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First Record of *Orobdella tsushimensis* (Hirudinida: Arhynchobdellida: Gastrostomobdellidae) from the Korean Peninsula and Molecular Phylogenetic Relationships of the Specimens

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ABSTRACT

Specimens of the genus *Orobdella* Oka, 1895 from Korea, including various locations in the Korean Peninsula, were identified as *Orobdella tsushimensis* Nakano, 2011. Phylogenetic analyses using mitochondrial cytochrome oxidase subunit 1 (COI), ND1, $tRNA^{Cys}$, $tRNA^{Met}$, 12S rRNA, $tRNA^{val}$, and 16S rRNA markers show that the newly collected specimens form a monophyletic group with the known *O. tsushimensis* specimens. The genetic distance of COI of these specimens was in the range 0.4−6.6%. These results confirm that the newly collected specimens belong to *O. tsushimensis*. This is the first record of the genus *Orobdella* from the Korean Peninsula.

Keywords: Hirudinida, Gastrostomobdellidae, *Orobdella tsushimensis*, molecular phylogeny, first record, Korean Peninsula

INTRODUCTION

The genus *Orobdella* Oka, 1895 is a terrestrial macrophagous leech taxon that is distributed in the Far East. At present 11 species of this genus have been described: 10 from Japan (Oka, 1895; Richardson, 1975; Nakano, 2010, 2011a, 2011b, 2012a, 2012b, 2012c); and the other from Taiwan (Nakano and Lai, 2012). Additionally, *Orobdella whitmani* Oka, 1895 has been reported from Primorsky Krai, Russia (Gilyarov et al., 1969), but the precise taxonomic status of the Russian specimens still remains uncertain (Nakano, 2012b). Yun (1997) provided a Korean leech list, but *Orobdella* leeches were not included in this list. Recently, quadrannulate *Orobdella tsushimensis* Nakano, 2011 has been recorded from Gageodo Island, which is located in the Yellow Sea, southwest of the Korean Peninsula (Nakano and Seo, 2012). However, *Orobdella* leeches have never been reported from the Korean Peninsula.

Recently, additional specimens of the quadrannulate *Orobdella* were collected from various places in Korea, including locations in the Korean Peninsula. This is the first record of the genus *Orobdella* from the peninsula. The identification and a description of the newly collected specimens are herein presented. In addition, their phylogenetic relationships are reconstructed using mitochondrial cytochrome oxidase subunit 1 (COI), ND1, $tRNA^{Cys}$, $tRNA^{Met}$, 12S rRNA, $tRNA^{val}$, and 16S rRNA ($tRNA^{Cys}$-16S) sequence data.

MATERIALS AND METHODS

Leeches were collected from various places in Korea (Fig. 1). Altitudes and coordinates for localities were obtained using a Garmin eTrex GPS unit.

For DNA extraction, botryoidal tissue was taken from the posterior part of the body around the caudal sucker of the specimens. All of the specimens were preserved in 70% ethanol. Four measurements were taken: body length (BL) from the anterior margin of the oral sucker to the posterior margin of the caudal sucker; maximum body width (BW); caudal

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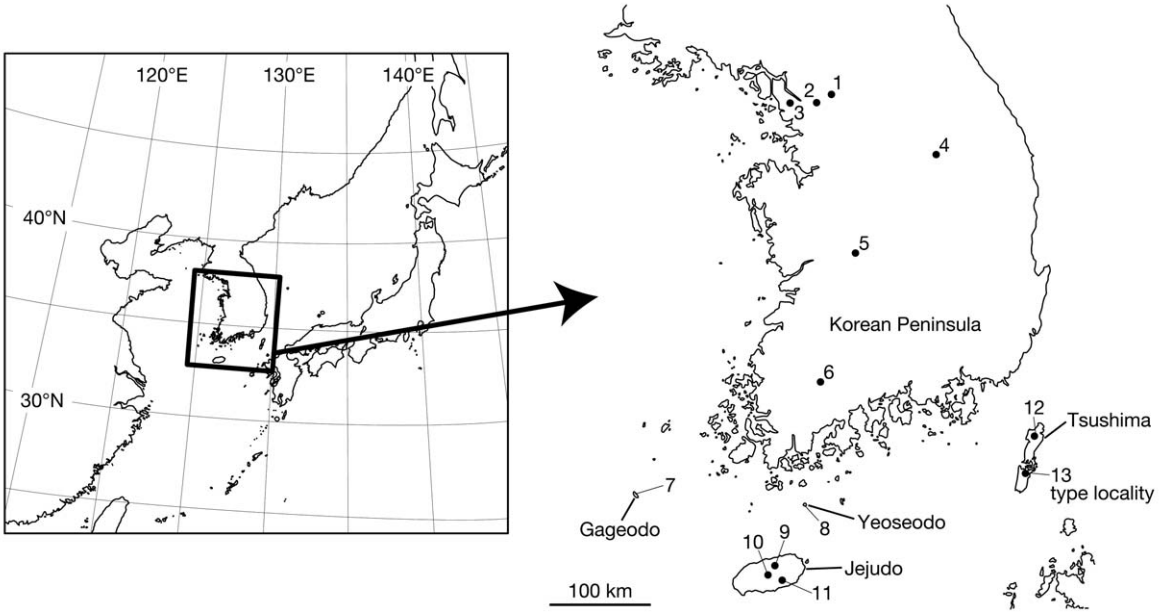

