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Genetic Identification and Molecular Phylogeny of *Pseudodiaptomus* Species (Calanoida, Pseudodiaptomidae) in Korean Waters

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Five species of the genus *Pseudodiaptomus*, *P. inopinus*, *P. poplesia*, *P. marinus, P. nihonkaiensis*, and *P.* sp. occur in Korea nearshore waters. Although the four species except for *P.* sp. have been classified into Lobus and Ramosus groups, two for each group, based on morphological characters, this classification had yet to be confirmed by molecular characters. Here, we determined molecular characters and phylogenetic relationships of the five species in order to evaluate the morphology-based groupings and the species identifications. For this, a 625-bp DNA region of the mitochondrial gene cytochrome oxidase subunit I (mtCOI) was sequenced and compared among the species. Intraspecific variation of the sequences is less than 0.6%, while interspecific variation ranges from 17.6–26.7%, indicating every species, including *P.* sp., is a genetically distinct, valid species. Phylogenetic trees of the mtCOI DNA reveal that the Lobus-group species including *P. inopinus* and *P. poplesia* form a well-supported clade and that *P.* sp. belongs to this group. On the other hand, the Ramosus group clade consisting of *P. marinus* and *P. nihonkaiensis* is not well supported by bootstrap analyses, suggesting that further evaluation of the validity of this group assignment is needed.

Key words: Crustacean, Copepoda, *Pseudodiaptomus*, molecular phylogeny, mitochondrial cytochrome oxidase I, mtCOI

INTRODUCTION

Copepods of the genus *Pseudodiaptomus* Herrick, 1884 mostly inhabit estuarine and coastal marine waters. They are distributed in a broad geographical area between latitudes 40°N and 40°S (Walter, 1986a, b, 1987, 1989). There are 76 species reported in the genus that are classified into eight groups, including seven well-defined species groups and a group of unassigned species. This classification was made on the basis of morphological characters such as the antennules, fifth legs, and habitus of both genders (Walter, 1986a). In Korean waters, four desribed species and an undescribed species of this genus occur, including *P. inopinus* and *P. poplesia* in the Lobus group, *P. marinus* and *P. nihonkaiensis* in the Ramosus group, and *Pseudodiaptomus* sp. (Soh *et al*., 2001). The Lobus species group lives in fresh and/or brackish waters, while the Ramosus species group is restricted to the more saline waters of the coast.

The undescribed species, *Pseudodiaptomus* sp., can be recognized as a separate species in that it has a genital structure distinct from the other species of the genus. Individuals of *Pseudodiaptomus* sp. used to be identified as *P. inopinus*, since they have the same body segment pattern and fifth-leg structure. However, a difference is found in the genital structure. Recent studies have reported that pseudodiaptomid copepods show interspecific variation in genital structure that can be used for species identification and determining phylogenetic relationships among the species groups (Ohtsuka *et al*., 1994, 2000; Cuoc *et al*., 1997; Barthélémy, 1999, Soh *et al*., 2001; Walter *et al*., 2002). Nonetheless, the identification of *Pseudodiaptomus* sp. and its phylogenetic relationships with other species need to be

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confirmed by other means such as molecular characters.

The validity of the species groupings also needs to be evaluated by means of cladistic analysis with molecular characters. Although the morphological characters provide some clues for classification, it has been difficult to understand the phylogenetic relationships among the species and the species groups only with morphological characters because of the limited number of characters. Mitochondrial genes are known to have enough nucleotide sequence variation to discriminate species in copepods (Miller *et al*., 1999;

