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Description of *Tisbe alaskensis* sp. nov. (Crustacea: Copepoda) Combining Structural and Molecular Traits

Supawadee Chullasorn[1], Hans-Uwe Dahms[2,3], Kyun-Woo Lee[2], Jang-Seu Ki[2], Nikolaos Schizas[4], Pawana Kangtia[1], Heum Gi Park[5], and Jae-Seong Lee[2,6,*]

[1]*Department of Biology, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand*

[2]*Department of Molecular and Environmental Bioscience, Graduate School, Hanyang University, Seoul 133-791, Korea*

[3]*Department of Green Life Science, Sangmyung University, Seoul 110-743, Korea*

[4]*Department of Marine Sciences, University of Puerto Rico, Mayagüez Campus, Isla Magueyes, Laboratories, P.O. Box 9013, Mayagüez, PR 00681, USA*

[5]*Faculty of Marine Bioscience & Technology, Kangnung-Wonju National University, Gangneung 210-702, Korea*

[6]*National Research Lab of Marine Molecular and Environmental Bioscience, Department of Chemistry, and Research Institute for National Sciences, College of Natural Sciences, Hanyang University, Seoul 133-791, Korea*

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Supawadee Chullasorn, Hans-Uwe Dahms, Kyun-Woo Lee, Jang-Seu Ki, Nikolaos Schizas, Pawana Kangtia, Heum Gi Park, and Jae-Seong Lee (2011) Description of *Tisbe alaskensis* sp. nov. (Crustacea: Copepoda) combining structural and molecular information. *Zoological Studies* **50**(1): 103-117. The purpose of this paper is to provide a species description of *Tisbe alaskensis* sp. nov. that combines a visual description with molecular identification that allows a meaningful characterization. Morphological details of this species are described from laboratory stocks raised from individuals collected in the harbor at Juneau, Alaska. The description revealed the following morphological autapomorphic characters: the spiniform outer seta of P1 endopod-3 bears short but stiff spinules along the outer border and an oblique spinule row at the outer corner; the middle spiniform seta carries a tuft of spinules at the outer tip; and there are some spinules scattered on the anterior surface of the female P5 exopod, but no ornamentation on the male P5 exopod. In addition, we determined the 18S ribosomal DNA sequence of *T. alaskensis* sp. nov. and compared it against publicly available sequences. http://zoolstud.sinica.edu.tw/Journals/50.1/103.pdf

Key words: Species description, Phylogenetic systematics, 18S rDNA, External morphology, Tisbidae.

Describing a new taxon such as a new species is a challenging issue for biologists. The classical use of morphological traits for species identification has several limitations. The importance of any particular morphological trait reflects the judgement of the taxonomist. Phenotypic variations of 1 or more traits under study may result in 2 or more names applied to the same taxon (Dodson and Lee 2006), or a group of morphological traits might not vary between 2 or more species that can be recognized by other attributes (Lee and Frost 2002, Ki et al. 2009). Moreover, taxonomically important morphological traits are often expressed only in a particular life stage or gender, as for example in the Copepoda, where species identification is mainly based on adult appendages (Ferrari and Dahms 2007). Thus, a high level of expertise is often required to correctly identify species with the accuracy required in a wide array of studies. Morphological criteria for diagnosing species vary from genus to genus; similarly, there is no consensus as to how much

*To whom correspondence and reprint requests should be addressed. Tel: 82-2-22200769. Fax: 82-2-22999450.
E-mail:jslee2@hanyang.ac.kr; hansdahms@smu.ac.kr; hgpark@gwnu.ac.kr

divergence between taxa results in reproductively isolated species. Despite these shortcomings, traditional morphology remains the only way to establish a binomial name for eukaryotes, since a visual description and subsequent deposition of a holotype at an established institution remain necessary steps in naming new species.

Application of the biological species concept (Mayr 1942) was a major advance in understanding species, because the concept makes use of reproductive compatibility or isolation among individuals to make predictions about other aspects, including morphology, of the biology of different species. The biological species concept has been applied directly to elucidate species through crossbreeding experiments (Volkmann 1975 1979), or indirectly to infer reproductive isolation from divergence in secondary sex characters (Fleminger 1967). Breeding experiments are limited to a few "model" genera (e.g., *Tigriopus* - see Dahms et al. 2007, Raisuddin et al. 2007; and *Tisbe* - see Dahms et al. 1991a b, Chullasorn et al. 2009), that are currently under culture in several laboratories. There are very few opportunities to conduct interbreeding experiments between morphologically similar species to confirm reproductive isolation. This is especially true for copepods that inhabit inaccessible habitats (e.g., deep waters) or are rare. Nevertheless, the biological species concept remains the standard for defining sexually reproducing species (Dahms et al. 2007).

Recent technological advances in sequencing nucleotides of nucleic acids and in computer technologies have brought many changes to the field of taxonomy. The use of molecular tools to identify species dates from Kangethe et al. (1982). The ease of nucleotide sequencing allows species identifications using short segments of nucleic acids, and this "DNA barcoding" was developed through an international initiative (Hebert et al. 2003). Although variations in nucleic acid sequences brought back the problem of subjective judgment that hampers morphological taxonomy, the Consortium for the Barcode of Life (CBOL, http://barcoding.si.edu) supports global standards and coordinated research in DNA barcoding. Thus DNA barcoding techniques are expected to increasingly be employed in taxonomy (Cameron et al. 2006).

The taxon *Tisbe* Lilljeborg, 1853 presently contains at least 54 recognized species (Boxshall and Halsey 2004) and occurs worldwide especially in shallow marine waters. The taxonomy of *Tisbe* has been comparatively well studied (Dahms and Qian 2005) since its representatives are easy to collect, keep, and rear in the laboratory. Species of *Tisbe* were formerly thought to be cosmopolitan, eurythermic, and euryhaline. However, it was shown by crossbreeding experiments that some *Tisbe* species are a complex of sibling species, distinguished by minute morphological details, e.g., *T. holothuriae/T. battagliai* (Volkmann-Rocco 1972b, Volkmann 1975), and *T. bulbisetosa/T. inflatiseta* or *T. gracilis/T. cucumariae* (Volkmann 1979). To distinguish them, it is essential that all morphological details are taken into consideration (see Dahms 1991a). In this paper the morphology of a new species of *Tisbe* is described and a 1766 bp sequence of its 18S ribosomal (r)DNA is characterized as a barcode for the new species.

MATERIALS AND METHODS

Specimens

Specimens of *Tisbe* were collected from the harbor at Juneau, Alaska, USA by a plankton net that was introduced to the substratum. Sediment samples were stirred up in a beaker and decanted over a screen. Specimens of the new *Tisbe* were kept in cultures with pasteurized seawater at 32‰ salinity. Two-thirds of the water in the culture vessels was renewed once a week. The diatoms *Isochrysis galbana*, *Chaetoceros*, and *Tetraselmis* were used as food.

