

Reassessing the Taxonomic Subdivision of the *Jesogammarus jesoensis* Complex (Crustacea: Amphipoda: Anisogammaridae) in Northern and Central Japan

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The taxonomic subdivision of certain Japanese freshwater amphipods into *Jesogammarus jesoensis* (Schellenberg, 1937), *J. hokurikuensis* Morino, 1985, *J. fujinoi* Tomikawa and Morino, 2003, and *J. shonaiensis* Tomikawa and Morino, 2003 was reassessed using both morphological and molecular data. Putative diagnostic morphological characters did not exhibit consistent geographic distributions, but eight geographically consistent monophyletic clades were detected based on COI and COI+12S rRNA sequence data. Uncorrected COI nucleotide divergences between the clades ranged from 7.0% to 16.4%, a level usually considered to represent inter-specific differences in crustaceans. No morphological features that differentiate the molecular clades were found. While the formal nomenclatural handling of these findings remains a problem for the future, for now we suggest the use of the term “*J. jesoensis* complex” to represent taxonomic entity consisting of the nominal taxa *J. jesoensis*, *J. fujinoi*, *J. shonaiensis*, and *J. hokurikuensis*, and potentially a number of hitherto cryptic taxa as well.

Key Words: *Jesogammarus jesoensis*, Amphipoda, COI, 12S rRNA, species complex, freshwater, phylogeny, taxonomy, Japan.

Introduction

The anisogammarid genus *Jesogammarus* is distributed in fresh and brackish waters of Japan, Korea, and China, and currently 17 species in two subgenera, *Jesogammarus* and *Annanogammarus*, have been described (Morino 1985; Tomikawa 2007; Tomikawa and Morino 2012). Four Japanese species, *J. (J.) jesoensis* (Schellenberg, 1937), *J. (J.) hokurikuensis* Morino, 1985, *J. (J.) fujinoi* Tomikawa and Morino, 2003, and *J. (J.) shonaiensis* Tomikawa and Morino, 2003, inhabit cold freshwaters and their distributions have been described as allopatric: *J. jesoensis* is widely distributed in Hokkaido and northern Honshu (Morino 1985) while *J. hokurikuensis*, *J. fujinoi*, and *J. shonaiensis* are restricted to northern Honshu (Morino 1985; Tomikawa and Morino 2003). These four species are morphologically similar and share the following characteristics: a few (two or three) clusters of setae on the posterior margins of peduncular articles 4 and 5 of antenna 2, no robust setae on palp article 1 of the mandible, the presence of serrate robust setae on the palmar margin of female gnathopod 2, the posterior accessory lobe of the coxal gills being subequal to or longer than the anterior one, and the absence of a pair of setae on the posterodorsal margins of pereonites 5 to 7 (Schellenberg 1937; Morino

1985; Tomikawa and Morino 2003; Tomikawa *et al.* 2003). They have been distinguished from each other by different combinations of the following morphological characters: the dorsal armature of the pleonites, the dorsal armature of the urosomites, and the armature of the outer ramus of uropod 2 (Morino 1985; Tomikawa and Morino 2003). Kusano and Ito (2003), however, reported high morphological variation in these three supposedly diagnostic characters between and within populations of *J. jesoensis* in Hokkaido. This renders the morphological definitions of *J. jesoensis*, *J. hokurikuensis*, *J. fujinoi*, and *J. shonaiensis* unsatisfactory, and the taxonomic validity of the latter three species doubtful. However, no targeted study has been conducted to reassess the taxonomic relationships among these four nominal species.

Recently, it has been widely recognized that mitochondrial DNA characters are useful in the classification of amphipods (Hou *et al.* 2007; Tomikawa *et al.* 2007, 2012, 2014a, b). In this study, we investigated the morphological variation of the supposedly diagnostic characters together with partial sequences of the cytochrome *c* oxidase subunit I (COI) and 12S rRNA genes in the mitochondrial genome to reassess the taxonomic validity and practical delineation of the four species *J. jesoensis*, *J. hokurikuensis*, *J. fujinoi*, and *J. shonaiensis*.

Materials and Methods

Sampling. A sixty individuals in total were collected from five *Jesogammarus* (*Jesogammarus*) populations in Hokkaido and nine in Honshu, from a geographical range that roughly covered the supposed distributions of *J. jesoensis*, *J. hokurikuensis*, *J. fujinoi*, and *J. shonaiensis* (Fig. 1, Table 1). Specimens collected from the type localities of *J. fujinoi*

and *J. shonaiensis*, and from Takinami, Fukui city, Fukui Prefecture, which is close to the type locality of *J. hokurikuensis* (a spring brook at Shimizu, Niu; currently in the city of Fukui), were included in this study. Schellenberg (1937) initially described *J. jesoensis* based on specimens from “Jeso (Ezo)” which is the old name of Hokkaido, but he did not indicate the precise locality there. To allow for this uncertainty, specimens collected from several localities in Hokkaido were examined in the present study. Since unequivocal