Fig. 1. Map showing the collection localities of the specimens examined in this study (nos. 1–11), Tsushima Island, Japan, and the type locality of *Orobdella tsushimensis* Nakano, 2011.

sucker length (CL) from the anterior to the posterior margin of the sucker; caudal sucker width (CW) from the right margin to the left margin of the sucker. Examination, dissection, and drawings of the specimens were accomplished using a stereoscopic microscope equipped with a drawing tube (Leica M125; Leica, Wetzlar, Germany). Specimens used in this study have been deposited in the Zoological Collection of Kyoto University (KUZ).

The numbering convention is based on Moore (1927): body somites are denoted by Roman numerals and the annuli in each somite are given alphanumeric designations.

The extraction of genomic DNA and DNA sequencing methods for COI and $tRNA^{Cys}$-16S followed Nakano (2012b). For obtaining $tRNA^{Leu}$ and ND1 ($tRNA^{Leu}$-ND1) sequence data, the procedure above was modified as follows: the primer sets for $tRNA^{Leu}$-ND1 were LND300 and HND1932 (Light and Siddall, 1999) or HND1932-in (5′-AATGGAGCACGA TTAGTTTC-3′) (in this study); the PCR reaction mixture for $tRNA^{Leu}$-ND1 was heated to 94°C for 5 min, followed by 35 cycles of 94°C (10 s), 45°C (20 s), and 72°C (39 s) and a final extension at 72°C for 6 min; the sequencing reaction mixture for $tRNA^{Leu}$-ND1 was incubated at 96°C for 2 min, followed by 40 cycles of 96°C (10 s), 50°C (5 s), and 60°C (39 s). Obtained sequences have been deposited in GenBank. Sequences used for molecular phylogenetic analyses are listed in Table 1; 16 sequences of COI, 3 those of $tRNA^{Leu}$-ND1 and 15 those of $tRNA^{Cys}$-16S, which were published in the previous studies (Nakano, 2012a, 2012b; Nakano and Lai, 2012; Nakano and Seo, 2012; Nakano and Gongalsky, 2014), were obtained from GenBank.

Sequences of mitochondrial COI and ND1 ($tRNA^{Leu}$ region was removed from each sequence) were aligned by eye because there were no indels. Mitochondrial $tRNA^{Cys}$-16S sequences were aligned using MAFFT L-INS-I (Katoh et al., 2005) and then refined with GBLOCKS (Castresana, 2000). The length of the aligned COI sequences was 1,266 bp, that of ND1 was 579 bp, and that of $tRNA^{Cys}$-16S was 818 bp (288 characters were eliminated). The concatenated sequences thus yielded a total of 2,663 bp positions. Pairwise comparisons of Kimura-2 parameter (K2P) distance (Kimura, 1980) of the COI sequences were calculated using MEGA5 (Tamura et al., 2011).

Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI) models. ML phylogenies were calculated using TREEFINDER v October 2008 (Jobb et al., 2004) with the tool package PHYLOGEARS v 2.0 (Tanabe, 2008), and then nonparametric bootstrapping (Felsenstein, 1985) was conducted with 500 replicates. The best-fit models for each partition were selected based on the Akaike Information Criterion (Akaike, 1974) by using KAKUSAN4 (Tanabe, 2011): for the 1st position of COI, TN93 with gamma distribution (+G) and proportion of invariant sites (+I); for the 2nd position of COI, TVM+I; for the 3rd position of COI, HKY85+G; for the 1st position of ND1, GTR+G+I; for the 2nd position of ND1, J1+G; for the 3rd position of ND1, J2+G; and for

Table 1. Samples used for the phylogenetic analyses, with the information on voucher and GenBank accession numbers

Voucher	Locality (no.)	COI	$tRNA^{Leu}$-ND1	$tRNA^{Cys}$-16S	Voucher	Locality	COI	$tRNA^{Leu}$-ND1	$tRNA^{Cys}$-16S
Orobdella tsushimensis					*Orobdella ketagalan*				
KUZ Z616	Seoul, KOR (1)	AB828566	AB828545	AB828574	KUZ Z208 (H)	Taipei, TWN	AB704787[a]	AB828563	AB828582
KUZ Z618	Gwangju, KOR (6)	AB828567	AB828546	AB828575	*Orobdella koikei*				
KUZ Z620	Jejudo, KOR (10)	AB828568	AB828547	AB828576	KUZ Z156 (H)	Hokkaido, JPN	AB679688[b]	AB828560[e]	AB679689[b]
KUZ Z622	Seoul, KOR (2)	AB828569	AB828548	AB828577	*Orobdella mononoke*				
KUZ Z625	Incheon, KOR (3)	AB828570	AB828549	AB828578	KUZ Z224 (H)	Yakushima, JPN	AB698866[c]	AB828564	AB698867[c]
KUZ Z626	Gangwon, KOR (4)	AB828571	AB828550	AB828579	*Orobdella octonaria*				
KUZ Z627	Daejeon, KOR (5)	AB828572	AB828551	AB828580	KUZ Z181 (T)	Kanagawa, JPN	AB679708[b]	AB828562	AB679709[b]
KUZ Z628	Yeoseodo, KOR (8)	AB828573	AB828552	AB828581	*Orobdella shimadae*				
KUZ Z215	Gageodo, KOR (7)	AB693165[d]	AB828565	AB693166[d]	KUZ Z128 (H)	Okinawajima, JPN	AB679676[b]	AB828557	AB679677[b]
KUZ Z133	Tsushima, JPN (12)	AB679660[b]	AB828553	AB679661[b]	*Orobdella whitmani*				
KUZ Z134 (H)	Tsushima, JPN (13)	AB679662[b]	AB828554	AB679663[b]	KUZ Z45 (T)	Gifu, JPN	AB679668[b]	AB828556[e]	AB679669[b]
Orobdella esulcata					Outgroup				
KUZ Z29 (H)	Kumamoto, JPN	AB679664[b]	AB828555	AB679665[b]	*Erpobdella japonica* Pawłowski, 1962				
Orobdella dolichopharynx					KUZ Z178	Nagano, JPN	AB679654[b]	AB828542	AB679655[b]
KUZ Z120 (H)	Amamioshima, JPN	AB679680[b]	AB828558	AB679681[b]	*Gastrostomobdella monticola* Moore, 1929				
Orobdella ijimai					UNIMAS/A3/BH01/10	Kuching, MYS	AB679656[b]	AB828543	AB679657[b]
KUZ Z110 (T)	Tochigi, JPN	AB679672[b]	AB828559	AB679673[b]	*Mimobdella japonica* Blanchard, 1897				
Orobdella kawakatsuorum					KUZ Z179	Amamioshima, JPN	AB679658[b]	AB828544	AB679659[b]
KUZ Z167 (T)	Hokkaido, JPN	AB679704[b]	AB828561[e]	AB679705[b]					

Sources: [a]Nakano and Lai(2012); [b]Nakano(2012b); [c]Nakano(2012a); [d]Nakano and Seo(2012); [e]Nakano and Gongalsky(2014). For the locality number, see Fig. 1.
COI, cytochrome oxidase subunit 1; KOR, Korea; H, holotype; TWN, Taiwan; JPN, Japan; T, topotype; UNIMAS, The Universiti Malaysia Sarawak; MYS, Malaysia.