Morphological examination

Specimens were fixed in 5% formaldehyde and suspended in W 15 (embedding medium, Carl Zeiss, Jena, Germany). Before dissection, the habitus of *T. alaskensis* sp. nov. was drawn from whole mounts, and total body length measurements were made from specimens mounted in W 15. Dissected parts were mounted on slides in glycerin. Broken glass fibers were added to prevent the animal and appendages from being compressed by the coverslip and to facilitate rotation and manipulation, allowing observation from all angles. All drawings were prepared using a camera lucida on a Nikon HFX-DX compound microscope (Hamamatsu, Japan) at a magnification of 1000x. Measurements were made with an ocular micrometer. Scale bars in the illustrations are in microns (μm). The naupliuseye was not figured because it loses its red color

and shape soon after being embedded, and no color pattern was discernible after embedding. References used for the morphological description and study of this species of *Tisbe* were Huys and Boxshall (1991) and Gómez et al. (2004). Abbreviations used in the text include: R, rostrum; A1, antennule; A2, antenna; Md, mandible; Mx1, maxillule; Mx2, maxilla; Mxp, maxilliped; La, labrum; P1-P4, 1st-4th swimming legs; P5, leg 5; P6, leg 6; enp, endopod; exp, exopod; and ae, aesthetasc.

Polymerase chain reaction (PCR) amplification and DNA sequencing

For genomic DNA preparations, we isolated single individuals of *T. alaskensis* sp. nov., that were transferred to 200 μL thin-walled PCR tubes containing 5 μL of TE buffer. The tubes were maintained at 55°C for 10 min with in an iCycler thermoblock (Bio-Rad, Foster City, CA, USA), and were subsequently cooled down to 4°C. Then a PCR was carried out with eukaryote 18S-rDNA-targeting primers (Cop-18F24, 5'-TGGTTGATCC TGCCAGTAG-3' and Cop-18R2300, 5'-TAATGAT CCTTCCGCAGGTTC-3') which amplified nearly the entire 18S rDNA sequence. The PCR was performed with 25 μl reaction mixtures containing 15.8 μl sterile distilled water, 2.5 μl 10x Ex PCR buffer (TaKaRa, Kyoto, Japan), 2.5 μl of the dNTP mix (2 mM), 1 μl of each primer (10 μM), 0.2 μl Ex *Taq* polymerase (1.0 U), and 2 μl of the template. PCR cycling was performed in a Bio-Rad iCycler (Bio-Rad) at 94°C for 3 min, followed by 35 cycles at 94°C for 20 s, 55°C for 30 s, and 72°C for 2 min, with a final extension at 72°C for 5 min. The resulting PCR products were purified with the QIAquick PCR purification kit (Qiagen, Mannheim, Germany). DNA was sequenced by the PCR and internal walking primers, using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA, USA) and an automated DNA sequencer (Model 3700, Applied Biosystems, Foster City, CA, USA).

RESULTS AND DISCUSSION

SPECIES DESCRIPTION

Order Harpacticoida Sars, 1903
Family Tisbidae Stebbing, 1910
Genus *Tisbe* Lilljeborg, 1853
***Tisbe alaskensis* sp. nov.**
(Figs. 1-11)

Type locality: Juneau Harbor, Alaska, USA. The type material was collected on 13 Oct. 2007.

Type specimens: One female holotype (USNM 1136877) dissected on 7 slides, and 1 male allotype (USNM 1136878) dissected on 6 slides, 1 female paratype (USNM 1136879), and 1 male paratype (USNM 1136879) preserved in alcohol deposited in the Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC, USA.

Adult female (holotype): Habitus (Fig. 1) podoplean, tapering posteriorly; greatest width at posterior margin of cephalic shield; with marked distinction between 4th and 5th urosomites. Total body length 817 μm, measured from anterior margin of rostrum to posterior margin of caudal rami. Rostrum (Figs. 1A, 3A) completely fused to cephalic shield, with 2 sensillae of unequal length on each side of tip.

Urosome (Fig. 2) 5-segmented. First urosomite (P5-bearing somite) without ornamentation; 2nd and 3rd urosomites (genital double-somite) fused dorsally and ventrally, with a dorsolateral cuticular bar demarcating fusion line, each urosomite with a hyaline frill ventrally. Anal somite (as the 5th urosomite of podopleans) expanded posteriorly as a pseudoperculum. Caudal rami nearly as long as wide, with 7 setae each and small spinules at base of each caudal seta, except for seta V (Fig. 2A). Setae I and II of unequal length located on outer margin of ramus; seta III long, arising at outer distal corner; setae IV and V well-developed, arising at distal corner, seta V longest; seta VI nearly as long as seta III, arising at inner distal corner; seta VII nearly as long as seta II, located dorsally, close to seta V.

Antennule (Fig. 3B) distinctly 7-segmented, with large aesthetasc on segment 4 and small aesthetasc on distal segment. First segment with 1 bipinnate seta and row of spinules along inner margin. Armature formula: 1-(1), 2(11), 3-(9), 4-(4+ae), 5-(2), 6-(7), 7-(6+ae).

Antennae (Fig. 3C) biramous, with separate coxa and basis, latter well-developed, as long

as proximal segment of endopod, ornamented with rows of spinules on surface, with 1 bipinnate abexopodal seta at inner distal corner. Exopod 4-segmented, armature: 1-(1), 2-(1), 3(1), 4-(3). First 3 segments with 1 bipinnate seta each distally, inserted 4th segment with 3 bipinnate setae distally, and row of spinules near insertion of innermost seta. Endopod 2-segmented, 1st segment with 1 smooth seta; 2nd segment with inner ornamentation and 2 spinules proximal to outer armature. Armature consisting of 1 normal and 1 geniculate spine on inner margin, 4 geniculate elements, 1 unipinnate seta + 1 smooth seta (fused at base), and 1 bipinnate seta inserted distally.

Mandible (Fig. 4A) with well-developed coxa, longitudinal row of spinules on anterior surface, and small spinules proximally along posterior margin. Gnathobase with well-developed cutting teeth, and 1 bipinnate spinulose seta. Basis with 2 small spinules, and armed with 1 bipinnate spinulose inner seta close to origin of endopod. Exopod 1-segmented, with many rows of spinules along oral surface and on outer margin of exopod, and 1 distal outer bipinnate spinulose seta and 2 smooth setae with a forked tip; 1 distal and another subdistally inserted on inner margin. Endopod 1-segmented, with row of spinules along oral margin and oblique row of spinules on oral surface, and armed with 3 lateral setae (1 with forked tip), 6 apical slender elements forming 2 sets of fused setae with 3 elements each.

