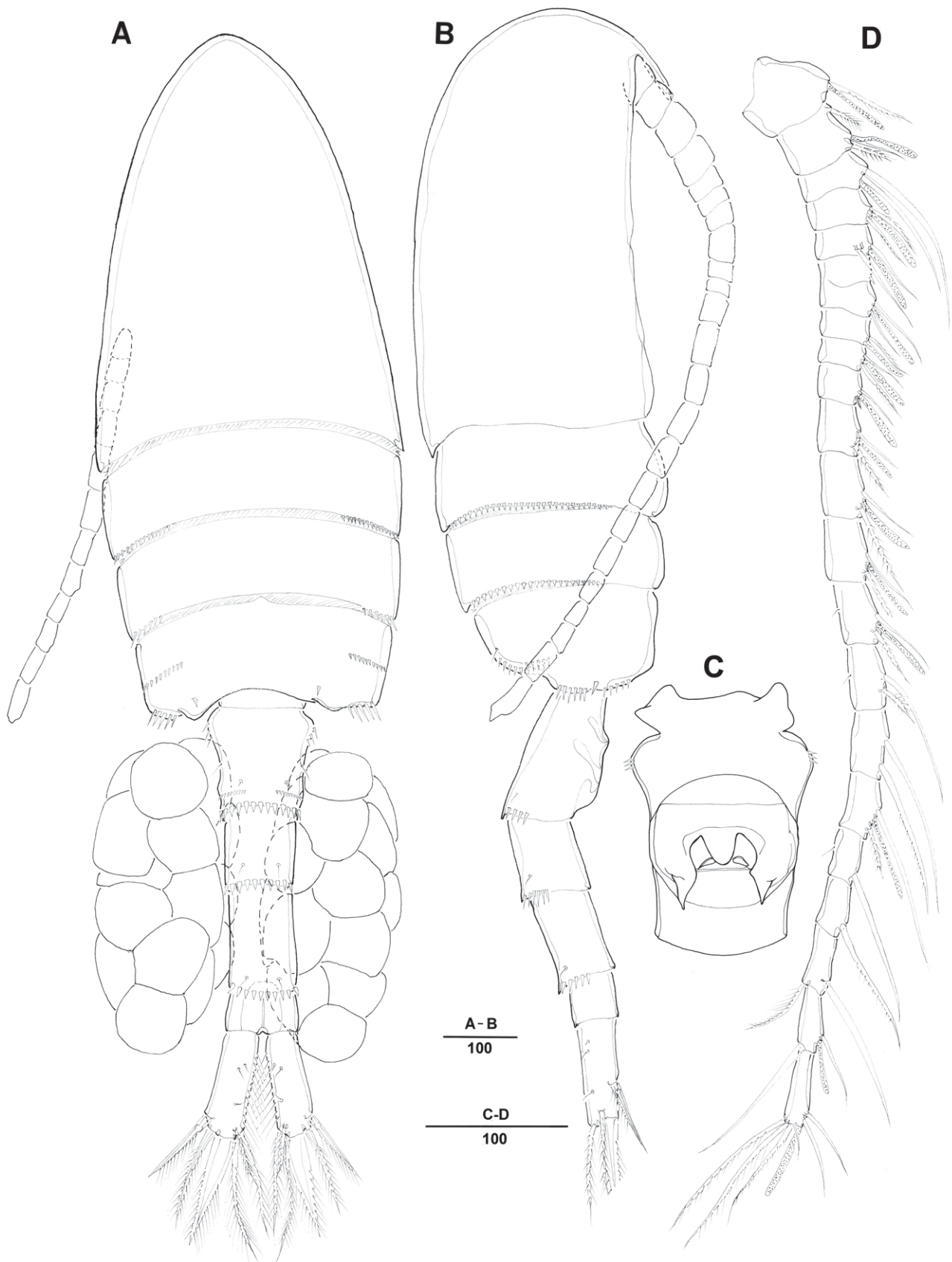


FIGURE 2. *Pseudodiaptomus koreanus* **sp. nov.** female (holotype): A, habitus, dorsal; B, habitus, right lateral; C, genital double somite, ventral; D, antennule. Scale bars in μm .

Other materials examined. Undissected 20 females and 20 males in alcohol (NIBR0000245893) from Beolgyo stream (34°50'31.14"N 127°21'10.38"E), southern Korea, collected by H. Y. Soh on 15 May 2008.

Description. Female. Body length 1.30-1.45 mm (mean±sd=1.36±0.03, n=6), with paired egg sacs. Prosome/Urosome ca. 1.71. First pediger coalesced with cephalosome; second and third pedigers with row of fine spinules along posterolateral margins; fourth and fifth pedigers completely fused with rounded corners, posterolateral corner on each side with row of spinules and with small bump posteromedially (Fig. 2A, B). Urosome of 4 free somites: genital double-somite, 1.1 times wider than long, greatest width anterior in dorsal view with several spinules on each anterolateral projection (Fig. 2A). In ventral view posterolateral process of female lateral genital flaps, wide and extend well short of posterior border of genital double-somite (Fig. 2C); pair of uncovered gonopores and rounded posteriorcentral projection between those (Fig. 3A, white arrowhead) located posteroventrally; anal somite shortest. Each somite except anal somite serrated along posterior margin. Caudal rami nearly symmetrical, with six setae; outer terminal seta IV basally swollen (Fig. 2A).

Antennules symmetrical, 22-segmented (Fig. 2D); ancestral segments II-VI and XXVI-XXVIII completely fused; segment X and XI incompletely fused with suture line; setal formula as follows: I-1+aesthetasc, II-VI-3+aesthetasc, VII-2+aesthetasc, VIII-1+aesthetasc, IX-2+aesthetasc, X-1 (spiniform element), XI-2+aesthetasc, XII-2 (1 spiniform)+aesthetasc, XIII-2+aesthetasc, XIV-2+aesthetasc, XV-2+aesthetasc, XVI-2+aesthetasc, XVII-2+aesthetasc, XVIII-2+aesthetasc, XIX-2, XX-2, XXI-2+aesthetasc, XXII-1, XXIII-1, XXIV-1+1, XXV-1+1+aesthetasc, XXVI-XXVIII-6+aesthetasc.