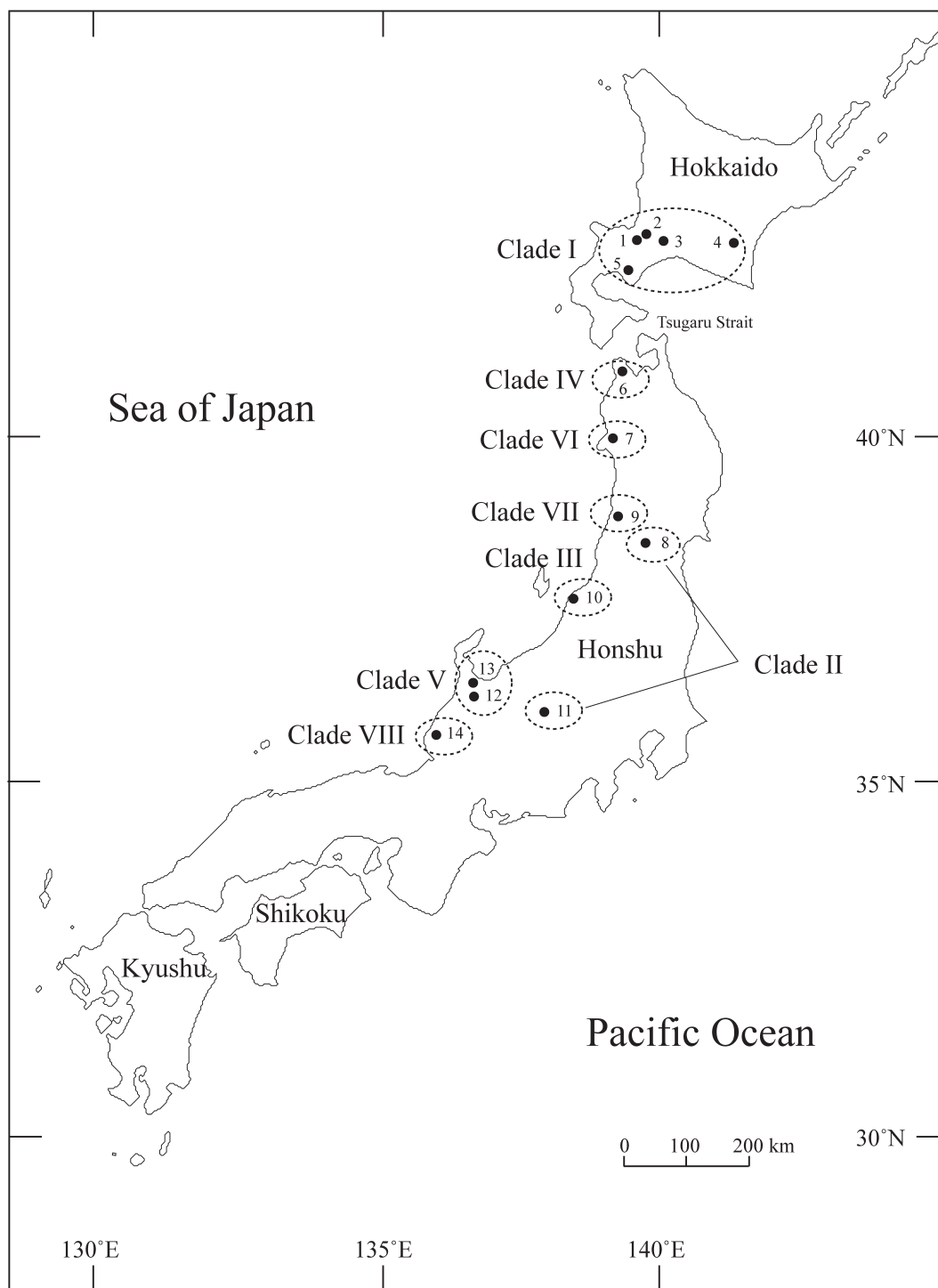


Fig. 1. Localities of sampling sites of *Jesogammarus jesoensis* complex in Japan. Numerals 1 to 14 refer to the sample sites in Table 1. Clade numbers I to VIII near sites enclosed with dashed ovals the same as in Figs 3–5.

Table 1. Specimens, population numbers, localities, and habitats of *Jesogammarus jesoensis* complex amphipods examined in this study. PN: pleonite, UM: urosomite, UP: uropod. Clades I to VIII correspond to those in Figs 3 and 4. PN and UM types are as shown in Fig. 2. AB numbers in the right hand column are DDBJ DNA sequence accession number.