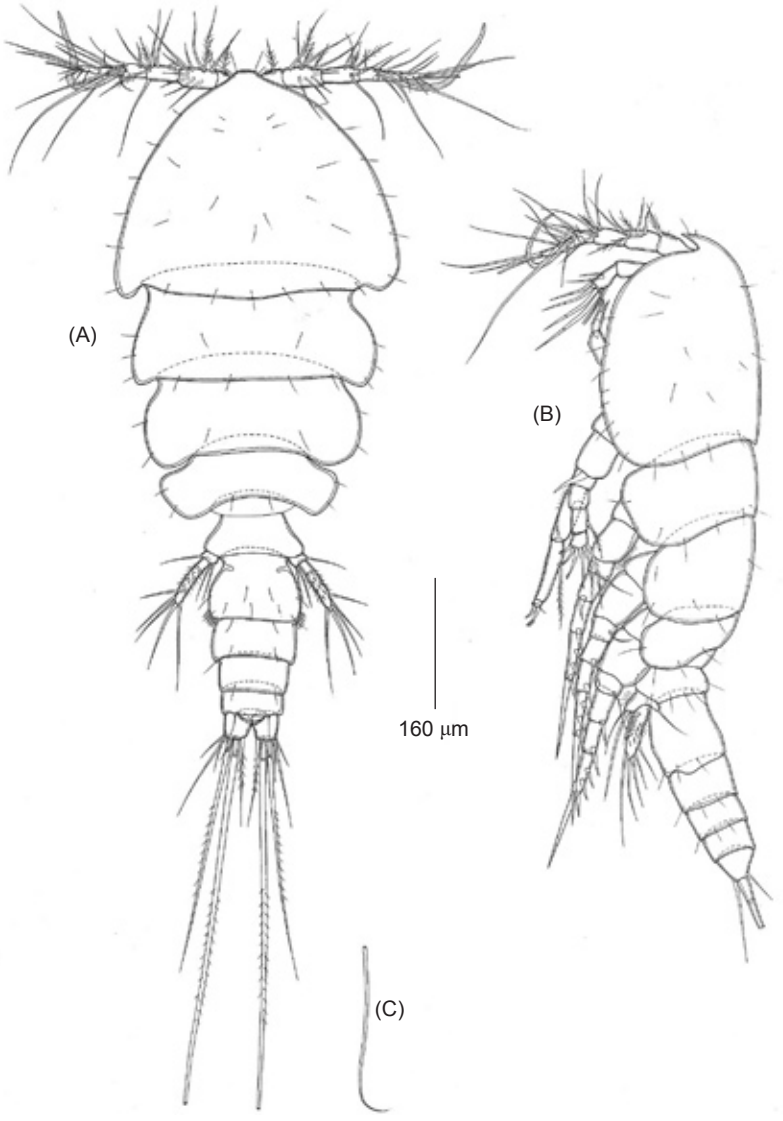

Fig. 1. *Tisbe alaskensis*. Female. Habitus in dorsal view (A). In left lateral view (B). Extension of the caudal seta (C).

Maxillule (Fig. 4B) with long developed praecoxal arthrites with many rows of spinules and armed with 2 strong setae on aboral surface, and 6 strong smooth elements: 5 distal and 1 insertion at inner corner. Coxa without ornamentation, with 5 slender setae distally. Basis inserted on distal margin of coxa with 2 smooth setae fused at their bases. Exopod represented by 1 well-developed smooth seta. Endopod 1-segmented, with 3 smooth setae.

Maxilla (Fig. 4C). Syncoxa well-developed with 1 distal endite bearing 1 smooth and 1 very small seta, and with 1 row of long spinules at outer margin. Allobasis drawn out into a strong claw, with 1 strong unipinnate spinulose spine at midlength, and with some outer spinules proximally.

Maxilliped (Fig. 4D). Praecoxa well-developed, with a row of slender spinules on inner margin and some spinules near insertion of coxa. Coxa small, with some spinules at inner distal corner. Basis with 2 rows of spinules along outer and anterior surfaces, and some spinules apically. Endopod 1-segmented, small, and short, with a long unipinnate claw, 1 bipinnate seta, and 3 unequal-length smooth setae at midlength.

P1 (Fig. 5A). Praecoxa and coxa with oval-shaped intercoxal sclerite, with a few spinules on each side. Coxa with several transverse rows of spinules on posterior surface. Basis with 1 outer and 1 inner bipinnate spine, several rows of spinules on posterior surface, a row of spinules