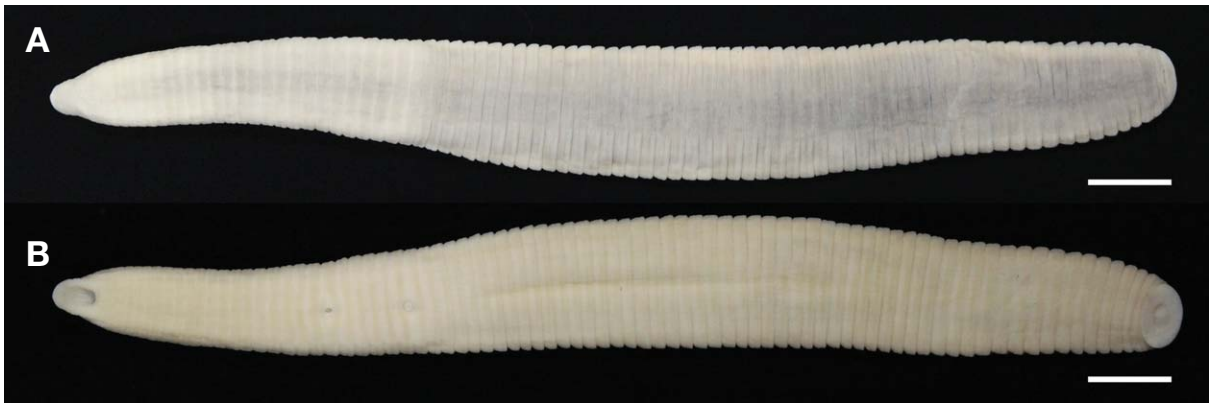

Fig. 2. *Orobdella tsushimensis* Nakano, 2011 from Seoul, KUZ Z616. A, Dorsal view; B, Ventral view. Scale bars: A, B=5 mm.

tRNA^{Cys}-16S, GTR+G was selected. BI and Bayesian posterior probabilities (BPPs) were estimated using MRBAYES v 3.2 (Ronquist et al., 2012). The best-fit models for each partition were identified under the Bayesian information criterion (Schwarz, 1978) and also by using KAKUSAN4: for the 1st position of COI, GTR+G+I; for the 2nd position of COI, F81+I; for the 3rd position of COI, HKY85+G; for the 1st position of ND1, GTR+G+I; for the 2nd position of ND1, HKY85+G; for the 3rd position of ND1, HKY85+G; and for tRNA^{Cys}-16S, GTR+G was selected. Two independent runs of four Markov chains were conducted for 4 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using TRACER v 1.5 (Rambaut and Drummond, 2009), and based on these results, the first 10,001 trees were discarded.

Nodes with bootstrap (BS) values higher than 70% were considered as sufficiently resolved (Hillis and Bull, 1993). Nodes with BPPs higher than 95% were considered statistically significant (Leaché and Reeder, 2002).

SYSTEMATIC ACCOUNTS

Order Arhynchobdellida Blanchard, 1894
Suborder Erpobdelliformes Sawyer, 1986
[1]*Family Gastrostomobdellidae Richardson, 1971
[2]*Genus *Orobdella* Oka, 1895

[3]****Orobdella tsushimensis*** **Nakano, 2011 (Figs. 2, 3)**

Orobdella tsushimensis Nakano, 2011a: 41–46, figs. 2–4 (near the Orobashi Bridge at Kechi Dam, alt. 69 m, 34° 15′17″N, 129° 17′17″E, Tsushima, Tsushima Island, Nagasaki Prefecture, Japan, holotype, KUZ Z134); Nakano and Seo, 2012: 237–239, figs. 2–4.