Individual code number or species	Map locality (Fig. 1)	Sampling site	Habitat	PN type	UM type	UP2, robust setae on outer ramus	Clade no.	12S	COI
164	1	Hokkaido Univ., Sapporo, Hokkaido, Japan	spring brook	2	S	absent	I	AB973883	AB974366
165	1	Hokkaido Univ., Sapporo, Hokkaido, Japan	spring brook	2	S	absent	I	—	AB974367
166	1	Hokkaido Univ., Sapporo, Hokkaido, Japan	spring brook	3	A	absent	I	—	AB974368
167	1	Hokkaido Univ., Sapporo, Hokkaido, Japan	spring brook	2	A	absent	I	AB973884	AB974369
168	1	Hokkaido Univ., Sapporo, Hokkaido, Japan	spring brook	2	S	absent	I	AB973885	AB974370
206	2	Nopporo, Sapporo, Hokkaido, Japan	spring brook	2	A	absent	I	AB973886	AB974371
207	2	Nopporo, Sapporo, Hokkaido, Japan	spring brook	2	S	absent	—	—	—
208	2	Nopporo, Sapporo, Hokkaido, Japan	spring brook	2	S	absent	I	AB973887	AB974372
209	2	Nopporo, Sapporo, Hokkaido, Japan	spring brook	2	S	absent	I	AB973888	AB974373
169	3	Rankoshi, Eniwa, Hokkaido, Japan	spring brook	2	A	absent	I	—	—
170	3	Rankoshi, Eniwa, Hokkaido, Japan	spring brook	2	A	absent	I	—	—
171	3	Rankoshi, Eniwa, Hokkaido, Japan	spring brook	2	A	absent	I	AB973889	AB974374
172	3	Rankoshi, Eniwa, Hokkaido, Japan	spring brook	2	A	absent	I	—	AB974375
173	3	Rankoshi, Eniwa, Hokkaido, Japan	spring brook	2	A	absent	I	—	—
194	4	Obihiro, Hokkaido, Japan	spring brook	0	A	absent	I	AB973890	AB974376
195	4	Obihiro, Hokkaido, Japan	spring brook	1	S	absent	I	AB973891	AB974377
196	4	Obihiro, Hokkaido, Japan	spring brook	0	S	absent	I	AB973892	AB974378
197	4	Obihiro, Hokkaido, Japan	spring brook	1	S	present	—	—	—
198	4	Obihiro, Hokkaido, Japan	spring brook	1	A	absent	I	AB973893	AB974379
204	5	Lake Toya, Hokkaido, Japan	lake shore	3	A	absent	I	AB973894	AB974380
205	5	Lake Toya, Hokkaido, Japan	lake shore	2	S	absent	I	AB973895	AB974381
215	6	Nakazato, Aomori Pref., Japan	spring brook	2	A	present	IV	AB973896	AB974382
216	6	Nakazato, Aomori Pref., Japan	spring brook	2	A	absent	IV	AB973897	AB974383
217	6	Nakazato, Aomori Pref., Japan	spring brook	2	A	absent	IV	AB973898	AB974384
218	6	Nakazato, Aomori Pref., Japan	spring brook	2	C	present	IV	AB973899	AB974385
219	6	Nakazato, Aomori Pref., Japan	spring brook	2	A	absent	IV	AB973900	AB974386
210	7	Kotooka, Akita Pref., Japan	spring brook	2	A	present	VI	AB973901	AB974387
211	7	Kotooka, Akita Pref., Japan	spring brook	2	A	present	VI	—	AB974388
212	7	Kotooka, Akita Pref., Japan	spring brook	2	A	absent	VI	AB973902	AB974389
213	7	Kotooka, Akita Pref., Japan	spring brook	2	A	absent	—	—	—
214	7	Kotooka, Akita Pref., Japan	spring brook	2	A	present	—	—	—
184	8	Gobanmiki, Yamagata, Yamagata Pref., Japan*	spring	0	C	absent	—	—	—
185	8	Gobanmiki, Yamagata, Yamagata Pref., Japan*	spring	0	C	absent	II	—	AB974390
186	8	Gobanmiki, Yamagata, Yamagata Pref., Japan*	spring	0	C	absent	—	—	—
187	8	Gobanmiki, Yamagata, Yamagata Pref., Japan*	spring	0	C	absent	—	—	—
188	8	Gobanmiki, Yamagata, Yamagata Pref., Japan*	spring	0	C	absent	II	AB973903	AB974391
189	9	Kuromori, Sakata, Yamagata Pref., Japan**	spring brook	3	S	absent	—	—	—
190	9	Kuromori, Sakata, Yamagata Pref., Japan**	spring brook	3	S	absent	VII	—	AB974392
191	9	Kuromori, Sakata, Yamagata Pref., Japan**	spring brook	3	S	absent	—	—	—
192	9	Kuromori, Sakata, Yamagata Pref., Japan**	spring brook	3	S	absent	VII	AB973904	AB974393
193	9	Kuromori, Sakata, Yamagata Pref., Japan**	spring brook	3	S	absent	—	—	—
199	10	Honsagata, Niigata, Niigata Pref., Japan	spring brook	3	S	present	III	AB973905	AB974394
200	10	Honsagata, Niigata, Niigata Pref., Japan	spring brook	3	A	present	III	AB973906	AB974395
201	10	Honsagata, Niigata, Niigata Pref., Japan	spring brook	3	S	present	—	—	—
202	10	Honsagata, Niigata, Niigata Pref., Japan	spring brook	3	S	absent	—	—	—
203	10	Honsagata, Niigata, Niigata Pref., Japan	spring brook	3	S	absent	—	—	—
179	11	Azumino, Nagano Pref., Japan	spring brook	2	C	absent	II	—	AB974396
180	11	Azumino, Nagano Pref., Japan	spring brook	2	C	absent	—	—	—
181	11	Azumino, Nagano Pref., Japan	spring brook	2	C	absent	—	—	—
182	11	Azumino, Nagano Pref., Japan	spring brook	2	C	absent	II	AB973907	AB974397
183	11	Azumino, Nagano Pref., Japan	spring brook	2	C	absent	II	—	AB974398
174	12	Taira, Toyama Pref., Japan	spring brook	2	C	present	—	—	—
175	12	Taira, Toyama Pref., Japan	spring brook	1	C	present	—	—	—
176	12	Taira, Toyama Pref., Japan	spring brook	2	C	present	V	AB973908	AB974399
177	12	Taira, Toyama Pref., Japan	spring brook	2	C	absent	V	AB973909	AB974400
178	12	Taira, Toyama Pref., Japan	spring brook	2	C	present	—	—	—
385	13	Hizume, Takaoka, Toyama Pref., Japan	spring brook	3	C	present	V	AB973910	AB974401
386	13	Hizume, Takaoka, Toyama Pref., Japan	spring brook	3	C	present	V	AB973911	AB974402
383	14	Takinami, Fukui, Fukui Pref., Japan***	spring brook	1	C	present	VIII	AB973912	AB974403
384	14	Takinami, Fukui, Fukui Pref., Japan***	spring brook	2	C	present	VIII	AB973913	AB974404
<i>Jesogammarus (J.) spinopalpus</i>	—	Jikkoku, Onjuku, Chiba Pref., Japan	spring brook	—	—	—	—	AB973914	AB974405
<i>Eogammarus kygi</i>	—	Naibetsu R., Chitose, Hokkaido, Japan	river	—	—	—	—	AB973915	AB974406

*Type locality of *J. (J.) fujinoi*. **Type locality of *J. (J.) shonaiensis*. ***Close to type locality of *J. (J.) hokurikuensis*.