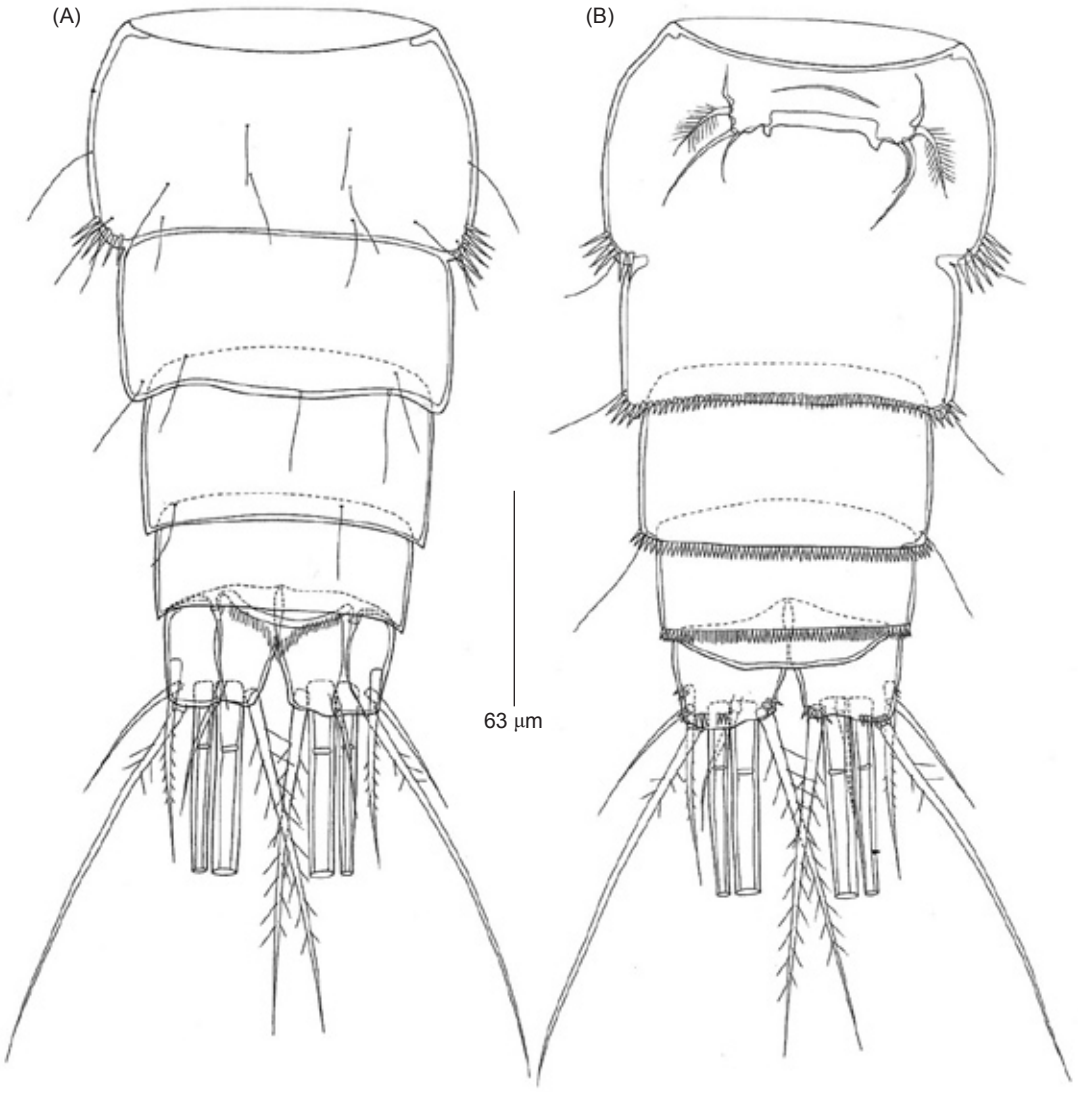

Fig. 2. *Tisbe alaskensis*. Female. Urosome in dorsal view (A) and in ventral view (B).

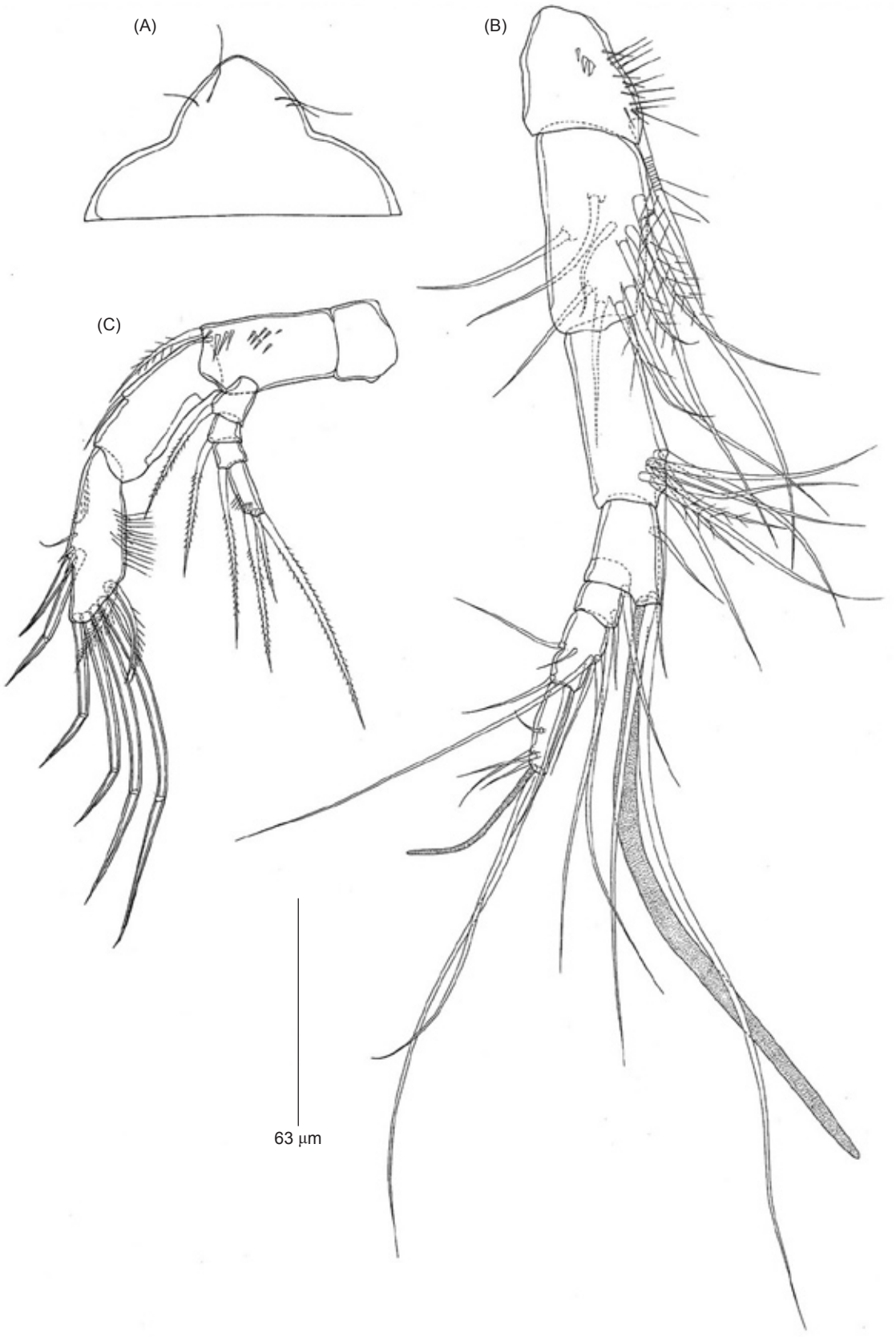

Fig. 3. *Tisbe alaskensis*. Female. Rostrum (A), antennule (B) and 2nd antenna (C).

Fig. 4. *Tisbe alaskensis*. Female. Mandible (A), maxillule (B), 2nd maxilla (C), and maxilliped (D).

juxtaposed to insertion of spines, and some setules along inner distal margin. Exopod 3-segmented; exp-1 with 2 longitudinal rows of spinules along outer margin and on posterior surface, and 1 long, strong, bipinnate spine on outer distal corner; exp-2 with 1 inner plumose seta and 1 outer spine with a tuft-like comb of spinules at outer distal margin; exp-3 short, with 4 unequally long outer spines, bearing a tuft-like comb of spinules at outer distal margin and 2 long setae: 1 outer unipinnate and inner plumose and 1 inner plumose seta inserted distally. Endopod 3-segmented; distinctly longer than exopod; enp-1 slightly shorter than enp-2, with a row of spinules along outer margin, some spinules on posterior surface, and 1 long plumose-seta inserted at inner margin; enp-2 with a row of spinules along outer margin, and 1 inner pinnate seta inserted at inner margin; enp-3 very small, with some spinules at outer distal corner on posterior surface, 1 tiny seta at inner distal corner, and 2 spines of unequal length: inner spine longer, with a tuft-like comb of spinules at distal inner margin and outer one with a spinule tuft of a larger spine.