Antenna (Fig. 4A): coxa with single seta; basis and first endopod fused into allobasis, each having 2 setae, respectively; second endopod with 9 and 7 setae on subterminal and terminal lobes, respectively; exopod 6-segmented, with setation of 1, 5 1, 1, 1, 3.

Mandible (Fig. 4B): basis with 4 setae; endopod 2-segmented, first segment with 4 setae, second having 9 setae armed with two rows of setules; exopod 5-segmented, first to fourth segments each with single seta, fifth segment with 2 setae.

Maxillule (Fig. 4C): praecoxal arthrite with 15 elements; coxa with 4 setae on endite and 9 setae on epipodite; basis with 4 and 5 setae on proximal and distal endite, respectively, with single seta on exite; endopod 3-segmented, with setal formula 4, 4, 7; exopod unisegmented, with 11 setae.

Maxilla (Fig. 4D): praecoxa elongated, proximal endite with 4 setae, distal endite with 3 setae; two coxal endites with 3 setae each; basis with sclerotized stout seta in addition to single short and 2 long setae; endopod 4-segmented with 2, 3, 2, 2, respectively.

Maxilliped (Fig. 4E): syncoxa with setal formula of 0, 2, 3, 4 on endites; basis bearing 3 setae, with first endopodal segment separated; endopod 6-segmented, with setal formula of 2, 3 (including 2 bifurcate setae), 2 (both bifurcate setae), 2, 2+1, 4.

Legs 1-4 (Fig. 5, Fig. 6A) each with 3-segmented endopodal segments; first and second endopodal segments of legs 1 to 4 with spinules on outer distal corner; terminal spines on third exopodal segment of legs 1 to 4 with row of hairs on inner margin and teeth on other border along distal 3/4 of medial margin.

Seta and spine formula as follows:

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

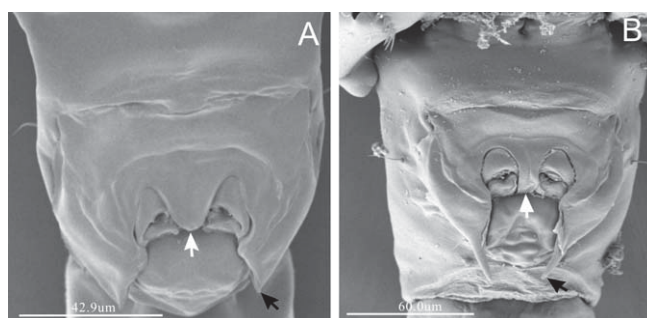


FIGURE 3. SEM photographs of the female genital double somite of *Pseudodiaptomus koreanus* sp. nov. (A) and *P. inopinus* (B). White arrows indicate process between gonopores; black arrows indicate the posterior process of genital flaps.