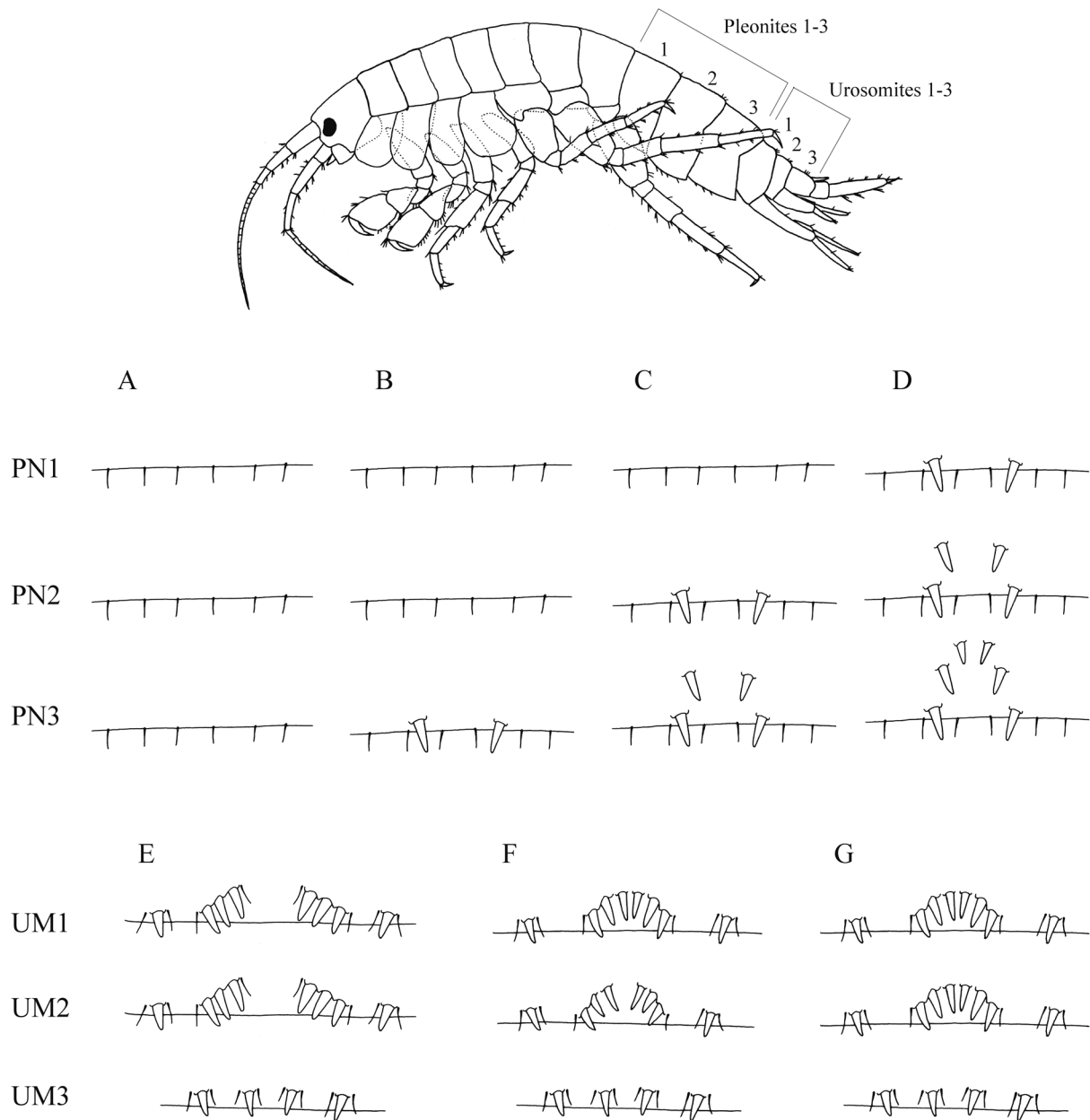


Fig. 2. Different types of dorsal posterior armature of pleonites (PN1–3; A–D) and urosomites (UM1–3; E–G) of *Jesogammarus jesoensis* complex. A, type 0; B, type 1; C, type 2; D, type 3; E, type S; F, type A; G, type C. Typology and type numbering follow Kusano and Ito (2003).

cal identification of the four nominal species is difficult due to high morphological variability, we made no effort to assign the present specimens individually to nominal species categories before analysis.

Samples were fixed and preserved in 70–90% ethanol. All 60 individuals were examined morphologically as detailed below. Subsequently, we tried to determine partial sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) and 12S rRNA genes of all 60 individuals. PCR amplification failed in some individuals, though, and in the end only 39 individuals for COI and 31 individuals for 12S were sequenced (Table 1). Two other anisogammarid species, *J. spinopalpus* Morino, 1985 and *Eogammarus kygi* (Derzhavin, 1923) were selected as operational outgroup taxa.

DNA extraction, PCR amplification and sequenc-

ing. Total genomic DNA was extracted from pereopod musculature of each amphipod by using the DNeasy blood and tissue Kit (Qiagen, Hilden, Germany). Partial regions of the COI and 12S rRNA genes (about 400 and 200 bp, respectively) were amplified by the polymerase chain reaction (PCR) using the following primer pairs: Am-COI-H[5'-CGTCG(AGT)GG(CT)AT(ACG)CC(ACGT)CT(AGT)A(AG)(ATC)CCTA-3'] and Am-COI-T[5'-CG(AG)GC(CGT)TA(CT)TT(CT)AC(CT)TC(ATC)GC(AC)ACTAT-3'] for COI (Tomikawa *et al.* 2007), and Am-12S-H[5'-(AGT)AA(AT)TTAC(AT)(AT)TTAA(AG)TCC(AG)CCTA-3'] and Am-12S-T[5'-AAATTAGAT(AG)ATTTGGCGGCT-3'] for 12S (Tomikawa *et al.* 2007). PCR reaction mixtures containing 0.5 µl of template solution, 2 mM MgCl₂, 2.5 mM of each dNTP, 10 pmol of each primer,

and 0.05 µl of Taq polymerase (5 U/µl; TaKaRa Ex Taq®) in 1X buffer provided by the manufacturer were performed in 10 µl volumes in an PC-320 thermal cycler (ASTEC). Amplification conditions were as follows: initial denaturation for 7 min at 94°C; 35 cycles of denaturation for 45 s at 94°C, annealing for 1 min at 30–42°C depending on the primer set, and extension for 1 min at 72°C; and final extension for 7 min at 72°C. Amplification products were purified by Boom *et al.*'s (1990) method. Sequencing reactions were performed according to the manufacturer's instructions using the BigDye Terminator Cycle Sequencing Reaction Kit ver. 3.1 (Applied Biosystems, CA, USA). Sequencing reaction products were purified by ethanol precipitation. Labeled fragments were analyzed using an ABI 3130x Genetic Analyzer (Applied Biosystem). Sequences were obtained from both strands of the gene segments for verification using the same primers. The nucleotide sequences have been submitted to the DNA Data Bank of Japan (DDBJ) nucleotide-sequence database (linked to the EMBL and GenBank databases) under accession numbers AB973883–973915 and AB974366–974406 (Table 1).