P2 (Fig. 5B). Praecoxa and coxa with trapezoid-shaped intercoxal sclerite with some spinules on each side. Coxa with several rows of spinules on posterior surface and at outer margin. Basis with 1 outer plumose seta. Basis with a row of small spinules on outer margin near insertion of outer plumose seta and exopod, a row of short spinules on posterior surface and a row of setules along inner margin. Exopod 3-segmented; exp-1 and exp-2 with 1 outer pinnate and 1 non-annulate spine, plumose seta at each inner margin, and a row of spinules along outer margin; exp-3 with 3 outer pinnate spines, 1 outer unipinnate and 1 inner plumose terminal seta, and 3 annulate and plumose setae (1 terminal and 2 lateral) at inner margin. Endopod 3-segmented; enp-1 with 1 non-annulate plumose seta at inner margin, ornamented with a row of spinules along outer margin; enp-2 with some spinules on posterior surface and 1 tube pore on outer distal corner, with a row of spinules along outer margin, and 2 annulate and plumose setae at inner margin;

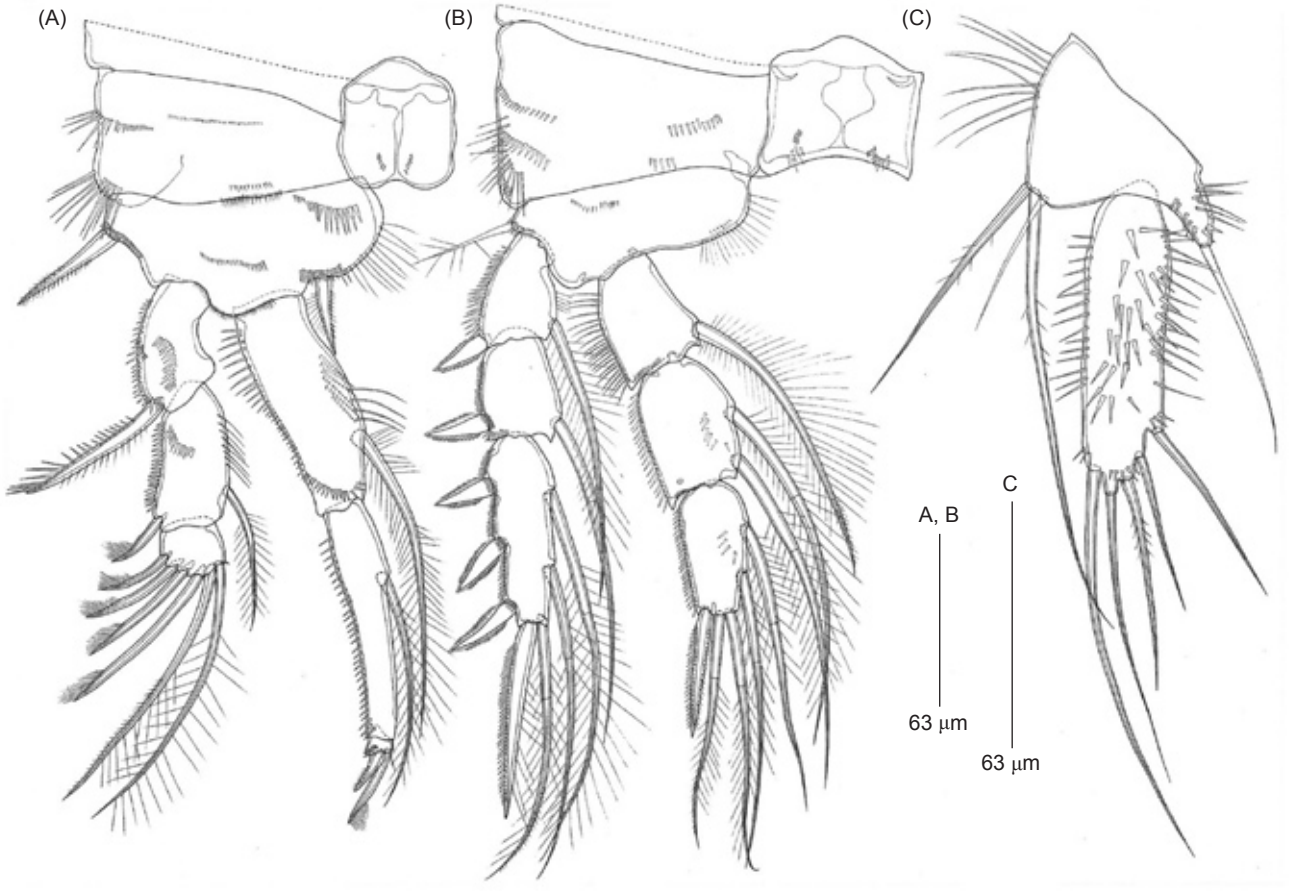

Fig. 5. *Tisbe alaskensis*. Female. Swimming leg 1 (P1) (A), swimming leg 2 (P2) (B), and P5 (C).

enp-3 with some spinules on posterior surface, a row of spinules along outer margin, 1 terminal pinnate spine, and 4 annulate and plumose setae (2 terminal and 2 lateral).

P3 (Fig. 6A) as P2, except all segments of exp-1 with 1 annulate plumose seta at inner margin; exp-2 without spinules on posterior surface; and exp-3 with 4 annulate and plumose setae. Enp-2 without tube pore on posterior surface, and with 5 annulate plumose setae (2 terminal and 3 lateral).

P4 (Fig. 6B) as P3. Exp-3 ornamented with 3 oblique rows of small spinules. Endopod-3 armed with 4 annulate plumose setae.

Armature formula of P1-P4 of *Tisbe alaskensis* sp. nov.

Fig. 6. *Tisbe alaskensis*. Female. Swimming leg 3 (P3) (A) and swimming leg 4 (P4) (B).

