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DESCRIPTION OF LATE-INSTARS OF *BRYOTHINUSA KOREANA* AHN AND JEON (COLEOPTERA: STAPHYLINIDAE: ALEOCHARINAE) BY ASSOCIATION OF LIFE STAGE BASED ON DNA SEQUENCE DATA

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ABSTRACT

Late-instars of *Bryothinusa koreana* Ahn & Jeon are described for the first time based upon DNA sequence data of larvae and adults. Twelve larvae were collected on Geoje Island, Korea, in association with adults of *B. koreana*. The partial cytochrome oxidase II gene (410 bp) was sequenced from the larvae and from several identified adult *Bryothinusa* specimens, including *B. koreana*. The intraspecific p-distances of adult *B. koreana* were from 0 to 4.44%, and interspecific pairwise distances were 10.77% to 13.47%. The sequence results of the larvae were similar to those of adult *B. koreana*. Based on these results, the larvae were identified as *B. koreana* and diagnostic characters of the species are provided, with illustrations of features.

Key Words: *Bryothinusa koreana*, DNA identification, Korea, larval description, Staphylinidae

RESUMEN

Se describen por la primera vez los últimos instares de *Bryothinusa koreana* Ahn & Jeon basado en los datos de las secuencias de ADN de las larvas y adultos. Se recolectaron doce larvas en la Isla Geoje, Korea, en asociación con adultos de *B. koreana*. Se hizo una secuencia del gene citocromo-oxidasa II de la larva y de varios especimenes identificados de *Bryothinusa*, incluyendo *B. koreana*. La distancia -p interespecifica de los adultos de *B. koreana* fue de 0 a 4.44%, y la distancia interespecifica de los pares fue de 10.77% a 13.47%. Los resultados de las secuencias de larvas fueron similares a las de los adultos de *B. koreana*. Basado en estos resultados, las larvas fueron identificadas como *B. koreana* y se preveen caracteres diagnósticos de las especies con ilustraciones de sus características.

The genus *Bryothinusa* Casey contains 26 species and is distributed throughout the Pacific Basin and the Red Sea. This intertidal genus has the greatest number of species in the staphylinid subfamily Aleocharinae. Typically adults and larvae are found under stones along coasts, but some are found in estuarine habitats (Ahn & Ashe 2004). To date the only described larva in the genus *Bryothinusa* is represented by *B. catalinae* Casey (Moore & Roth 1979).

Aleocharine larvae provide information for phylogenetic and evolutionary studies (Ashe 1986; Ahn & Ashe 1996). However, very few immature aleocharines have been described because of the difficulty of making larval-adult associations (Ashe & Watrous 1984). Larvae can be reared to adults in the laboratory, allowing larval identification and association with adults, but rearing is time intensive, and it is difficult to achieve the appropriate rearing conditions to successfully obtain adults.

Recently, DNA sequencing has become straightforward, inexpensive, and is an obvious alternative for the identification of immature staphylinids as well as other insects (Caterino & Tishechkin 2006; Hebert et al. 2003; Tautz et al. 2003; Blaxter 2004). A partial sequence of the cytochrome oxidase II (410bp) gene is sufficient to make a confident association between life stages of staphylinid beetles (Jeon & Ahn 2005, 2007).

In this paper, we describe late-instars of *B. koreana* Ahn & Jeon based upon the association of larval and adult DNA sequences in the genus *Bryothinusa*. We also provide morphological diagnostic characters with illustrations of features and discuss differences between *B. koreana* and *B. catalinae*.

MATERIALS AND METHODS

Twelve larvae (identity unknown at time of collection) and many adults of *B. koreana* were

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collected together under stones within a habitat range of about 10 m, on Geoje Island, Korea. A partial sequence of the CO II gene (410 bp) was generated from the unknown larvae and five identified adult specimens of *Bryothinusa* to confirm that the unknown larvae are *B. koreana*. Three *B. koreana* adults, each from different populations (from Gangin, Jindo Is., and Geoje Is.), were included in order to examine intraspecific variation. The sequences of *B. nakanei* (Sawada) from Korea and unidentified *Bryothinusa* species from Philippines were also generated to study interspecific variation among the genus *Bryothinusa*. *Brachypronomaea esakii* Sawada was included to root the cladogram (Table 1).