Phylogenetic analyses. The nucleotide sequences were aligned with the multiple alignment algorithm in CLUSTAL W (Thompson *et al.* 1994) with default settings. Phylogenetic relationships were reconstructed by the Neighbor-joining (NJ) (Saitou and Nei 1987), equally weighted maximum parsimony (MP), and maximum likelihood (ML) methods with MEGA 5.03 software (Tamura *et al.* 2011). Alignment gaps were treated as missing data in all analyses. The two gene segments were first analyzed separately, then the two data sets were analyzed together. In the NJ analysis, Kimura's 2-parameter model (Kimura 1980) of nucleotide substitution was used to estimate genetic distances. The MP tree was obtained using the Close-Neighbor-Interchange algorithm starting with trees obtained with the random addition of sequences (10 replicates). The ML analysis used the Tamura 3-parameter (T92+G) model (Tamura 1992) for COI, 12S, and COI+12S, which was determined to be the best-fit model using the Bayesian information criterion (BIC) in MEGA 5.03. To estimate statistical support for branching patterns, 10,000 bootstrap replications (Felsenstein 1985) were performed for the NJ, and 1,000 for the MP and ML analyses. Uncorrected pairwise distances for COI sequences (357–372 bp) were calculated using MEGA 5.03.

Morphological observation. The dorsal armature of the pleonites and urosomites was observed under a stereomicroscope (Olympus SZX7). Dissected appendages were mounted on glass slides in gum-chloral medium and examined with differential interference using a compound light microscope (Nikon Ni).

Results

Molecular phylogeny. A total of 343–372 bp of COI and 202–228 bp of 12S rRNA partial mitochondrial gene sequences was obtained from 39 individuals for COI and 31 individuals for 12S rRNA. There were no indels or stop co-

dons in the COI sequence but two insertions were found in the 12S sequences. For the COI data set, all the codon positions were used in the phylogenetic analyses.

The aligned COI data set of 39 samples was 313 bp long, revealing 19 different haplotypes, with 109 variable sites and 108 parsimony-informative sites for these 19 haplotypes. Nucleotide frequencies among the 19 haplotypes were biased with 64.7% A+T (mean: A=38.5%, C=15.9%, G=19.4%, T=26.2%).

In the ML tree (Fig. 3), eight well-supported mitochondrial clades or lineages were recognized. The clades were geographically discrete and well-defined. The relationships among clades I to VIII were, however, not clear, as reflected in low bootstrap values. The NJ and MP trees were identical in topology to the ML tree.

Excluding gap sites, the aligned 12S data set of 31 samples was 182 bp long, revealing 14 haplotypes, with 42 variable sites and 33 parsimony-informative sites for ingroup taxa. Nucleotide frequencies among the 14 haplotypes were biased with 68.9% A+T (mean: A=35.8%, C=20.7%, G=10.4%, T=33.1%). The 12S ML tree (Fig. 4) differs from the COI tree in several ways. Monophyly of some clades found in the phylogenetic analyses based on the COI data set was not well supported by bootstrap values. Clade VIII was a basally branching clade within the ingroup. The NJ and MP trees were identical in topology to the ML tree, but the monophyly of clade I was supported only in the MP tree, with a moderate bootstrap value (72).

Excluding gap sites, the aligned combined data set of 31 samples was 510 bp long, revealing 18 haplotypes, with 151 variable sites and 133 parsimony-informative sites for ingroup taxa. The structure of the ML tree (Fig. 5) agreed with that of the COI tree; clade VIII was a basally branching clade within the ingroup. The NJ and MP trees were identical in topology.

The uncorrected pairwise differences of partial COI gene sequences among clades ranged from 7.0% to 16.4% (Table 2). Clade II showed strong differentiation even within the clade, up to 4.6%.

Morphological variation. Four types of dorsal armature of pleonites and three types of dorsal armature of urosomites were found in our observed specimens (Fig. 2, Tables 1 and 3). Mapping of the three characters (armature of the posterior dorsal margins of the pleonites and urosomites, and presence or absence of robust setae on the outer ramus of uropod 2) on the phylogenetic tree (Fig. 5) suggested that the character states are distributed independently of molecular clade identity; they were also related to body length or sex in our specimens.

Discussion

Jesogammarus jesoensis, *J. hokurikuensis*, *J. fujinoi*, and *J. shonaiensis* were initially distinguished from each other by the dorsal armature of pleonites 1–3 (Fig. 2A–D) and urosomites 1–3 (Fig. 2E–G), and by the presence or absence of a robust marginal seta on the outer ramus of uropod 2. In our