	Exopod	Endopod
P1	I-0; I-1; III, I1, 1	0-1; 0-1; I, I, 1
P2	I-1; I-1; III, I1, 2	0-1; 0-2; I, 2, 2
P3	I-1; I-1; III, I1, 3	0-1; 0-2; I, 2, 3
P4	I-1; I-1; III, I1, 3	0-1; 0-2; I, 2, 2

P5 (Fig. 5C). Baseoendopod small, triangular, with scattered slender spinules near base of outer smooth seta, and with 3 inner setae of unequal lengths: inner one small, middle one longest and spinulose, and outer one spinulose. Exopod 3 times longer than wide, with spinules along inner and outer margins and on anterior surface, with 5 setae: 3 outer (2 smooth and 1 spinulose), 1 terminal, and 1 inner (longest).

P6 (Fig. 2B) represented by a small lobe, bearing 3 setae of unequal lengths: 1 outer pinnate, middle one longest, and inner one small.

Habitus (Fig. 7). Total body length from tip of rostrum to posterior margin of caudal rami 609 μm.

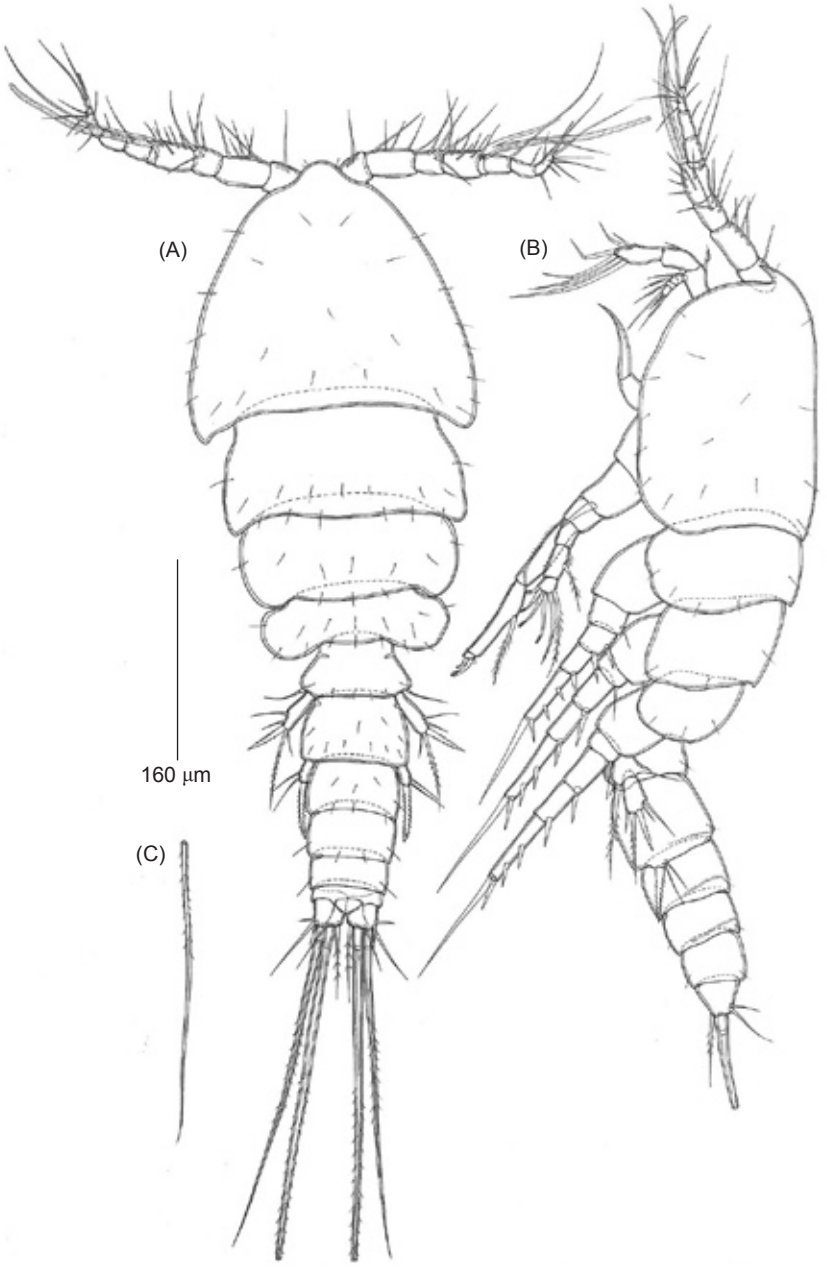

Fig. 7. *Tisbe alaskensis*. Male. Habitus in dorsal view (A) and in left lateral view (B).

General body shape and ornamentation as in female, except for genital double-somite (Fig. 8).

Antennule (Fig. 9B) haplocerate, 8-segmented, with 1 large aesthetasc on segment 4 and 1 very small one on distal segment. Armature formula: 1-(1), 2-(15), 3-(9), 4-(9+ae), 5-(2), 6-(1), 7-(2), 8-(8+ae).

Maxilliped (Fig. 9C) sexually dimorphic. Praecoxa well-developed with some slender spinules on outer edge. Coxa small with a few spinules on inner corner. Basis well-developed with some spinules along outer and inner edges. Endopod small with long claw, and 3 smooth setae of unequal lengths on inner and outer edges.

P1 (Fig. 10A) as in female, except for exp-1 with 1 spine bearing tuft-like comb of spinules at outer edge of tip.

P2 (Fig. 10B) as in female, except for enp-2 showing 1 tube pore at distal margin near base of outer terminal annulate seta.

P5 (Fig. 10C). Baseoendopod small, with a few spinules near base of outer spinulose seta;