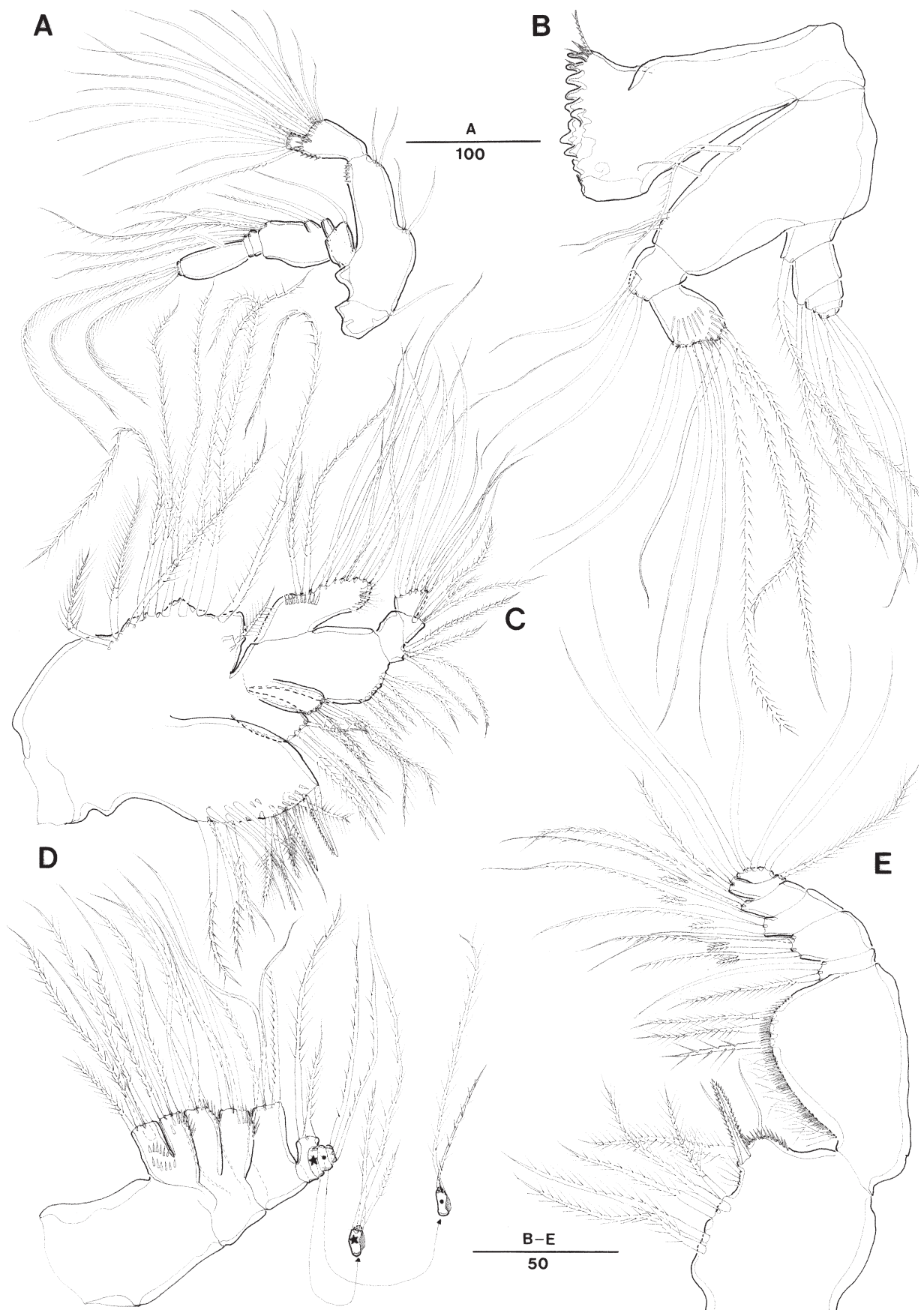


FIGURE 4. *Pseudodiaptomus koreanus* sp. nov. female (holotype): A, antenna; B, mandible; C, maxillule; D, maxilla; E, maxilliped. Scale bars in μm .

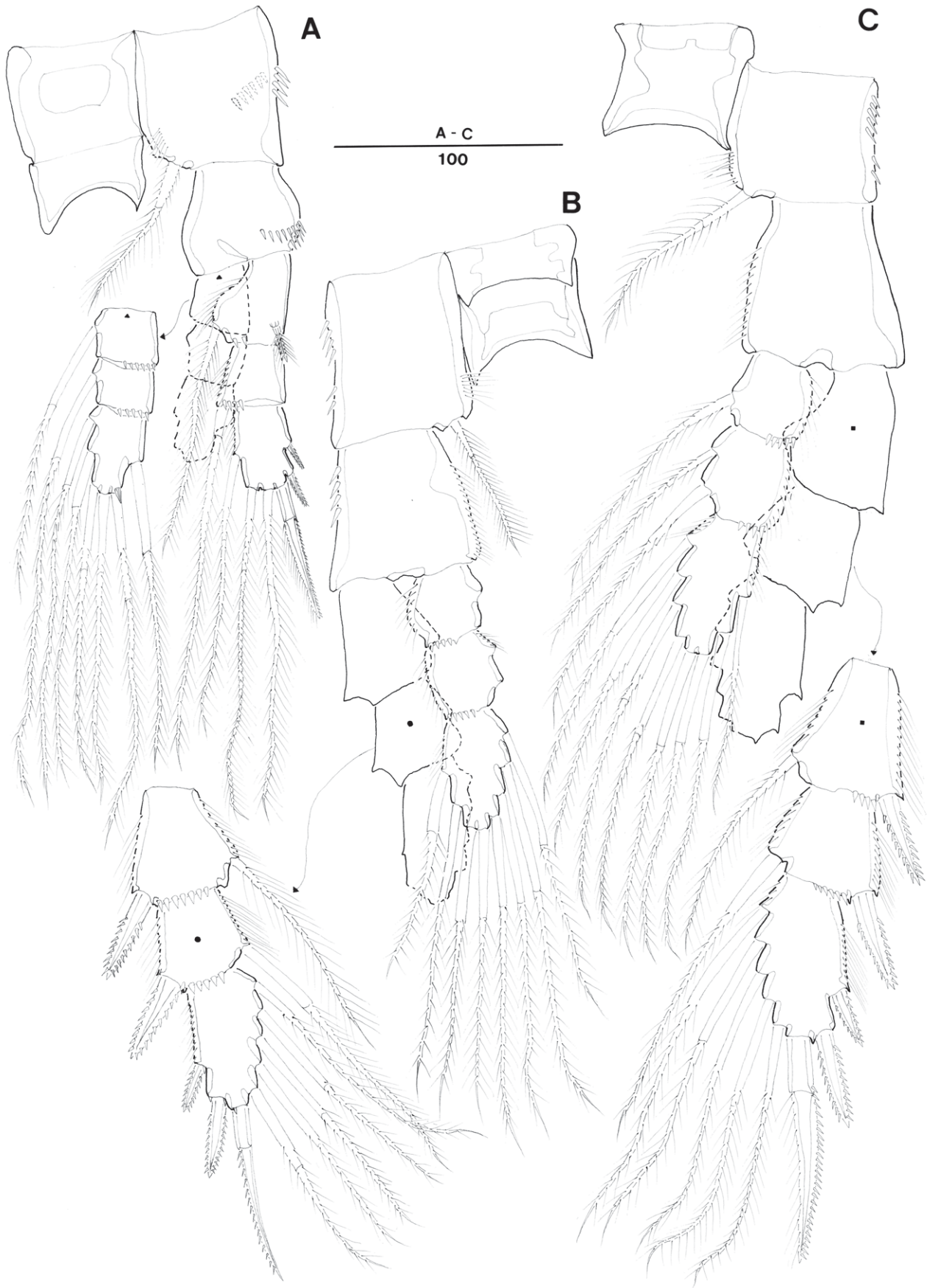


FIGURE 5. *Pseudodiaptomus koreanus* sp. nov. female (holotype): A, leg 1; B, leg 2; C, leg 3. Scale bar in μm .

Fifth leg symmetrical, uniramous (Fig. 6B): intercoxal sclerite completely incorporated into both coxae; basis with small posterior outer seta; exopod 3-segmented, first segment furnished with spinules on inner margin, with outer spine and mediodistal blunt projection; second and third segments incompletely fused, second segment with short outer spine and larger serrate inner spines, third segment with long terminal spine and smaller inner spine.

Male. Body length 1.09–1.19 mm (mean±sd=1.10±0.01, n=10). Cephalosome and first pediger, and fourth and fifth pedigers completely fused; first to fifth pediger with row of posterolateral spinules (Fig. 7A, B). Urosome of 5 free somites: genital somite with genital opening on left; second urosomite with ventral transverse row of spinules (Fig. 7C); genital and anal somites naked, second to fourth somites armed with posterodorsal spinules.