Material examined (see Fig. 1 for the locality number). Korea: 3 specimens, Seoul: Nowon-gu, Mt. Suraksan, alt. 118 m, 37° 41′08″N, 127° 04′07″E (locality no. 1), 4 Jul 2012, Seo HY, KUZ Z615, Z616, dissected, and KUZ Z617; 2 specimens, Jongno-gu, Mt. Bukhansan, alt. 447 m, 37° 37′ 28″N, 126° 57′41″E (locality no. 2), 6 Jul 2012, Seo HY, KUZ Z621 and Z622, dissected; 2 specimens, Jeollanam-do: Sinan-gun, Gageodo Island, alt. 429 m, 34° 04′49″N, 125° 96′27″E, (locality no. 7), 10 Jul 2012, Seo HY, KUZ Z623 and Z624; 1 specimen, Wando-gun, Yeoseodo Island, alt. 46 m, 33° 59′03″N, 126° 55′19″E (locality no. 8), 20 Jul 2012, Seo HY, KUZ Z628, dissected; 1 specimen, Jejudo Island: Jeju-si, alt. 384 m, 33° 26′47″N, 126° 33′49″E (locality no. 9), 17 Jun 2012, Seo HY, KUZ Z614; 1 specimen, Seogwipo-si, alt. 183 m, 33° 18′05″N, 126° 37′51″E (locality no. 11), 27 Sep 2012, Seo HY, KUZ Z629; 1 specimen, Jeju-si, Mt. Hanlasan, alt. 1,601 m, 33° 21′56″N, 126° 30′48″E (locality no. 10), 2 Jul 2012, Kang SG, KUZ Z620, dissected; 1 specimen, Gwangju Metropolitan City: Dong-gu, Mt. Mudeungsan, alt. 533 m, 35° 07′11″N, 126° 58′56″E (locality no. 6), 1 Jul 2012, Seo HY, KUZ Z618, dissected; 1 specimen, Dong-gu, Mt. Mudeungsan, alt. 375 m, 35° 07′21″N, 126° 58′34″E (locality no. 6), 24 Jun 2012, Seo HY, KUZ Z619; 1 specimen, Incheon Metropolitan City: Gyeyang-gu, Mt. Gyeyangsan, alt. 63 m, 37° 33′33″N, 126° 43′30″E (locality no. 3), 11 Oct 2012, Seo HY, KUZ Z625, dissected; 1 specimen, Gangwon-do: Wonju-si, Mt. Chiaksan, alt. 790 m, 37° 18′07″N, 128° 03′39″E (locality no. 4), 5 Oct 2012, Seo HY, KUZ Z626, dissected; 1 specimen, Daejeon Metropolitan City: Seo-gu, Mt. Changtaesan, alt. 245 m, 36° 11′38″N,