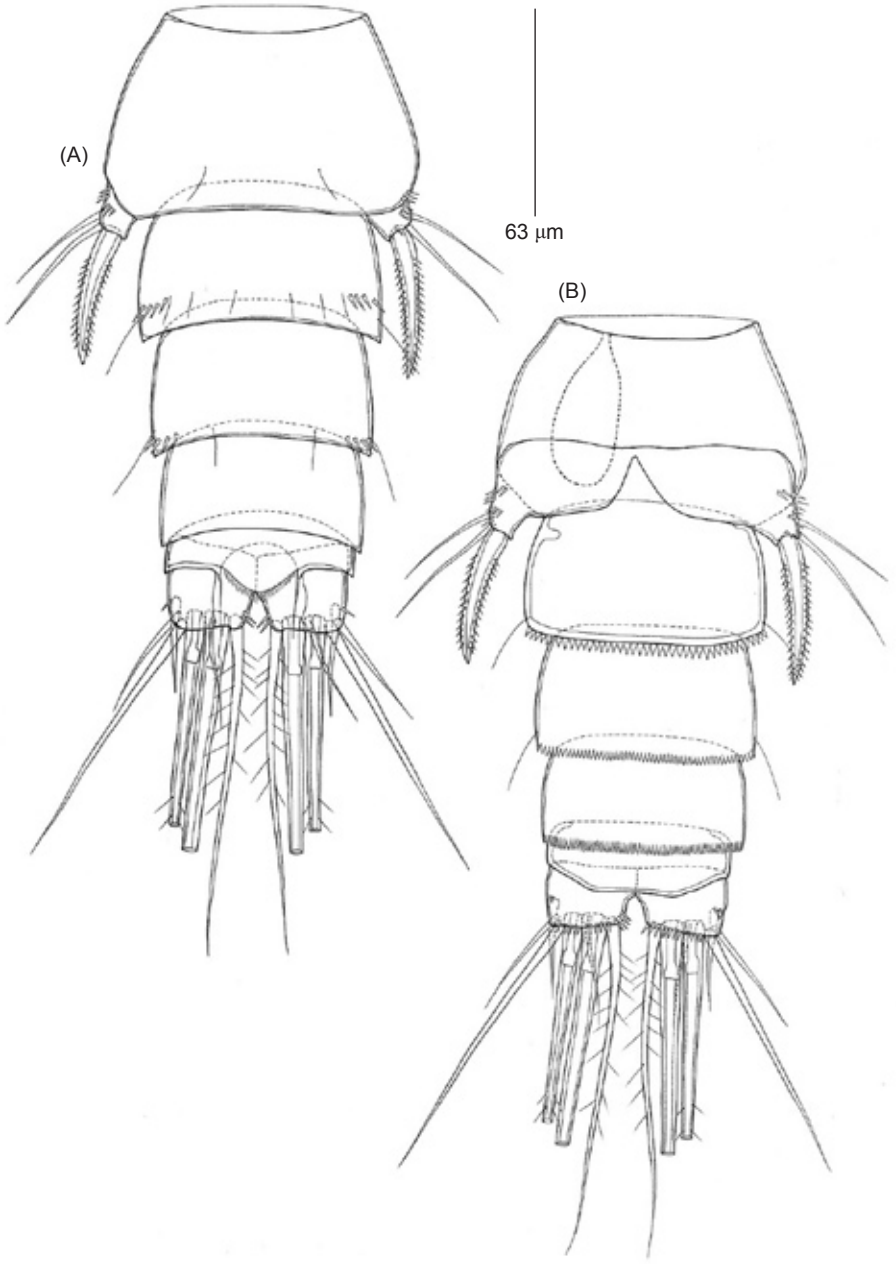

Fig. 8. *Tisbe alaskensis*. Male. Urosome in dorsal view (A) and in ventral view (B).

inner lobe without ornamentation, with 1 long spinulose seta and 1 very small smooth seta. Exopod about 1.5-times longer than wide, with tiny spinules along inner and outer margins and 5 elements of unequal lengths: middle spinulose spine, 2 outer setae (1 outermost spinulose longer and 1 slender), and 2 inner setae (1 innermost smooth and 1 long spinulose).

Adult male (allotype): Sexually dimorphic in antennule, maxilliped, P1, P2, P5, and P6. P3 and P4 as in female (Fig. 11).

P6 (Fig. 8) represented by a small lobe with 1 strong pinnate spinulose inner seta, and 2 outer slender setae nearly equal in length, and with spinules at base of setae.

Etymology: The species name refers to the State of Alaska (USA) where the specimens were found.

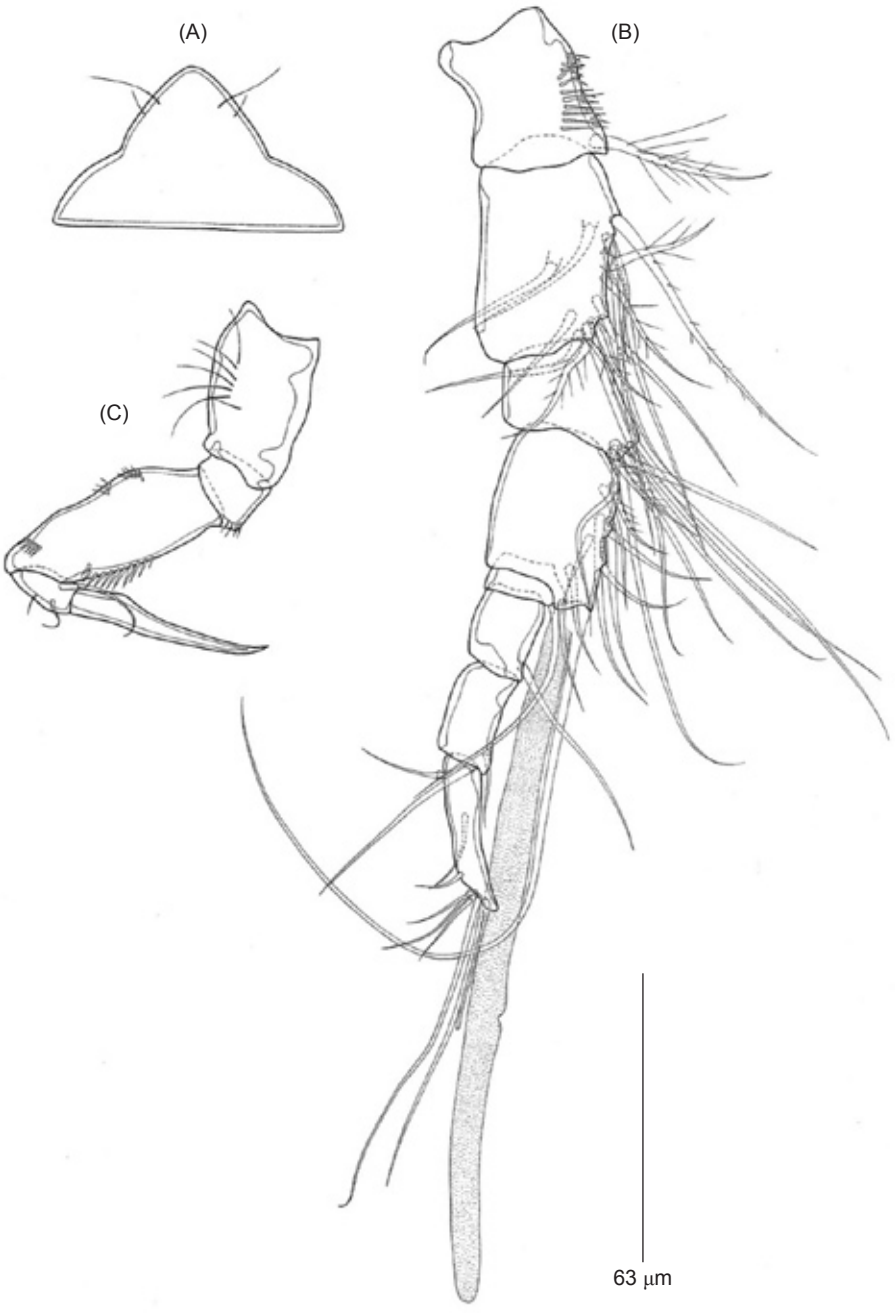

Fig. 9. *Tisbe alaskensis*. Male. Rostrum (A), 1st antenna (B) and maxilliped (C).