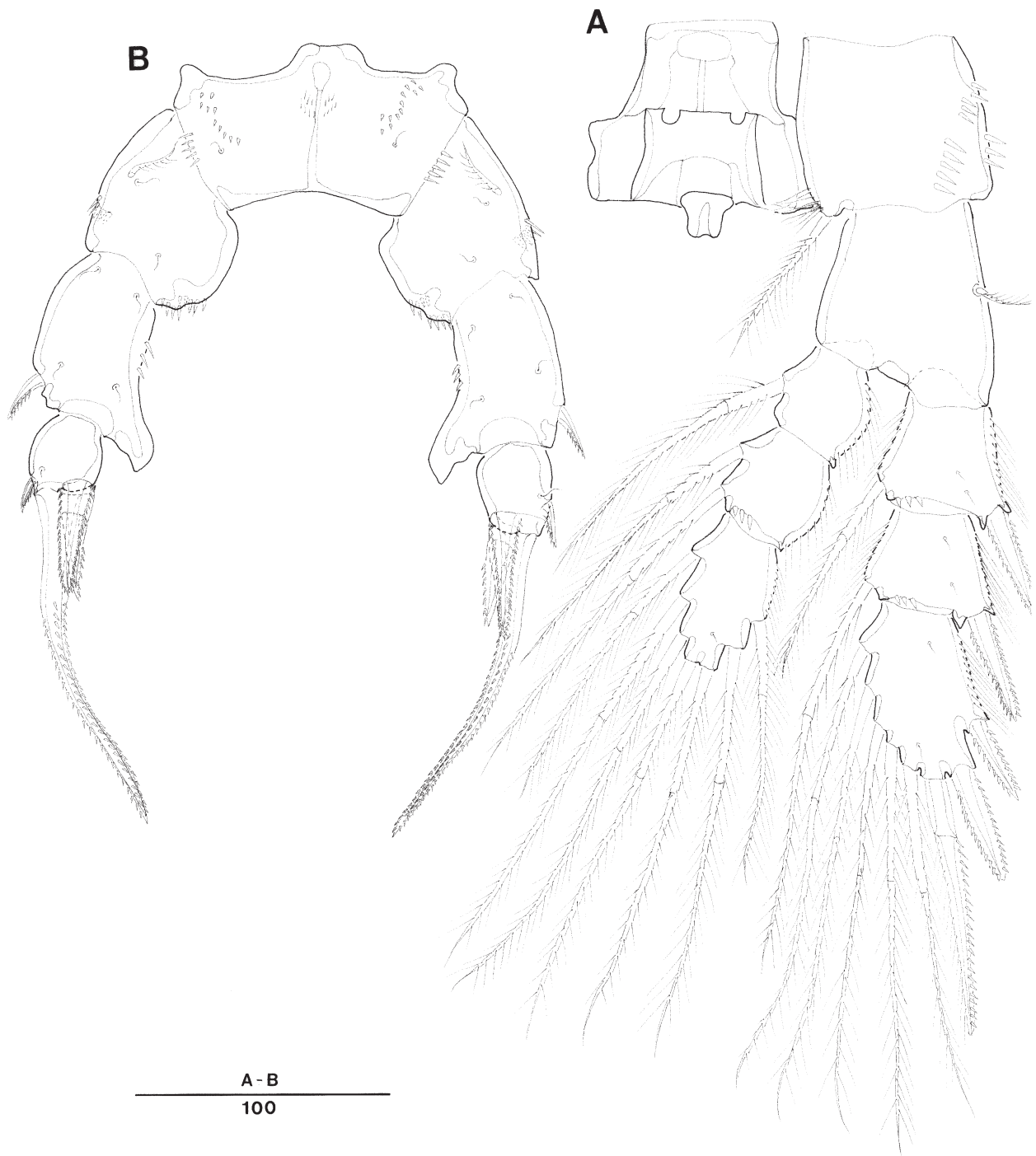


FIGURE 6. *Pseudodiaptomus koreanus* sp. nov. female (holotype): A, leg 4; B, leg 5. Scale bar in μm .