Preparation of permanent microscopic slides for late instars was performed with the techniques described by Ashe (1986). Terms and the chaetotaxic system for late-instars follow Ashe & Watrous (1984). Materials for this study were deposited in the Chungnam National University Insect Collection (CNUIC, Daejeon), Korea.

DNA Extraction, Amplification, and Sequencing

For adults, total genomic DNA was extracted from muscles in the head and pronotum to prevent contamination by DNA of parasites or gut contents. Genitalia were preserved to confirm the species identification. After grinding the specimens in liquid nitrogen, we followed the manufacturer's protocol for the DNeasy Tissue Kit (QIAGEN, Hilden, Germany). For larvae, DNA was extracted from muscles in the head and pronotum. The remaining cuticle was used as a voucher specimen of the sample deposited in CNUIC.

The CO II region examined in this study was amplified by a set of primers C2J 3400 (Simon et al. 1994) and TKN 3782 (Brent et al. 1999). PCR was performed in 50 µL with 1-10 µL of the genomic DNA with 1 or 2 units of *Taq*-polymerase and 3 mmol $MgCl_2$, 1.5 mmol dNTPs, and 50 pmol of each primer. The amplification involved 2 min of denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s of primer annealing at 45°C-55°C, and 1 min of extension at 72°C, followed by a final 4-min extension at 72°C. PCR products were cleaned of enzymes and remaining primers with a PCR Product Purification Kit (Roche, Indianapolis, Indiana, USA) and recovered in 20 µL of H_2O.

Amplified DNA was sequenced by a Perkin Elmer ABI377 Automated Sequencer (Applied Biosystems Inc., Foster City, California, USA) and confirmed with both sense and anti-sense strands. Partial CO II sequences (410 bp) of *B. koreana* and related species have been deposited in GenBank under accession numbers EF079108-EF079114 (Table 1).

RESULTS

As the CO II gene is a protein coding region, alignment was performed by SeqPup (Gilbert, 1995). Parsimony analysis was conducted with PAUP* (Swofford 2003) with the Branch and Bound tree search option, and branch support values were estimated by bootstrapping. The analysis resulted in a single most parsimonious cladogram with a length of 126, a consistency index of 0.84 and a retention index of 0.70 (Fig. 1). Average CO II sequence differences among populations of *B. koreana* were 2.38% (0-4.44%). The maximum intraspecific distance in *B. koreana*

TABLE 1. SPECIES, COLLECTION INFORMATION, AND GENBANK ACCESSION NUMBERS FOR CYTOCHROME OXIDASE II SEQUENCES FROM THIS STUDY.

Species	Collection information	GenBank accession
Bryothinusa koreana (adult)	Korea: Jeonnam Prov., Gangjin-gun, 19 IV 2004, K.-J. Ahn, S-J. Park, D.-H. Lee, S.-M. Choi	EF079110
B. koreana (adult)	Korea: Jeonnam Prov., Jindo, Imboe-myeon, Geumgab beach, 21 VIII 2001, K.-J. Ahn	EF079112
B. koreana (adult)	Korea: Gyeongnam Prov., Geoje Isl., Gabae-ri, 30 VI 2001, C.-W. Shin	EF079109
B. koreana (larva)	Korea: Gyeongnam Prov., Geoje Isl., Gabae-ri, 30 VI 2001, C.-W. Shin	EF079111
B. nakanei (adult)	Korea: Jeonnam Prov., Gangjin-gun, 19 IV 2004, K.-J. Ahn, S-J. Park, D.-H. Lee, S.-M. Choi	EF079108
Bryothinusa sp. (adult)	Philippines: Camotes Isl., San Francisco, Puertobellu, 14 XII 2003, M.-J. Jeon	EF079113
Brachypronomaea esakii (adult)	Japan: Chatan-cho, Okinawa-honto, Nansei shoto, 26 V 2002, M. Moriguchi, K. Miyagi, and G. Masaki	EF079114