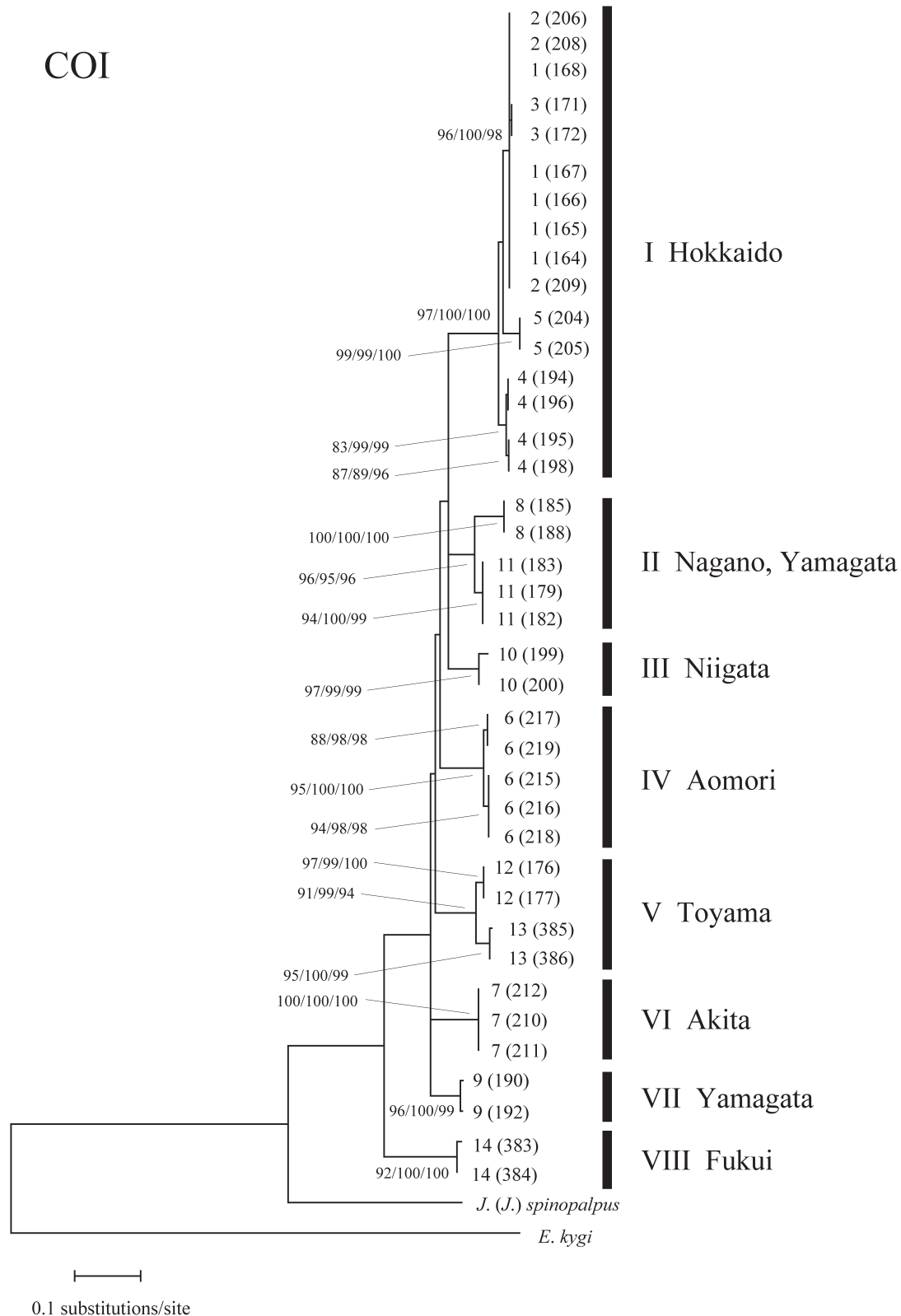


Fig. 3. Maximum likelihood (ML) tree for the mitochondrial COI sequence data (313 bp) of the *Jesogammarus jesoensis* complex, based on the T92+G substitution model. Number sequences beside internal branches indicate the bootstrap probabilities of the ML, NJ, and MP trees, respectively, for nodes with greater than 70% support. Locality codes (1 to 14) of individuals are indicated beside the terminal branches, and code numbers of individuals are shown in parentheses. Sampling data are given in Table 1. Clades I–VIII appear to be restricted geographically to the named prefectures.