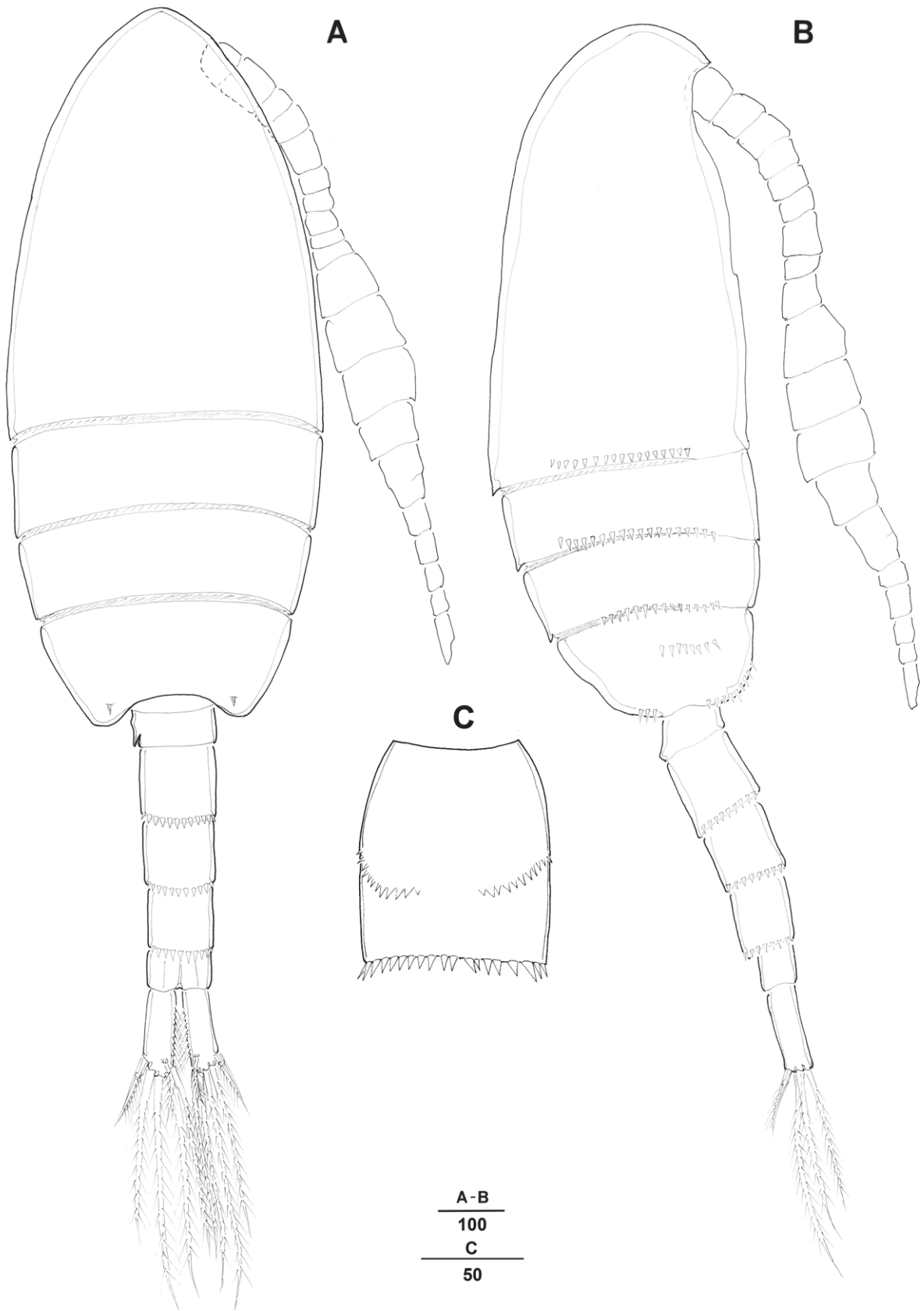


FIGURE 7. *Pseudodiaptomus koreanus* sp. nov. male (paratype): A, habitus, dorsal; B, habitus, right lateral; C, second urosomite, ventral view. Scale bars in μm .

Left antennule same as in female. Right antennule 21-segmented, geniculated (Fig. 8A); ancestral segments II to IV, XXII and XXIII, and XXIV and XXVIII completely fused; segments VIII and IX incompletely separated. Setation and segmentation as follows: I-1+aesthetasc, II-IV-3+aesthetasc, V-2+aesthetasc, VI-1, VII-2+aesthetasc, VIII-1, IX-2+aesthetasc, X-1 (spiniiform), XI-2+aesthetasc, XII-2 (1 spine)+aesthetasc, XIII-2+aesthetasc, XIV-2+aesthetasc, XV-2+aesthetasc, XVI-2+aesthetasc, XVII-2+aesthetasc, XVIII-2+aesthetasc, XIX-1+process+aesthetasc, XX-1+process, XXI-1, XXII-XXIII-2+process, XXIV-XXVIII-9+2aesthetasc. Other appendages except for fifth leg nearly similar to those of female.

Fifth leg asymmetrical (Fig. 8B, C); coxa with spinule patch on both surfaces, left basis and endopod completely fused, produced into 2 large medial processes. In finger-shaped morph (Fig. 8C) larger medial process of left basoendopodal segment curved, with patch of minute spinules at mid-length; proximal endopodal segment having inner seta proximally widest on central part; distal segment with 5 setae and serrated outer spine covered with hairs; right basis with outer seta and two unequal inner knobs; first exopodal segment with long stout distal outer spine and medial seta; second segment medially swollen with medial inner seta and spine; third segment elongate, with stout terminal spine, medial seta, and distal seta. In paddle-shape morph (Fig. 8B) patch of minute spinules absence at mid-length of medial process of left basoendopodal segment; proximal exopodal segment with inner seta proximally widest on proximal part; distal segment covered with hairs with 3 setae and serrated outer spine; two unequal inner lobes absence on right basis; width of second exopodal segment nearly equal.

Remarks. *Pseudodiptomus koreanus* sp. nov. is very closely related to *P. inopinus* Burckhardt, 1913 and *P. nansei* Sakaguchi and Ueda, 2010. However, this new species differs from *P. inopinus* in having broad, short posterior processes of the female lateral genital flaps, a rounded posterioventral projection between genital flaps (see Fig. 3A), the separation of ancestral segments XXI and XX–XXIII of the male right antennules, patch of minute spinules on medial process of basoendopodal segment of male left fifth leg, and longer proximal endopodal segment of male left fifth leg. *Pseudodiptomus koreanus* has generally single inner seta on the fifth exopodal segment of its antenna, but the inner seta is absent in some specimens. Also, in finger-shaped morph of male left leg (Fig. 8C) a larger medial process of male left basoendopodal segment has a patch of minute spinules at the mid-length, but it is absent in some specimens. *Pseudodiptomus koreanus* is distinguishable from *P. nansei* Sakaguchi and Ueda, 2010 by the presence of a row of fine spinules along the posterior margins of the second and third pedigers in both sexes, rounded posteromedial projection between both gonopores, the separation of ancestral segments XXI and XXII–XXIII of the male right antennule, and a row of fine ventral spinules on male second urosomite.

Pseudodiptomus koreanus is a dominant species in salinity between 5 and 15, in estuarine waters of southeastern Korea in spring and autumn (Park et al. 2005), while *P. inopinus* is restricted to western estuarine waters of Korea occurring at salinities <30 with *P. popesia* (Park 2005). However, Chang (2009) showed that *P. inopinus* occurs widely in the inland waters of Korea.

Etymology. The specific name *koreanus* refers to the apparent centre of distribution for this species.

Molecular diversity. A 266 base pair region of the ITS1 was obtained for three individuals of *Pseudodiptomus inopinus* collected from the western estuary and eight individuals of *P. koreanus* from the southeastern estuary of Korea. The ITS1 sequences differed by 12–14% between *P. inopinus* and *P. koreanus* sp. nov. (Table 1), by 5–6% between the southern and eastern individuals of the new species, and by 1.2% between two morphs of the male fifth legs, respectively.