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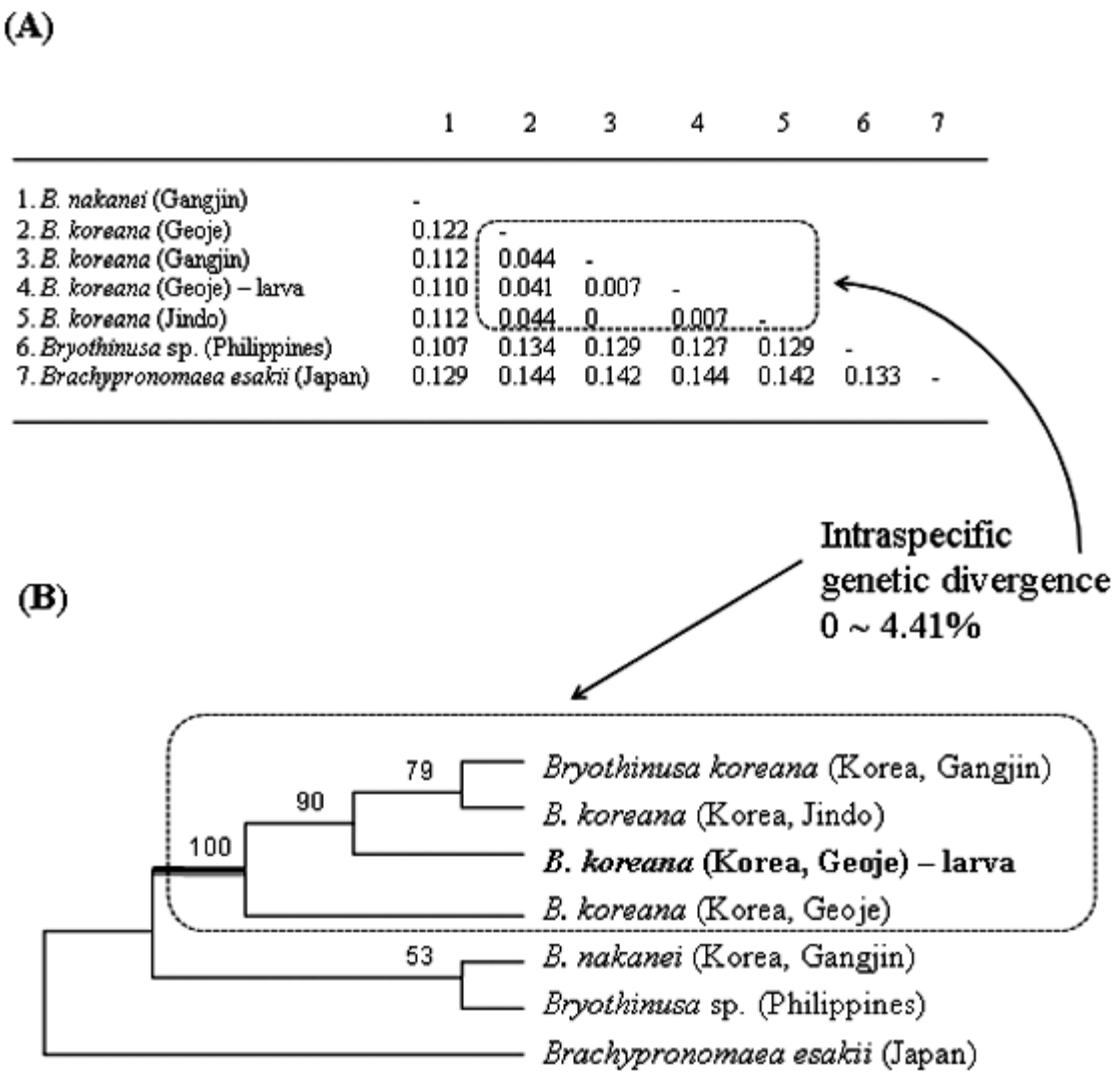

Fig. 1. Pairwise distances (A) and single most parsimonious tree (B) estimated from cytochrome oxidase II sequences (Tree length = 126, CI = 0.84, RI = 0.70). Parsimony bootstrap values (>50%) are given above the branches.

adults was 4.44% between the Gangjin, Jindo, and Geoje populations. The minimum interspecific distance within the genus *Bryothinusa* was 10.7% between *Bryothinusa* sp. from the Philippines and *B. nakanei* (Fig. 1). The CO II sequences from adults and larvae of *B. koreana* were within the range of intraspecific distance (0.7-4.1%). Therefore, we are describing below the larvae as probable late-instars of *B. koreana.*

Description of Late Instar of *Bryothinusa koreana* Ahn & Jeon

DESCRIPTION. Length 3.5-3.7 mm. General body shape elongate, flattened, nearly parallel sided, pale in color.