Korean name: [1]*왕산거머리과, [2]*왕산거머리속, [3]*왕산거머리

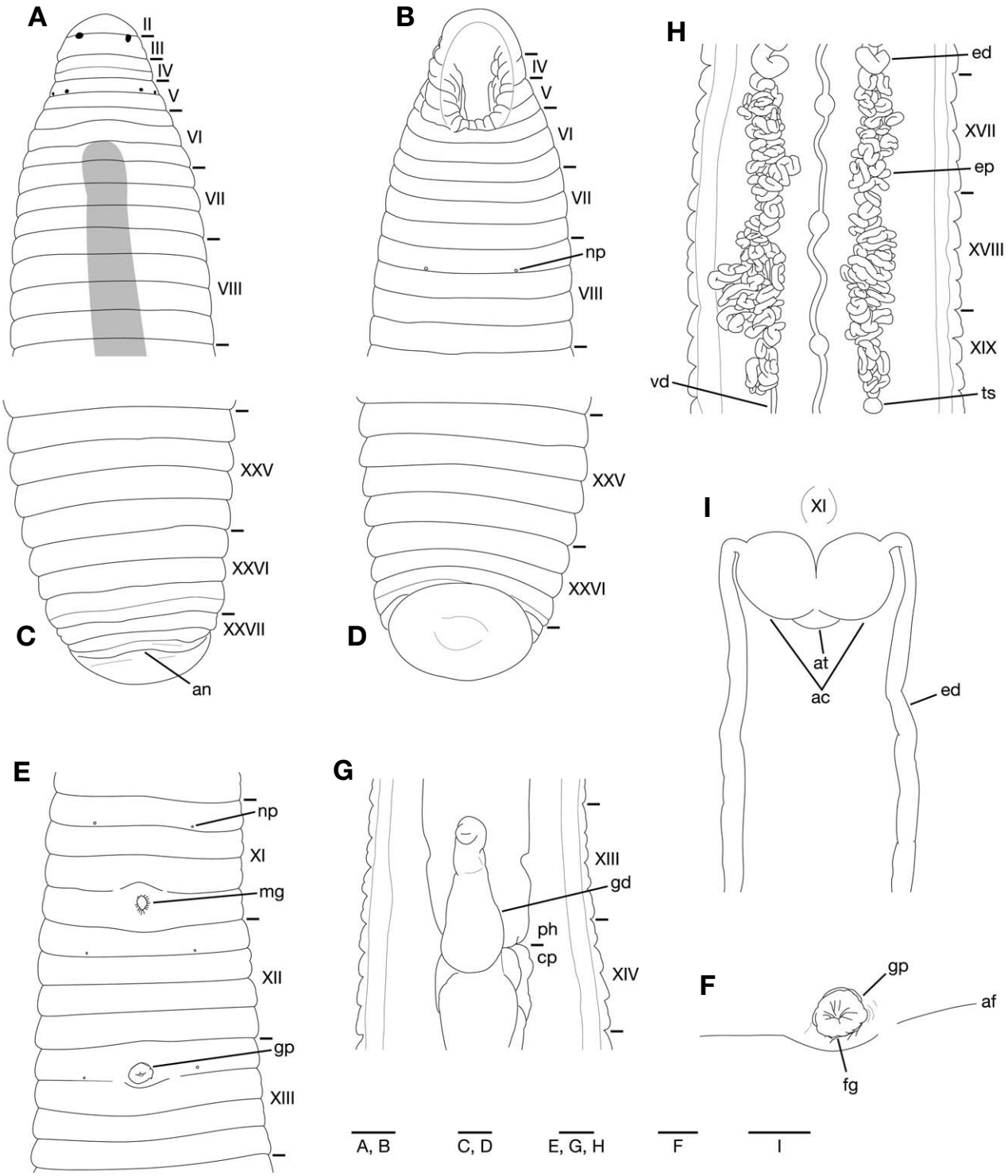

Fig. 3. *Orobdella tsushimensis* Nakano, 2011 from Seoul, KUZ Z616. A, Dorsal view of somites I–VIII; B, Ventral view of somites I–VIII; C, Dorsal view of somites XXV–XXVII and caudal sucker; D, Ventral view of somites XXV–XXVII and caudal sucker; E, Ventral view of somites XI–XIII; F, Ventral view of gastropore and female gonopore; G, Ventral view of gastroporal duct; H, Dorsal view of epididymides and ventral nervous system; I, Dorsal view of male atrium showing position of ganglion XI. ac, atrial cornua; af, annular furrow; an, anus; at, atrium; cp, crop; ed, ejaculatory duct; ep, epididymis; fg, female gonopore; gd, gastroporal duct; gp, gastropore; mg, male gonopore; np, nephridiopore; ph, pharynx; ts, testisac; vd, vas deferens. Scale bars: A–E, G–I=1 mm, F=0.5 mm.

127° 20′19″E (locality no. 5), 5 Oct 2012, Seo HY, KUZ Z627, dissected.

Description. Maximum BL 105.4 mm (KUZ Z622), BW 11.9 mm (KUZ Z628), minimum BL 33.2 (KUZ Z626), BW 4.3 mm (KUZ Z619). Caudal sucker ventral, ellipsoid, maximum CL 3.9 mm, CW 6.6 mm (KUZ Z622), minimum CL 1.6 mm (KUZ Z624), CW 2.0 mm (KUZ Z626).

Somites VIII–XXV quadrannulate, somite XXVI triannulate (Fig. 3A–E). Anus behind somite XXVII with no postanal annulus (Fig. 3C). Clitellum in XI b5 to XIII a2. Eyes often in 3 pairs, first pair dorsally in posterior margin of II, second and third pairs dorsolaterally in posterior margin of V (a1+a2) (Fig. 3A). Nephridiopores in 17 pairs in VIII–XXIV (Fig. 3B, E).

Pharynx reaching to XIII b5/b6-XIV b6 (Fig. 3G); each pharyngeal ridges reaching to crop straightly. Crop tubular, reaching to XIX b5/b6-XX a1/a2. Gastropore conspicuous in XIII a1/a2 (Fig. 3E, F). Gastroporal duct developed, bulbous, or bottle-shaped, slightly winding at junction with gastropore; joining with crop in XIII b6-XIV b6 (Fig. 3G).