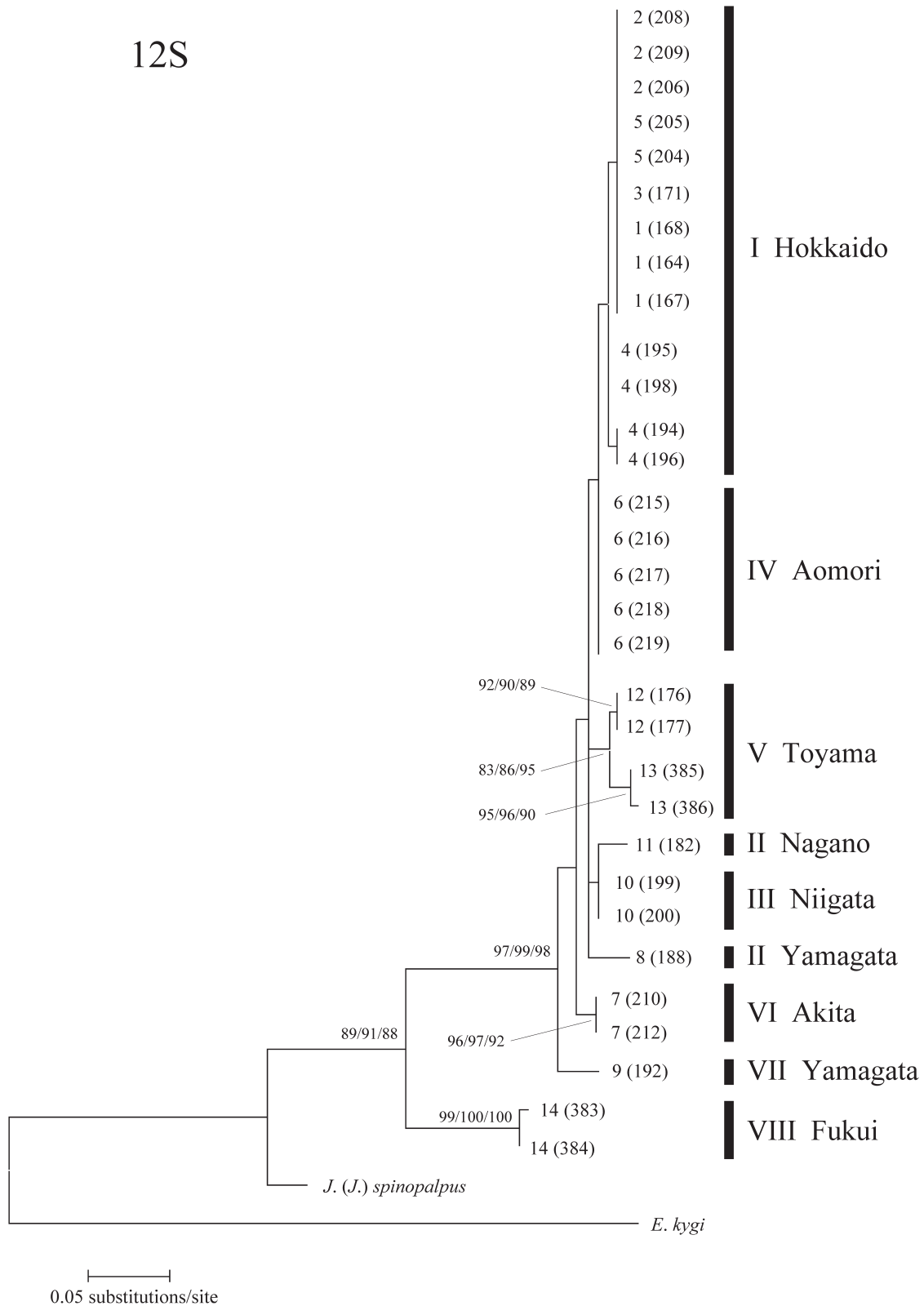


Fig. 4. Maximum likelihood (ML) tree for the mitochondrial 12S rRNA data (182 bp) of the *Jesogammarus jesoensis* complex, based on the T92+G substitution model. Number sequences beside internal branches indicate the bootstrap probabilities of the ML, NJ, and MP trees, respectively, for nodes with greater than 70% support. Locality codes (1 to 14) of individuals are indicated beside the terminal branches, and code numbers of individuals are shown in parentheses. Sampling data are given in Table 1. Clades I–VIII appear to be restricted geographically to the named prefectures.

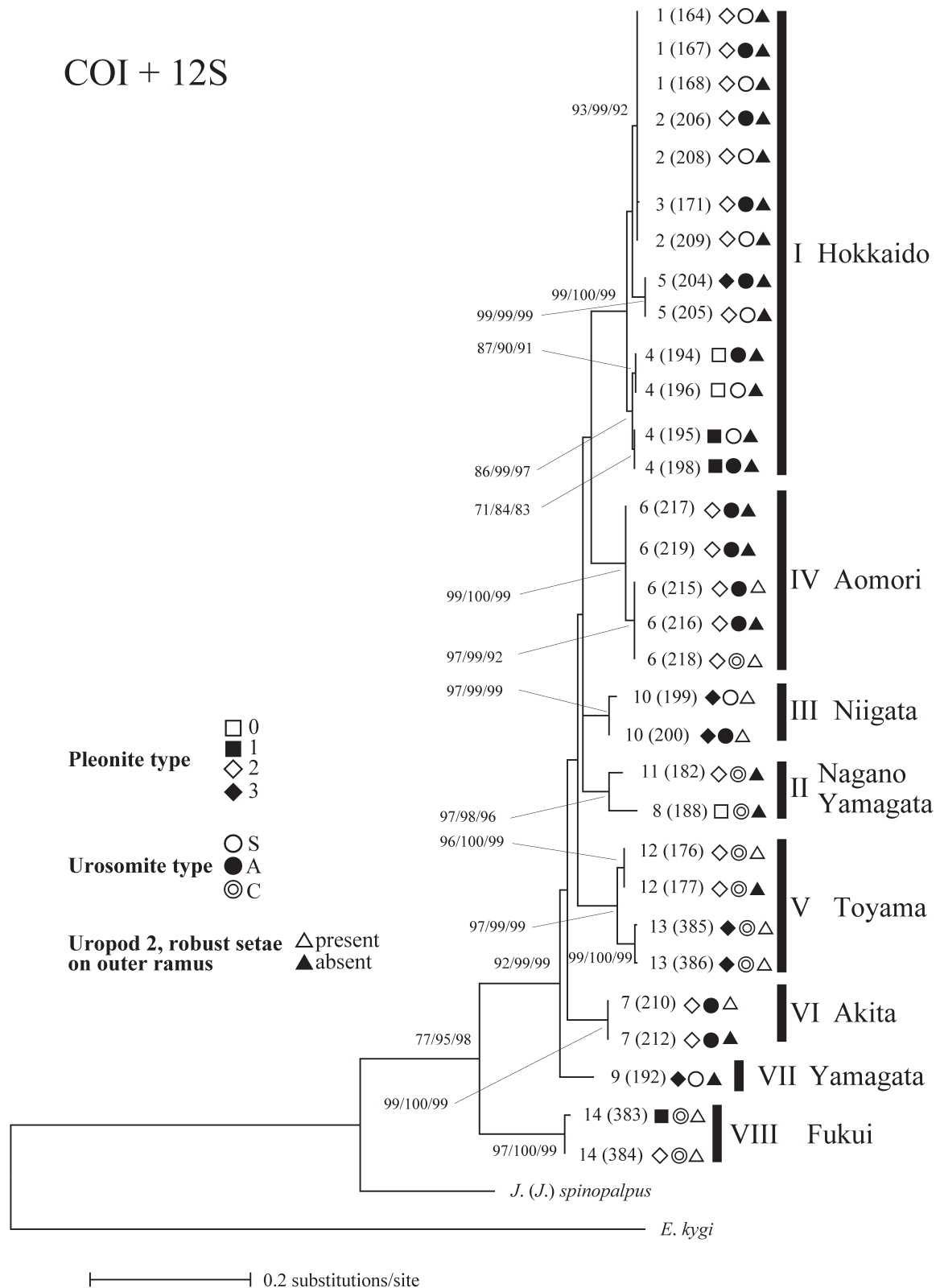


Fig. 5. Maximum likelihood (ML) tree for the combined COI and 12S rRNA data (510 bp) of the *Jesogammarus jesoensis* complex, based on the T92+G substitution model. Number sequences beside internal branches indicate the bootstrap probabilities of the ML, NJ, and MP trees, respectively, for nodes with greater than 70% support. Locality codes (1 to 14) and morphological character states of individuals are indicated beside terminal branches and code numbers of individuals are shown in parentheses. Sampling data are given in Table 1. Clades I–VIII appear to be restricted geographically to the named prefectures.