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                  1                                                                   70
P. inopinus       AATAGTGGGA ACTGGGCTGA GTATATTAAT TCGGTTCGAG TTGGGTCAAC CTGGGAGGTT AATTGGGGAC
P. poplesia       T..C..T..C ..C..... .G..GC.T.. ...A..T... C.T..G.... ....A..A.. ......T..T
P. sp.            G..G..T... ..A..C..A. .A.....G.. ...A..T... ..A..A.... .C..A..A.. ...C.....T
P. marinus        T..G.....T ..G..TT.A. .A...A.T.. ...CC.A... C....G...G ....ATCCC. T.....A...
P. nihonkaiensis  ...GA.C..C ..G..TT.A. .A..GA.T.. ...A..G... C.T......G .A..CTCT.. G.....A...
M. gerlachei      G.....A... ..A..C.... .GG.C..G.. ....C.A... C....G.... .G..CTCT.. .........T
                  71                                                                  140
P. inopinus       GACCAAATTT ATAATGTGGT GGTAACCGCA CACGCTTTTG TAATAATTTT TTTCATAGTT ATACCTATTT
P. poplesia       ..T....... .......TA. T..T..A..T .......... .......... ...T.....A .....C....
P. sp.            .......... .......T.. T..C..A..C .....C.... .G........ C..T..G..G ..G.......
P. marinus        ..T.....C. .C........ A..T..A... ..T......A .T........ ...T.....C ..G..A..C.
P. nihonkaiensis  ..T..G..C. .......... C........G .........A .T........ ...T.....A ..G.......
M. gerlachei      .....G.... .......T.. ...T..A..C ..T..A...A .T........ ...T.....A ..........
                  141                                                                 210
P. inopinus       TAATTGGTGG GTTTGGGAAT TGATTAGTCC CACTTATATT AGGAGCCGCA GACATAGCTT TCCCTCGTAT
P. poplesia       .......... ......A... ..G.....T. .C..A..... G..G.....G ..T..G..C. .......A..
P. sp.            .......... ......T..C ..G.....T. .TT.A..G.. G..C..G..T ..T..G.... .......G..
P. marinus        .......C.. ......A..C ..G....... .C..A..... ...G..A..G ........C. ....G..A..
P. nihonkaiensis  ....C..A.. ......A... ..G..G..A. .TT.G...C. ...G..A..C ..T.....C. .......A..
M. gerlachei      .......A.. ......A..C ..GC....A. .C..A..GC. G..G..T... .....G..C. .T.....GC.
                  211                                                                 280
P. inopinus       AAATAACATA AGATTTTGAT TTTTATTGCC TGCTTTGGTA ATGTTATTAT CTAGCTCACT GGTAGAGAGA
P. poplesia       G.....T... ..C....... .....C.... G..C..A... ..A....... .......TT. A........C
P. sp.            G.....T... ..G....... ..C.TC.... C.....A... ...C.TC.T. .......TT. A.....A..T
P. marinus        .......... ........G. .....A.C.. A..AC.AA.. ..A...C... .A.....T.. T..T..A...
P. nihonkaiensis  G.....T... .......... .CC..A.C.. ...CC.TA.T ..AC.T.... ....A..TT. ......A..G
M. gerlachei      ......T... ........G. .CC..G.C.. A...C.A... C.T...C.TA .A..GG.C.. A..T..A...
                  281                                                                 350
P. inopinus       GGAGCCGGTA CAGGTTGAAC TGTGTACCCA CCCCTCTCTA GTAATATTGC TCACGGGGGG AGCTCAGTTG
P. poplesia       ..G..T..G. .......... ...T.....C ..G..A.... .A..C..... .....CA..C .....T....
P. sp.            ..G..G..G. .G........ A..T.....C ..T.....G. .C..C..... C....CA... .....T..A.
P. marinus        ..G.....A. .T..A..G.. ...A.....C .....T.... .A.....C.. G....C...C ..A.....A.
P. nihonkaiensis  .....A..A. ....G..... C..T.....C ........A. .G.....C.. G....CC..C ..G..C....
M. gerlachei      ..C..A.... ....G..G.. G..T..T..T ..T..TG.C. AA...G.G.. G..T.CC..C ..G..T..A.
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                351                                                                 420
P. inopinus     ATTTTGCCAT TTTTTCTCTT CATTTGGCCG GAGTTAGTTC AATTTTAGGT GCGGTAAACT TTATTAGAAC
P. poplesia     .......T.. ...C.....A ..C..A..G. .T..G..A.. G........A ..T....... .C........
P. sp.          .C.....T.. .......... .......... .T..G..G.. .........A ..A..G.... ..........
P. marinus      .......A.. ...C..CT.G .....A..T. .T..C..C.. T........G ........T. ..........
P. nihonkaiensis .C........ .......... .....A..G. .T..G..A.. T...C....A ..T.....T. ..........
M. gerlachei    .C..C..A.. .........C .....A..G. .T...TCG.. T...C....A ..AA.T..T. .......T..
                421                                                                 490
P. inopinus     TGTGGGTAAC CTACGAACGT TTGGAATGGT ACTTGATCGG ATGCCTCTTT TCGTATGGTC GGTTTTAATT
P. poplesia     C..T..G... T.G.....C. .C..G..A.. TT.A...... .......... .T..T..AG. A.....G...
P. sp.          C..T..A... T.G.....T. ....T..A.. T.....C..A .......... .T..G...G. T..C..G...
P. marinus      ...A..A... .....GGT.. ....G..AA. TT.A...... ........A. ...CG...G. T.........
P. nihonkaiensis CC....A... ..T...GT.. ....C..... G.....C..A ..A....... ...CT..AG. T.........
M. gerlachei    CC....C..T ..T..GGTA. ........T. TT.G..C..T .....CT.A. .T.CT...G. .........C
                491                                                                 560
P. inopinus     ACTGCTGTCC TTTTATTGCT TTCACTGCCC GTGTTAGCTG GGGCAATTAC TATATTATTA ACAGACCGTA
P. poplesia     .....A...T .A.....AT. A..T..T..T ..C....... .C........ ...GC.C... ..........
P. sp.          ..A..A..G. .AC.GC.A.. G..T...... ..T.....C. .C........ A..G...... ..T.......
P. marinus      ......A.T. .A.....A.. A..T..A..G ...C.G..G. ....C..... ....C.CC.C ..G.....A.
P. nihonkaiensis ..C..AA.T. .....C.A.. A..T..C..T ..T..G.... .A..T..... ...GC.T... ..G.....A.
M. gerlachei    ...A..A..T .A..GC.T.. ...T..C..T ..T.....A. ....C..... A..G..G... ..........
                561                                                           625
P. inopinus     ACCTCAACTC TTCTTTTTAT GATCCAAGAG GGGGCGGGGA TCCAATTTTA TATCAGCACC TATTC
P. poplesia     .TT.A..... .........C .....T..G. ....T..A.. C..C..CC.. .........T ....T
P. sp.          .TT.A..T.. C.....C..C ..C..C.... .......A.. C......... .....A...T .G..T
P. marinus      ..T.A..... .......... ...G.T.... .C........ C..C.....G ..C....... .G..T
P. nihonkaiensis .T..T..... ......C... ...G.T.... ....G..A.. ...T...C.T ..C..A.... .G..T
M. gerlachei    ....A...A. AA........ ...GTTG.G. .T..A..... C..T.....G ..C.....TT ....T
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Fig. 1. DNA sequence alignment of the mitochondrial gene cytochrome oxidase subunit I sequences obtained from five Korean pseudodiaptomid copepod species.