HEAD (Figs. 2, 3). About 0.9 times as long as wide, 1 small stemma present on each side. Ecdysial sutures distinct and complete from antennal fossae anteriorly to base of head posteriorly. Antenna (Fig. 4) with 3 articles; article 1 about 0.6 times as long as wide, transverse, 5 campaniform sensilla present around apical margin, 1 campaniform sensillum present on middle of article 1, setae absent; article 2 about 2.0 times as long as article 1; article 3 about 0.4 times as long as article 2; article 2 with 1 solenidium in addition to sensory appendage, length of sensory appendage almost equal to article 2; article 2 and 3 each with 4 and 3 setae; article 2 with 1 long (IIS1) and 1 short (IIS2) so-

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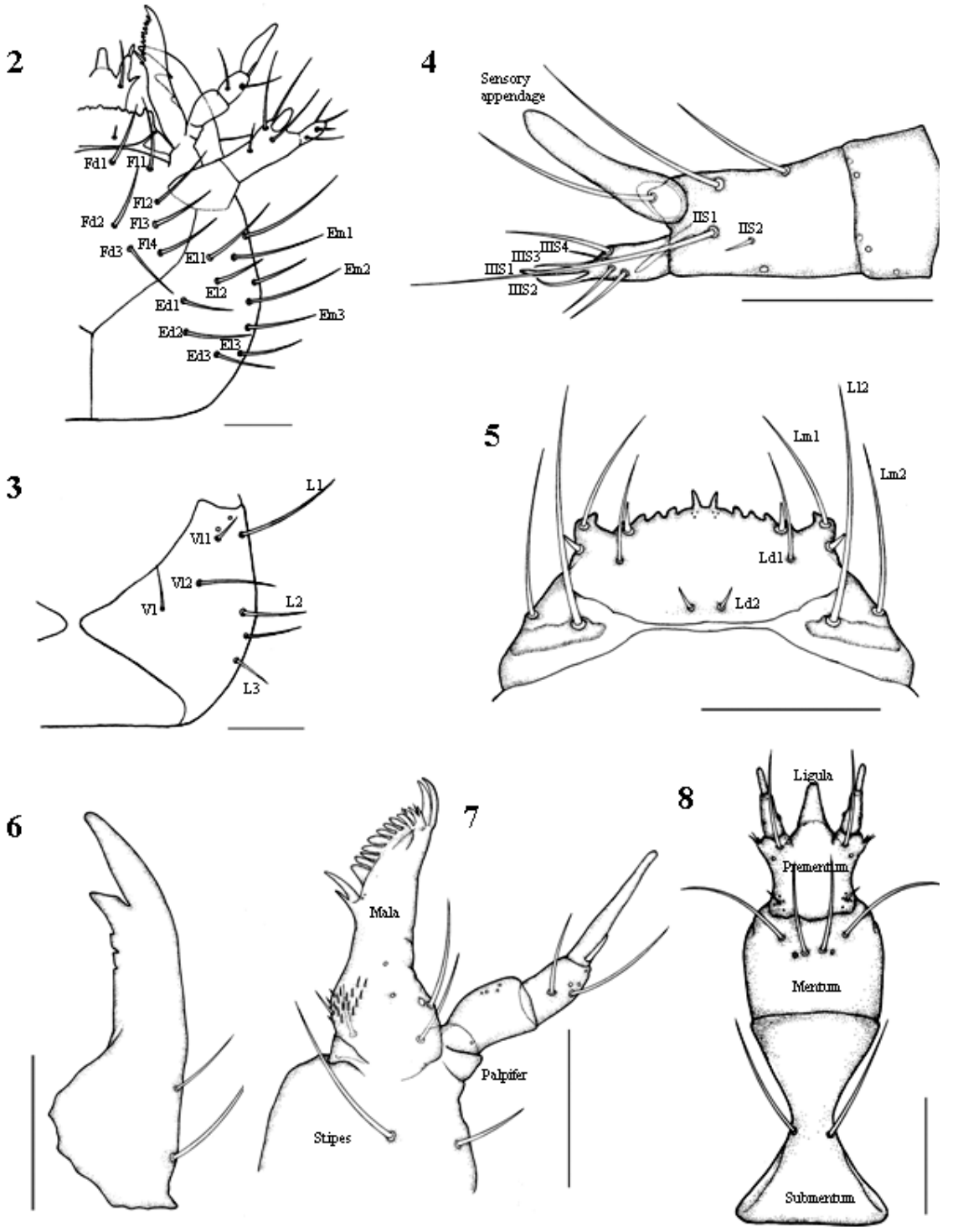