Male gonopore in XI b6 (slightly anterior to middle of annulus), female gonopore inconspicuous in XIII a1/a2, behind gastropore, gonopores separated by 1/2+5 annuli (Fig. 3E, F). Testisacs multiple, in XVIII b5-XX b6 to XXIV a1-XXVb6 (Fig. 3H). Paired epididymides in XV b5-XVIII a1/a2 to XVIII b5-XX a1/a2 (Fig. 3H). Ejaculatory ducts in XI b5 to XV b5-XVIII a1/a2; nearly straight in position anterior to ovisacs (Fig. 3I); coiled in position posterior to ovisacs (Fig. 3H). Pair of muscular atrial cornua, ovate in XI b5 and b6 (Fig. 3I) or fusiform in XI b6. Atrium short, muscular, globular, in XI b5 and b6. One pair of ovisacs, thin-walled, globular, in XIII a2 and b5. Oviducts thin-walled, both oviducts converging into common oviduct in XIII a2.

Distribution. Known from mountainous regions of the Korean Peninsula and its peripheral islands including Tsushima Island, Japan (Nakano, 2011a; Nakano and Seo, 2012).

Phylogenetic relationships (see Fig. 1 for the locality number). The ML tree with ln L=−17134.56 (Fig. 4) was identical in topology to the obtained BI tree (not shown). Monophyly of the genus *Orobdella* was confirmed (BS=100%, BPP=100%). Although *O. tsushimensis* formed a monophyletic lineage with the clade that consists of ([*O. esulcata* Nakano, 2010+*O. mononoke* Nakano, 2012]+[*O. ketagalan* Nakano and Lai, 2012+{*O. dolichopharynx* Nakano, 2011+*O. shimadae* Nakano, 2011}]), the phylogenetic position of *O. tsushimensis* was not statistically resolved (BS=45%, BPP=86%).

Monophyly of *O. tsushimensis* was confirmed (BS=100%, BPP=100%). Within *O. tsushimensis*, three lineages were recognized and defined. Lineage A contained only KUZ Z626 from Mt. Chiaksan, Gwangwon (locality no. 4). Lineage B consisted only of KUZ Z627 from Mt. Changtaesan, Daejeon (no. 5). Lineage C consisted of the other nine specimens. Lineages B and C formed a monophyletic lineage