Lee, 2000; Bucklin *et al*., 2003). They have been used for reconstructing phylogenetic trees and estimating species divergence times of other marine invertebrates (Lee, 2003; Lee *et al*., 2004). Using mitochondrial gene cytochrome oxidase subunit I (mtCOI) sequences, the present study aims to evaluate the validity of species and species-group assignments of the Korean pseudodiaptomid copepods and also to understand their phylogenetic relationships.

MATERIALS AND METHODS

Specimens

The five species of pseudodiaptomid copepods, *Pseudodiaptomus inopinus*, *P. poplesia, P. marinus, P. nihonkaiensis,* and *P.* sp., were sampled from several estuarine and coastal regions in Korean waters using a Norpac net (mouth aperture, 45 cm in diameter; mesh size, 200 μm) (Table 1). All samples were immediately preserved in 95% ethyl alcohol after the seawater was removed by a 45-μm sieve. The zooplankters were then sorted out and identified

Table 1. Collection information and GenBank accession numbers for the five pseudodiaptomid copepod species from Korean waters.

Species	Collection region	GenBank accession no.
P. inopinus	Mankyeong River estuary	AF536520
P. poplesia	Mankyeong River estuary	AF536521
P. sp.	Seomjin River estuary	DQ243938
P. marinus	Bangjukpo, Dolsando	AY145436
P. nihonkaiensis	Pyoseon, Jeju Island	AF536519

to the species level on the basis of their morphological characters. Three individuals of each species were used separately for DNA analysis.