A 624 base pair region of the mtCOI gene was obtained for 19 individuals of *P. koreanus* from the southeastern estuaries of Korea including two male morphs (GenBank Accession No. JQ714036–JQ714054), one individual of *P. inopinus* from the western estuary of Korea (GenBank Accession No. JQ714057), and two individuals of *P. inopinus* from the western Japan (GenBank Accession No. JQ714055, JQ714056). The mtCOI sequences differed by 0.6–3.8% between individuals of *P. koreanus* including two male morphs. Meanwhile, the mtCOI sequences differed by 14.3–22.0% between *P. koreanus* and *P. inopinus* and by 19.6–20.7% between Korean and Japan populations of *P. inopinus*. The mtCOI tree shows that *P. koreanus* is clearly separated from *P. inopinus* (Fig. 9). The MtCOI reference sequences were selected to represent the new species from the southeastern estuaries of Korea (GenBank Accession No. JQ714037) and *P. inopinus* from the Mankyung River estuary (GenBank Accession No. AF536520).

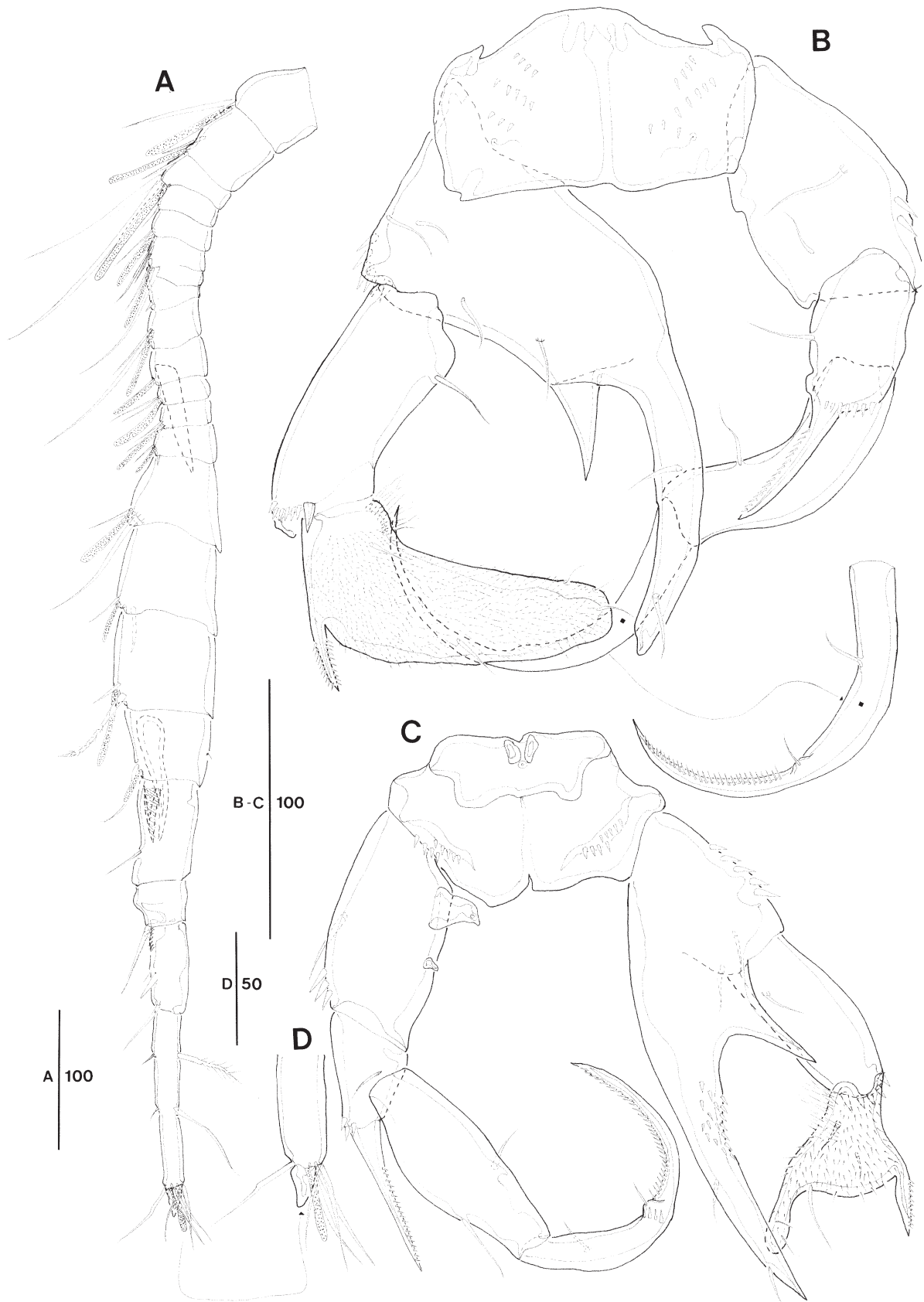


FIGURE 8. *Pseudodiaptomus koreanus* **sp. nov.** male (paratype): A, antennule; B, leg 5, paddle-shape; C, leg 5, paddle-shape; D, leg 5, detail. Scale bars in μm .