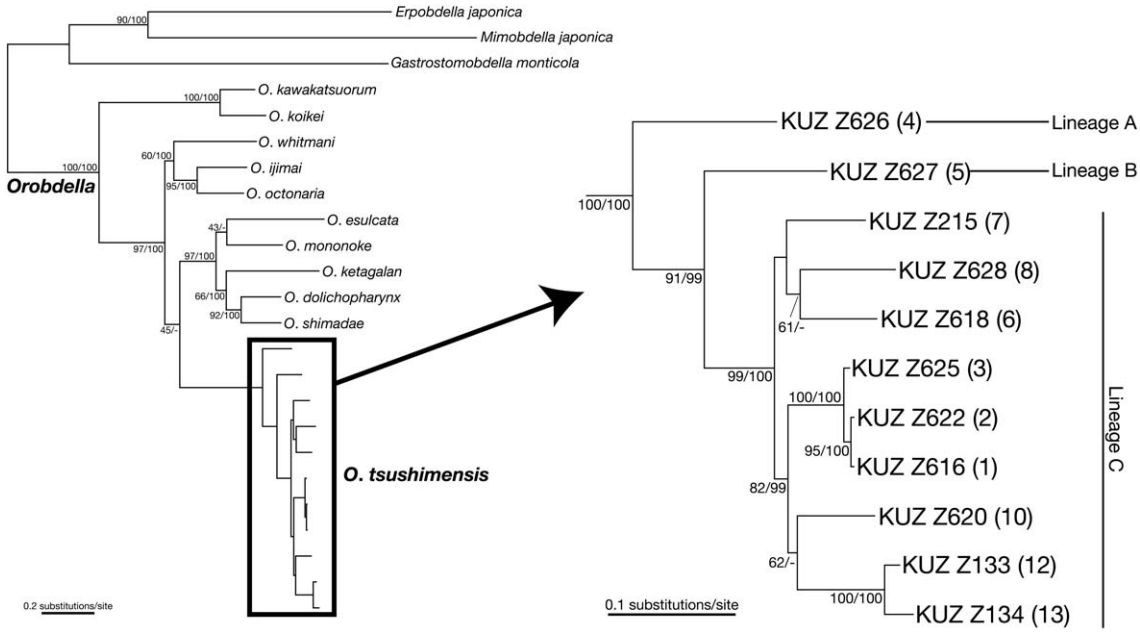

Fig. 4. The maximum likelihood (ML) tree of 2,663 bp of cytochrome oxidase subunit 1 (COI), ND1, $tRNA^{Cys}$, $tRNA^{Met}$, 12S rRNA, $tRNA^{val}$, and 16S rRNA. The numbers associated with the nodes represent bootstrap values for ML (BS)/and Bayesian posterior probabilities (BPPs). BSs higher than 40% and/or BPPs higher than 95% are indicated. For the locality number, see Fig. 1 and Table 1.

(BS=91%, BPP=99%). Within Lineage C, a monophyletic lineage (BS=100%, BPP=100%) of three specimens, KUZ Z616 (no. 1), Z622 (no. 2) and Z625 (no. 3) from around Seoul, KUZ Z620 from Jejudo Island (no. 10) and a monophyletic lineage (BS=100%, BPP=100%) of two specimens, KUZ Z133 (no. 12) and Z134 (no. 13), from Tsushima Island formed a monophyletic lineage (BS=82%, BPP=99%). KUZ Z215 (no. 7), Z618 (no. 6) and Z628 (no. 8) from the southwestern part of Korea, including Gageodo and Yeoseodo Islands, also formed a monophyletic lineage, but with low support values (BS=37%, BPP=58%).

Genetic divergence. The COI K2P distance within *O. tsushimensis* was between 0.4–6.6% (mean=5.0%).

Remarks. The present specimens newly collected from various places in Korea clearly belong to *O. tsushimensis* based on their possession of the following characteristics: somites VIII–XXV quadrannulate; gastropore conspicuous in XIII a1/a2; gastroporal duct developed, bulbous; male gonopore in middle of XI b6, female gonopore in XIII a1/a2, behind gastropore, gonopores separated by 1/2+5 annuli; paired epididymides in XV–XX, occupying more than 2 somites; atrial cornua developed, ovate or conical. One specimen, KUZ Z627, possesses tubular gastroporal duct and fusiform atrial cornua. However, these characteristics of this specimen seemed to be due to its immaturity, since this individual has undeveloped sperm ducts and undetectable testisacs. In Nakano and Seo (2012), *O. tsushimensis* has been diagnosed as having epididymides in XVII–XIX (occupying approximately 2 somites). However, the present specimens have their epididymides in somites XV–XX (occupying 8–12 annuli, i.e., 2–3 somites). Therefore, the diagnosis in Nakano and Seo (2012) should be amended to take this difference into account.

The molecular phylogenies obtained here showed that the specimens that were morphologically identified as *O. tsushimensis* form a well-supported monophyletic group. Additionally, the COI sequence divergence within these specimens was between 0.4–6.6% (mean=5.0%). For comparison, the intraspecific variation of COI sequences of *O. koikei* in Hokkaido, Japan was between 4.8–8.1% (mean=7.1%) (Nakano, 2012b). Thus, our molecular phylogenetic analyses and genetic distance data strongly support our morphological identification result that the *Orobdella* leeches newly collected in this study belong to *O. tsushimensis*.

The present molecular phylogenies showed that the specimen from Mt. Chiaksan (Lineage A; locality no. 4) first diverged from the others. Then, the specimen from Mt. Changtaesan (Lineage B; no. 5) diverged from the other leeches. It is noteworthy that the specimen from Jejudo Island (no. 10) and two specimens from Tsushima Island (nos. 12, 13) are monophyletic, which was statistically supported, with the three specimens from locations around Seoul (nos. 1–3), not with the specimens from adjacent localities (i.e., nos. 6–8). This geographic pattern of the phylogenetic relationships suggests a complex biogeographical history of *O. tsushimensis*. However, the present phylogenies failed to reconstruct fully robust phylogenetic relationships within Lineage C. Additional specimens of *O. tsushimensis* as well as suitable genetic markers are still needed to clarify the precise phylogenetic relationships and biogeographical history of this species.

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