Sequence determination

DNA amplification was done directly from each individual without extracting the genomic DNA. Each individual sample was rinsed in 0.7 ml distilled water for 8 to 12 h before use. This specimen was homogenized in 87 μl PCR buffer (10X Qiagen PCR buffer, 10 μl; distilled water, 77 μl) with a 1.5-ml tube-fit pestle and incubated under refrigeration for 6 hrs. The target segment of the mtCOI gene was amplified from the homogenized sample by the polymerase chain reaction (PCR). Amplification was performed in 50-μl reaction volumes containing 43.5 μl of the homogenized sample, 3 μl of 10 μM each primer solution, 1 μl of 10 mM each dNTP, and one unit Taq polymerase. PCR reactions were carried out in a Tgradient thermocycler (Whatman, Germany) using HotStarTaq™ DNA polymerase (Qiagen, Germany). Reaction conditions were as follows: initial heating at 95°C for 10 min; 45 cycles of 95°C (1 min), 38–52.5°C (2 min), 72°C (3 min); final extension at 72°C for 10 min. PCR primers were LCOI-1489 (5'-TTY TCT ACI AAT CAY AAR GAY ATT GG-3'), LCOI-1529 (5'-TA SCT GGG GCN TGG WCA GG-3'), LCOI-1529N (5'-TA GCN GGN GCN TGR TCN GG-3'), HCOI-2195 (5'-AC TTC AGG NKG MCM AAA AAM-3'), and HCOI-2198 (5'-TA AAC TTC AGG GTG ACC AAA AAA TCA-3'). These COI primers were named after the position numbers of the *Dorsophila yakuba* COI sequence (Clary and Wolstenholme, 1985).

PCR products were cloned into pCRII-TOPO vector using a TOPO TA Cloning Kit (Invitrogen, California). Transformed *E. coli* cells were cultivated on LB-ampicillin plates coated with X-gal for 18 to 24 h. White, positive clones were selected from the plates and at least three clones were further processed for sequencing the insert DNA. DNA sequencing was done in an Automated DNA Sequencer (Applied Biosystems Inc., Model 377) after sequencing reactions with Sp6 (5'-AT TTA GGT GAC ACT ATA G-3') and T7 primers (5'-TA ATA CGA CTC ACT ATA GGG-3').

Phylogenetic analysis

The mtCOI sequences were aligned using Clustal W (Thompson *et al*., 1994). Nucleotide differences and genetic distances among the sequences were estimated using the computer package MEGA (Kumar *et al*., 2004). Phylogenetic trees of the sequences were reconstructed by the minimum-evolution (ME) method using MEGA and by the maximum parsimony (MP) and maximum likelihood (ML) methods using the PAUP* program (version 4.0b10; Swofford, 1998). For the ME tree, Log-determinant distances (Lake, 1994; Lockhart *et al*. 1994) with the TrN model (Tamura and Nei, 1993) were used to circumvent long-branch attraction. The MP analysis incorporated the parameter of transition/transversion bias. The weight parameter, estimated from ML analysis of the sequences with Kimura two-parameter model (Kimura, 1980), turned out to be 1.5 times more weighting for transversional changes than for transitional changes. The ML analysis adopted the general time-reversible model with parameters of invariable sites and unequal rates among sites (GTR+I+G model). The robustness of each branch in the tree was evaluated by bootstrap analysis of 1,000 replicates. Heuristic searches were carried out for the analyses. A calanoid species, *Metridia gerlachei* (Metridinidae), which is considered to belong to a primitive group, Arietelloidea, among the planktonic calanoids, was used as the outgroup of the phylogeny.

RESULTS

Sequence differences

A 625-bp region of the mtCOI gene was sequenced from five species of pseudodiaptomid copepods (*Pseudodiaptomus inopinus, P. poplesia*, *P.* sp., *P. marinus, P. nihonkaiensis*) which occur in Korean waters. Three individuals were analyzed for each species. The sequences aligned well among the species without any indels (Fig. 1). Of the aligned 625 sites, there are 223 variable sites and 108 parsimony informative sites. Most of the variable sites (180 out of 223) are located at third-codon positions. Pairwise comparisons show that the nucleotide sequences differ by 17.6 to 26.8% between any two of the five species (Table 2). When distances were corrected for multiple substitutions using the Tamura and Nei model (1993), the range became 20.7 to 34.2%. Intraspecific variation, however, is limited to less than 0.6% (Table 2, diagonal).