Figs. 2-8. *Bryothinusa koreana*. 2, Head, dorsal aspect; 3, head, ventral aspect; 4, antenna, dorsal aspect; 5, Labium, dorsal aspect; 6, mandible, dorsal aspect; 7, maxilla, dorsal aspect; 8, labrum, dorsal aspect. Scales = 0.1 mm.

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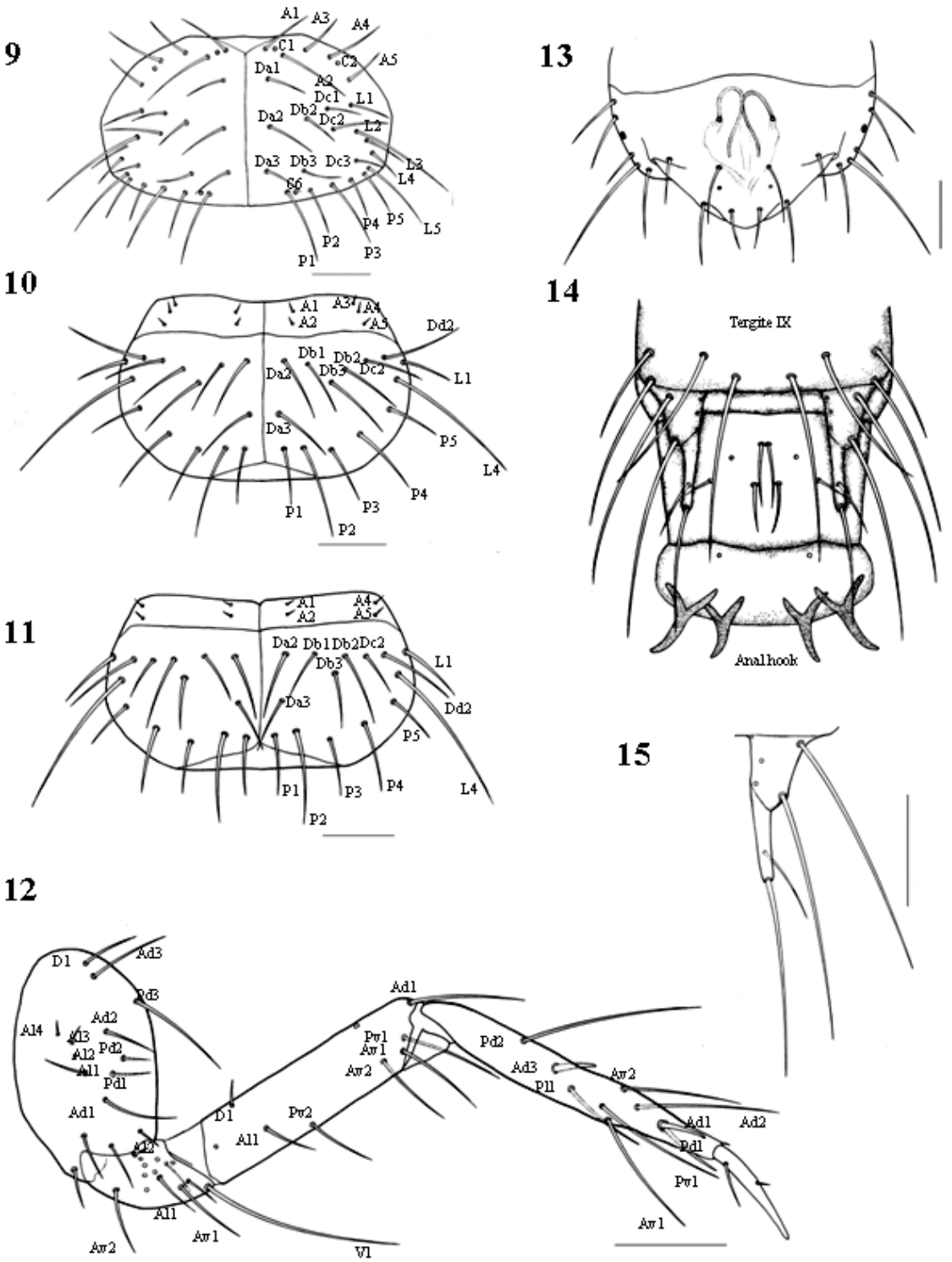