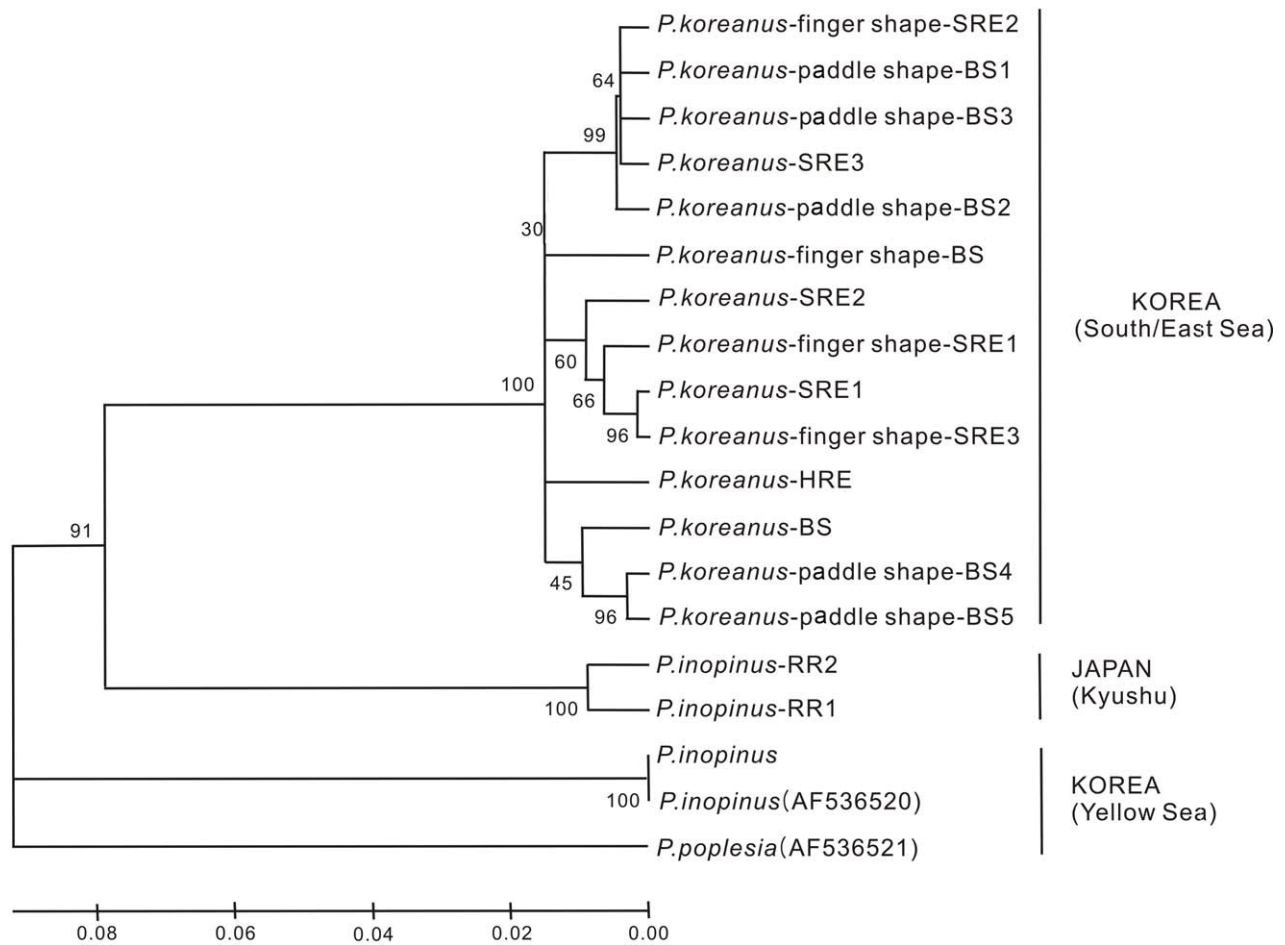


FIGURE 9. Cladistic tree based on mtCOI sequences of *Pseudodiptomus koreanus* sp. nov. from Korean estuaries and *P. inopinus* from Korean and Japanese estuaries. Numbers at branch points are bootstrap values (i.e., percentage of tree with that branch point among 1000 subreplicates). The axis scale indicates branch lengths in the same units as those of the evolutionary distances used to infer the tree. *Pseudodiptomus poplesia* (Shen, 1955) (GeneBank access no. AF536521) was used as an outgroup. The specimens correspond to those in Table 2.

TABLE 1. Pair distance for ITS1 rDNA gene of *Pseudodiptomus inopinus* and *P. koreanus* sp. nov. HRE, Hyunsan River estuary of the eastern Korea; SRE, Seomjin River estuary of the southern Korea; MRE, Mankyung River estuary of the western Korea

		Percent similarity				
		1	2	3	4	5
Percent divergence	1 <i>koreanus</i> -female-HRE (AY496260)		91.0	90.6	89.8	71.8
	2 <i>koreanus</i> -female-SRE (AY499003)	5.1		95.3	95.3	72.8
	3 <i>koreanus</i> -male-finger-SRE (AY499009)	5.5	0.4		94.9	70.8
	4 <i>koreanus</i> -male-paddle-SRE (AY499004)	5.9	0.8	1.2		71.8
	5 <i>inopinus</i> -female-MRE (AY5002279)	13.2	12.3	12.7	13.2	