Molecular phylogeny

The ME tree based on Log-Det distances using MEGA reveals that the two Lobus group species, *Pseudodiaptomus inopinus* and *P. poplesia*, and another species-level

Table 2. Pairwise percentage differences in mtCOI sequences among the five pseudodiaptomid species from Korean waters.

	1	2	3	4	5
1. P. inopinus	(0.00)	19.84	21.28	21.92	23.04
2. P. poplesia		(0.32)	17.60	21.92	21.76
3. P. sp.			(0.64)	26.72	23.52
4. P. marinus				(0.16)	20.00
5. P. nihonkaiensis					(0.32)

(diagonal: intraspecific variation)

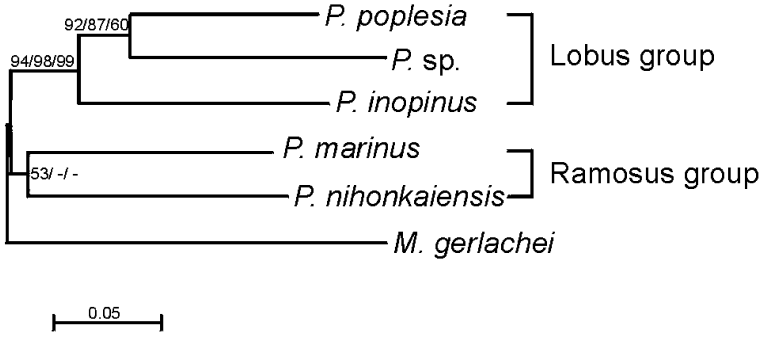

Fig. 2. Minimum evolution (ME) tree based on Log-Det distances of the mtCOI sequences (625 bp) from the five Korean pseudodiaptomid species and an outgroup species, *M. gerlachei*. Numbers near nodes are bootstrap values from the ME, MP, and ML analyses (1,000 replicates).

taxon, *Pseudodiaptomus* sp., form a clade separated from the other two Ramosus-group species, *P. marinus* and *P. nihonkaiensis* (Fig. 2). This clade is supported by bootstrap values higher than 90%. It is notable that *P.* sp. lies within this clade, clustered with *P. poplesia*. This cluster is also well supported by a high bootstrap value (92%). On the other hand, the two Ramosus-group species do not comprise a well-supported clade, as the robustness is only marginal (53% bootstrap value). Parsimony analysis of the sequences identified the ME tree as the maximum parsimony (MP) tree, with 599 steps in total. The MP tree shows monophyly of the Lobus-group species and *P.* sp., with a bootstrap value higher than 98%. Within this clade, *P*. sp. clusters with *P. poplasia*, also supported by a high bootstrap value (87%). However, the clade of the two Ramosus-group species is not confirmed by the 50% majority-rule consensus tree of MP bootstrap analysis, although it appears in the MP tree (Fig. 2). The ML tree (–lnL=2676.3255, alpha=0.35, I=0.35) shows the Lobus-group species and *P.* sp. as a monophyletic group, as in the ME and MP trees. This clade is strongly supported by a 99% bootstrap value. On the other hand, the phylogenetic position of *P.* sp within this clade is not resolved. Although the ML tree clusters *P. inopinus* and *Pseudodiaptomus* sp. together, in contrast to the ME and MP trees, this clustering is almost as likely as clustering *Pseudodiaptomus* sp. with *P. poplesia* (–lnL=2676.4239). The ML bootstrap analysis leaves the node of the three species as a polytomy. When the outgroup species *M. gerlachei* was removed and only the five pseudodiaptomid species were included in a ML analysis, *Pseudodiaptomus* sp. formed a cluster with *P. poplesia*, as the ME and MP trees show, supported by a bootstrap value of 60% (Fig. 2, parentheses). The Ramosus-group species do not comprise a single cluster in the ML tree. When this cluster was enforced, the likelihood value of the tree decreased significantly (–lnL=2679.3109).