Figs. 9-15. *Bryothinusa koreana*. 9, Pronotum, dorsal aspect; 10, mesonotum, dorsal aspect; 11, metanotum, dorsal aspect; 12, anterior leg, lateral aspect; 13, sternite VIII, ventral aspect; 14, tergite IX and X, dorsal aspect; 15, urogomphi, dorsal aspect. Scales = 0.1 mm.

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TABLE 2. DIFFERENCES BETWEEN THE LATE INSTARS OF *B. KOREANA* AND *B. CATALINAE*.

	B. koreana	*B. catalinae*
Labium	2nd palpomere half of the 1st (Fig. 5)	2nd palpomere as long as 1st (Moore & Roth 1979: Fig. 4)
Maxilla	stipes as long as palpus	stipes longer than palpus
Mandibles	4 internal teeth (Fig. 6)	5 internal teeth (Moore & Roth 1979: Fig. 3)
Urogomphi	shorter than pseudopod (Fig. 14)	longer than pseudopod (Moore & Roth 1979: Fig. 8)
Urogomphi: article 1	cylindrical (Fig. 15)	broadly triangular (Moore & Roth 1979: Fig. 8)

lenidium, article 3 with 4 apical solenidia (IIIS1-IIIS4).

MOUTHPARTS. Labrum (Fig. 5) with 5 distinct setae (Ld1, Ld2, Ll1, Lm1, Lm2) and 1 pair of campaniform sensilla on each side; Ll1 and Lm2 distinctly separated from main body of labrum by a suture; anterior margin saw-like; Ld1 very short, Ld2 present. Mandibles (Fig. 6) right and left nearly identical in size and shape, form elongate, slender with broader lobe mesally at molar region; 4 progressively smaller teeth; lateral surface with 2 setae in basal half. Maxilla (Fig. 7) with stipes broad at base, not distinctly separated from mala, surface with 2 large setae, 1 on disk and 1 near lateral margin; mala with apex acute, comb-like setae on mesal region, with 4 distinct setae and small spinous setae on basal region. Maxillary palpus with 3 articles; article 1 and 2 nearly equal in length; article 1 about 0.8 times as long as wide; article 2 around 0.6 times as long as wide; article 3 as long as article 1 and 2 combined; article 2 with 2 campaniform sensilla and 2 setae. Labium (Fig. 8) consisting of distinctly separated prementum, mentum, and submentum; ligula elongate, labial palps with 2 articles, article 1 about 2.0 times as long as article 2; submentum with 1 pair of setae; mentum with 2 pairs of setae and 1 pair of campaniform sensilla; prementum with 2 pairs of setae and 1 pair of campaniform sensilla.

THORAX. Pronotum transverse, lightly sclerotized, chaetotaxy as in Fig. 9. Mesonotum transverse, chaetotaxy as in Fig. 10. Metanotum (Fig. 11) similar to mesonotum. Anterior leg as in Fig. 12.

ABDOMEN. Abdominal tergite I-VIII transverse. Sternite VIII as in Fig. 13. Urogomphi (Figs. 14 and 15) 2-articled; article 1 more or less retangular, article 2 slender with 1 small seta ventrally, and 1 large seta arising from apex. Abdominal segment X 0.35 times as wide at base as at apex. Apex of abdomen with 4 small chitinized anal hooks located ventrally.

SPECIMENS EXAMINED: Korea: Gyeongnam Prov., Geoje City, Gabae-ri, 30 VI 2000, K.-J. Ahn, *ex* under stone on beach (CNUIC, 11); 30 VI 2001, C.-W. Shin (CNUIC, 1).

REMARKS. The differences between late instars of *B. catalinae* and *B. koreana* are presented in Table 2.

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