TABLE 2. Pairwise percent differences for mtCOI sequences between individual females and two types of male leg 5 of *Pseudodiaptomus koreanus* sp. nov. from Korean and Japanese estuaries. BS, Beolgyo stream, southern Korea; HRE, Hyungsan River estuary, eastern Korea; MRE, Mankyung River estuary, western Korea; RR, Rokkaku River, Kyushu, Japan; SRE, Seomjin River estuary, southern Korea. Numbers in parenthesis indicate GeneBank access numbers.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1 <i>P. koreanus</i> -SRE1 (JQ714036)																			
2 <i>P. koreanus</i> -SRE2 (JQ714037)	0.018																		
3 <i>P. koreanus</i> -SRE3 (JQ714038)	0.029	0.034																	
4 <i>P. koreanus</i> -finger shape-SRE1 (JQ714040)	0.014	0.022	0.029																
5 <i>P. koreanus</i> -finger shape-SRE2 (JQ714039)	0.003	0.014	0.026	0.011															
6 <i>P. koreanus</i> - BS (JQ714041)	0.027	0.032	0.034	0.029	0.024														
7 <i>P. koreanus</i> -finger shape-BS1 (JQ714043)	0.026	0.027	0.027	0.030	0.022	0.034													
8 <i>P. koreanus</i> -finger shape-BS2 (JQ714042)	0.030	0.035	0.008	0.032	0.027	0.035	0.032												
9 <i>P. koreanus</i> -paddle shape-BS1 (JQ714044)	0.029	0.034	0.006	0.030	0.026	0.034	0.030	0.008											
10 <i>P. koreanus</i> -paddle shape-BS2 (JQ714045)	0.027	0.032	0.008	0.029	0.024	0.029	0.029	0.010	0.008										
11 <i>P. koreanus</i> -paddle shape-BS3 (JQ714046)	0.032	0.037	0.010	0.034	0.029	0.037	0.034	0.011	0.010	0.011									
12 <i>P. koreanus</i> -paddle shape-BS4 (JQ714049)	0.019	0.027	0.032	0.024	0.016	0.018	0.029	0.034	0.032	0.030	0.035								
13 <i>P. koreanus</i> -paddle shape-BS5 (JQ714053)	0.022	0.026	0.035	0.027	0.019	0.021	0.034	0.037	0.035	0.034	0.038	0.006							
14 <i>P. koreanus</i> -HRE (JQ714054)	0.026	0.030	0.032	0.027	0.022	0.026	0.032	0.034	0.032	0.030	0.035	0.026	0.029						
15 <i>P. inopimus</i> -RR1 (JQ714056)	0.162	0.159	0.165	0.170	0.162	0.155	0.155	0.167	0.165	0.167	0.168	0.152	0.151	0.165					
16 <i>P. inopimus</i> -RR2 (JQ714055)	0.154	0.159	0.0157	0.162	0.154	0.147	0.147	0.159	0.157	0.159	0.157	0.144	0.143	0.157	0.018				
17 <i>P. inopimus</i> -MRE (JQ714057)	0.213	0.151	0.213	0.210	0.213	0.213	0.215	0.216	0.220	0.218	0.215	0.208	0.208	0.218	0.207	0.196			
18 <i>P. inopimus</i> (AF536520)	0.213	0.216	0.213	0.210	0.213	0.213	0.215	0.216	0.220	0.218	0.215	0.208	0.208	0.218	0.207	0.196	0.000		
19 <i>P. poplesia</i> (AF536521)	0.178	0.179	0.184	0.179	0.178	0.178	0.192	0.191	0.184	0.186	0.181	0.179	0.178	0.189	0.175	0.197	0.197		

Discussion

After recent studies of the species-specific characteristics on the female genital double-somite of pseudodiaptomiids (Ohtsuka et al. 2000; Soh et al. 2001; Walter et al. 2002), a dominant species in Korean estuaries (Chang & Kim 1986; Suh et al. 1991), *P. inopinus* Burckhardt, 1913, has been suspected not to be a single species because there are two types of the structure of the genital flaps. According to Burckhardt's (1913) original figure of the genital area of *P. inopinus*, the individuals from the western estuaries of Korea are very closely related to *P. inopinus* s. str. as follows: long processes of the female lateral genital flaps (see Fig. 3B, black arrow) and absence of posteriorcentral projection between gonopores (see Fig. 3A, white arrow). *Pseudodiaptomus inopinus* from the Sea of Japan is very similar to *P. koreanus*, but it differs in having several long spinules ventral to the bump on posterolateral corner on each side of the female last pediger, longer processes of the female lateral genital flaps, the fusion of ancestral segments XXI and XXII–XXIII of the male right antennule, and ventral transverse rows of spinules on the male second urosomite.

We sequenced the ITS1 region and mtCOI of *P. inopinus* and *P. koreanus* to investigate whether the observed morphological differences in the female genital structures are taxonomically significant. ITS 1 sequence between *P. inopinus* and *P. koreanus* differed by 12 to 14 %, compared with a range of 7 to 14 % among six fresh water calanoid copepod *Skistodiaptomus* Light, 1939 species (GeneBank Accession No. AY275453–AY275458). The mtCOI sequences between *P. koreanus* and *P. inopinus* from Korean waters differ by 14 to 22.0 %, between specimens of *P. koreanus* and *P. inopinus* from the Sea of Japan by 14–17 %, and between specimens of *P. inopinus* from Korea and Japan by 19 to 21 %. The intraspecific variation of the mtCOI is less than 3.8%. Considering that other copepods such as *Calanus* Leach, 1816 have interspecific divergences between 9 and 25% and intraspecific divergences between 1 and 4% (Bucklin et al. 2003), these data strongly indicate that *P. koreanus* is a separate, valid new species as suggested in Eyun et al. (2007) and *P. inopinus* from the mainland of Japan is also probably a new species. Sakaguchi and Ueda (2011) already detected two morphological groups based on the length of the posterior process of the genital flaps of *P. inopinus* in western Japan, and suggested that the species complex be a different species. These facts suggest that *P. inopinus* groups in the northeastern Asian geographical region, including the Chinese coasts (Shen & Tai 1962; Shen & Lee 1963; Chen & Zhang 1965; Shen & Song 1979), the South Korean coast (Chang & Kim 1986; Yoo & Lim 1989; Soh et al. 2001; Chang 2009), the Suifun River estuary near Vladivostok, Russia (Smirnov 1929), and the Japanese coast (Tanaka 1966; Mizuno & Miura 1984; Sakaguchi & Ueda 2010, 2011), probably include more extensive complex of species and need to be taxonomically reviewed. On the other hand, males of *P. koreanus* have either finger-shaped or paddle-shaped tip of the distal part of the left leg exopod, as in the estuarine pseudodiaptomid copepods *P. inopinus* and *P. poplesia* (Burckhardt, 1913; Shen & Sung 1979; Soh et al. 2001), and among other Centropagoidea (Fleminger 1967). Although the morphological differences between these morphs could be important enough to separate two forms into independent species, it has been considered as intraspecific variability because of the similarities of other morphological characters and sympatric occurrence (Shen & Song 1979; Chang & Kim 1986; Soh et al. 2001). The paddle-shaped morph of *P. inopinus* is known as a subspecies, *P. i. saccupodus* (Shen & Tai 1962). The ITS1 and mtCOI sequence analyses indicate that the sequence differences between the two male morphs are <1.2% for the ITS1 region and <3.8% for the mtCOI. These data suggest that two morphs probably belong to one species.

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