Differential diagnosis

In *T. alaskensis* sp. nov., the spiniform terminal seta of P1 enp-3 bears a peculiar ornamentation. Instead of the common spine-like seta, it bears a spinule row on the anterior face. The outer tip of the innermost seta has a tuft of spinules, whereas in *T. gracilis* there are only short stiff spinules. Along the outer border of the outermost seta of *T. alaskensis* sp. nov., there are short, stiff spinules. There are large surface spinules on the anterior face of P5 exp and base in the female and P5 exp in the male of *T. alaskensis* sp. nov. These are only known to a lesser extent from *T. furcata* (Dahms et al. 1991b).

Phylogenetic position of *T. alaskensis* sp. nov.

The 18S rDNA sequence of *T. alaskensis* sp. nov. is 1766 bp long (GenBank accession no.: FJ713566). Since very few harpacticoid species are represented in GenBank, it was essential to find homologous sequences using BLAST. The closest match (97.5% similarity) in GenBank was the 18S rDNA sequence of *T. furcata* (GenBank accession no.: AY692343). In addition, BLAST searches of the entire sequence revealed 95.7% similarity with *Itunella muelleri* (Harpacticoida; Canthocamptidae), followed by a 95.3% similarity with *Dactylopusia* sp. (Harpacticoida: Dactylopusiidae).

Tisbe alaskensis sp. nov. showed structural peculiarities that demarcate the structural diversity within the Tisbidae. Otherwise, comparatively small morphological differences make it difficult to distinguish between species of *Tisbe* and to evaluate the phylogenetic relationships of *Tisbe* species (Volkmann-Rocco 1971). Crossbreeding experiments were successfully employed by Volkmann-Rocco (1972b) and Volkmann (1979) to show that some *Tisbe* species are a complex of almost indistinguishable sibling species. Such sibling sister pairs include *T. holothuriae/T. battagliai* (Volkmann-Rocco 1972b, Volkmann 1975), and *T. bulbisetosa/T. inflatiseta* or *T. gracilis/T. cucumariae* (Volkmann 1979). Their morphological distinction was subsequently feasible after reproductive isolation was established (see Dahms 1991a). Detailed examination of taxa may yield sufficient morphological characters

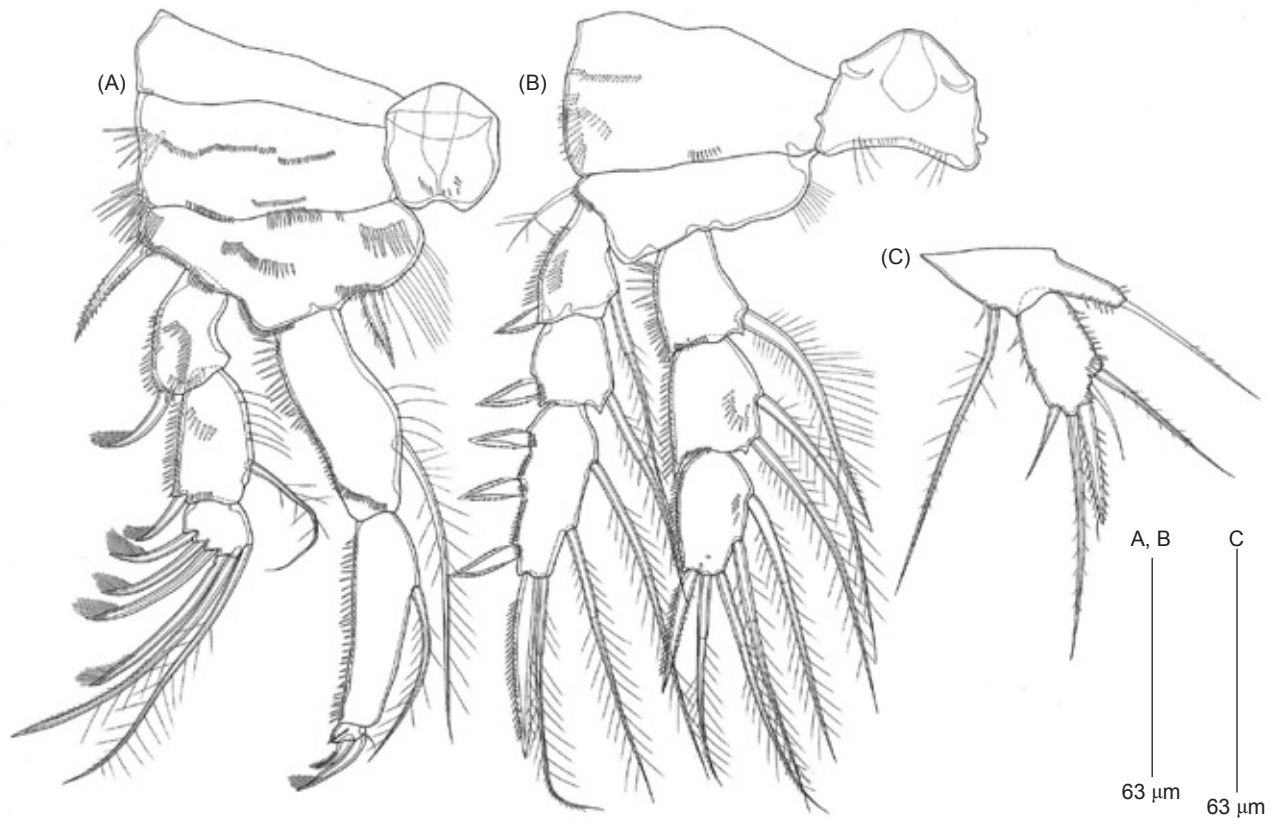

Fig. 10. *Tisbe alaskensis*. Male. Swimming leg 1 (P1) (A), swimming leg 2 (P2) (B), and leg 5 (P5) (C).

Fig. 11. *Tisbe alaskensis*. Male. Swimming leg 3 (P3) (A) and swimming leg 4 (P4) (B).

suitable for a robust cladistic analysis, particularly if this is combined with DNA sequences. Genetic information in the form of DNA barcoding will doubtlessly further enhance the description of new *Tisbe* species by providing a divergence estimate against congeneric species.

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