Table 2. Uncorrected pairwise differences (%: *p*-distance) of partial COI gene sequences within and among clades of the *Jesogammarus jesoensis* complex. Clades I to VIII correspond to those in Figs 3 and 4.

	Clade I	Clade II	Clade III	Clade IV	Clade V	Clade VI	Clade VII	Clade VIII
Clade I	0.0–3.8							
Clade II	9.0–11.4	0.0–4.6						
Clade III	8.5–10.5	8.4–10.5	1.1					
Clade IV	9.9–11.1	9.8–11.2	9.5–10.9	0.0–1.4				
Clade V	9.7–10.9	8.4–9.8	8.9–10.0	10.1–11.0	0.0–3.0			
Clade VI	11.2–11.8	9.3–10.9	9.8–10.1	10.8–11.0	10.7–11.2	0.0		
Clade VII	9.9–10.8	7.0–8.9	8.9–9.2	8.1–9.0	8.1–9.2	8.5	0.5	
Clade VIII	15.0–16.4	14.6–15.2	13.6–14.4	11.5–12.1	13.6–13.8	13.4–13.9	11.4–12.2	0.5

Table 3. Morphological character combinations among clades. PN: pleonite, with types numbered as in Fig. 2A–D; UM: urosomite, with types numbered as in Fig. 2E–G; UP: uropod. Clades I to VIII correspond to those in Figs 3 and 4.

PN type	UM type	UP, robust setae on outer ramus	Clade I	Clade II	Clade III	Clade IV	Clade V	Clade VI	Clade VII	Clade VIII
0	S	absent	○							
0	A	absent	○							
0	C	absent		○						
1	S	absent	○							
1	S	present	○							
1	A	absent	○							
1	C	present					○			○
2	S	absent	○							
2	A	absent	○					○		
2	A	present				○		○		
2	C	absent		○			○			
2	C	present					○			○
3	S	absent			○	○			○	
3	S	present			○	○				
3	A	absent	○		○					
3	C	present					○			

study, these diagnostic features did not exhibit consistent geographic distributions. Various character states were often found within the same population and were distributed independently of the specimens' molecular clade identities (Table 1, Fig. 5). From this, we conclude, in agreement with Kusano and Ito's (2003) suggestion, that these four nominal species cannot be distinguished from each other morphologically by the three putative diagnostic characters. Nevertheless, it is noteworthy that there is no overlap of morphological character combinations among the molecular clades I, II, VII, and VIII, which include specimens from the type localities of the nominal taxa (Table 3).

The molecular analyses did, however, detect high and geographically consistent molecular diversity (Figs 3–5, Table 2). The COI gene sequence has been used as a DNA barcoding system to discriminate known species and to discover unrecognized species (Hebert *et al.* 2003, 2004a, b). DNA barcoding has also been applied to amphipod taxa, and sequence divergence thresholds for distinguishing species have been suggested: 4% for marine and freshwater *Gammarus daiberi* (Rock *et al.* 2007), 3.5% for Chinese freshwater *Gammarus* species (Hou *et al.* 2009), and 3.75% for freshwater *Hyalella* species (Witt *et al.* 2006). In our study, the COI nucleotide divergence among the eight

clades ranged from 7.0% to 16.4%, whereas divergence within clades was only up to 4.6%. If the above-mentioned 3.5–4.0% threshold were applied strictly in the present situation, each clade would represent an independent species. Individuals of clades II and VII were collected from the type localities of *J. fujinoi* and *J. shonaiensis*, respectively, and clade VIII from near the type locality of *J. hokurikuensis*. This suggests that clades II, VII, and VIII correspond to the nominal species *J. fujinoi*, *J. shonaiensis*, and *J. hokurikuensis*, respectively. Schellenberg (1937) provided little information about the type locality of *J. jesoensis* in his original description, but clade I, which is known only from Hokkaido, most likely corresponds to *J. jesoensis*. The other clades may represent undescribed cryptic species. What matters here is the absence of morphological characters differentiating the molecular clades; taxonomic recognition of the latter would require revised diagnoses.

Clade II, which included individuals from the Yamagata and Nagano populations, seems to be divided into two monophyletic sub-clades. We tentatively treated them as a single clade because the COI nucleotide divergence between the two populations (4.6%) did not significantly exceed the 3.5–4.0% threshold value. For an objective evaluation, population genetic analyses based on a more substantial num-

ber of specimens, and from more localities, will be needed.

Other, higher thresholds for the recognition of distinct species have been proposed. For example, Lefébure *et al.* (2006) analyzed COI molecular divergences in various crustacean taxa, including amphipods, and arrived at a threshold of 16% for discriminating species. The values of genetic divergences among the clades in our study are all well below 16%, which suggest that molecular clades may all belong to the same species with a high degree of genetic variation.

In our opinion, molecular clades should not be named formally without morphological criteria. Hence we refrain from assigning the eight clades into independent species, some of which would be new species, for now. To clarify the taxonomic status of these clades, more detailed phylogenetic and morphological analyses are needed. For the time being, we suggest the use of the term “*J. jesoensis* complex” to encompass the nominal taxa *J. jesoensis*, *J. fujinoi*, *J. shonaiensis*, and *J. hokurikuensis*, along with the other four putative but as yet unnamed amphipod taxa.

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