DISCUSSION

Genetic species identification

Eleven species of the genus *Pseudodiaptomus* have been reported in northeastern Asia (Shen and Lee, 1963; Mizuno, 1984; Soh *et al*., 2001). These species are classified into three groups based on morphological and ecological characteristics (Walter, 1986a): the Lobus group (*P. inopinus, P. poplesia, P. bulbosus, P. forbesi, P. inflatus*), Ramosus group (*P. galleti, P. ishikakiensis, P. marinus, P. nihonkaiensis*), and Hyalinus group (*P. incisus, P. trihamatus*). In Korean waters, only four species are known to occur, *P. inopinus* and *P. poplesia* of the Lobus group and *P. marinus* and *P. nihonkaiensis* of the Ramosus group (Kim, 1985; Chang and Kim, 1986; Soh *et al*., 2001). None of the Hyalinus group has been reported. The Lobus- and Ramosus-group species are mainly discriminated by the number of egg sacs. The Lobus-group species have a pair of egg sacs, while Ramosus-group species have a single egg sac (Mizuno, 1984; Oka *et al*., 1991; Soh *et al*., 2001; Walter *et al*., 2002). Walter *et al*. (2002) suggested that the presence or absence of a genital opeculum covering the paired gonopores is correlated with the number of egg sacs. That is, in the Lobus group with a pair of egg sacs, the genital operculum is absent, while in the Ramosus group with a single egg sac it is present and the paired genital opercula partially or completely cover the gonopore.

Another species of the genus *Pseudodiaptomus, Pseudodiaptomus* sp., was recently identified in Korean waters (H. Y. Soh, unpublished data). Individuals of *Pseudodiaptomus* sp. occurring in the southern and eastern estuaries of Korea had been identified as *P. inopinus* by Chang and Kim (1986) since they share the same segment pattern and the fifth-leg structure. However, the genital structures of the two species are different enough that the two species can be recognized as distinct (Soh, unpublished data). The present study of mtCOI sequences corroborates the distinctness of *Pseudodiaptomus* sp. from both *P. inopinus* and *P. poplesia*. Nucleotide sequences of *Pseudodiaptomus* sp. differ from those of *P. inopinus* and *P. poplesia* by 21.28 and 17.60%, respectively, distances comparable to those between *P. inopinus* and *P. poplesia* (19.84%, Table 2; Fig. 1). Considering that intraspecific nucleotide variation is only at a level of 0.6% in these species, these differences are large enough for *Pseudodiaptomus* sp. to be described as a separate, valid species, and the description of this new species has been submitted elsewhere. In other copepods such as *Calanus*, interspecific mtDNA divergence ranges from 9 to 25% and intraspecific divergence from 1 to 4% (Bucklin *et al*., 2003).

In phylogenetic position, *Pseudodiaptomus* sp. seems to be closer to *P. poplesia* than to *P. inopinus*. Although morphological characteristics indicate a close relationship between *Pseudodiaptomus* sp. and *P. inopinus* as mentioned in the previous paragraph, the phylogenetic trees based on the mtCOI sequences show that *Pseudodiaptomus* sp. is the sister species of *P. poplesia* (Fig. 2). The ME and MP trees strongly support this relationship with bootstrap values higher than 87%. If a cluster of *Pseudodiaptomus* sp. and *P. inopinus* is enforced in the parsimony analysis, the tree becomes seven steps longer than the MP tree. The likelihood analysis without the outgroup species *M. gerlachei* produces a ML tree congruent with the ME and MP trees in this respect. The clade of *Pseudodiaptomus* sp. and *P. poplesia* is supported by a 60% ML bootstrap value.

Phylogenetic relationships and the validity of the species-group assignments

The ME, MP, and ML trees of the mtCOI sequences all reveal the monophyletic origin of the two Lobus-group species, *P. inopinus* and *P. poplesia*, and another species, *Pseudodiaptomus* sp. (Fig. 2). This clade is well supported by a bootstrap value higher than 94% and is clearly separate from the Ramosus-group species. Sequence comparisons of internal transcribed spacers (ITSs) are congruent with these results in that sequences of the three species are conserved and align well without many gaps, while comparisons between the species groups show poor alignment with many gaps (Soh *et al*., unpublished data). On the other hand, clustering of the two Ramosus-group species, *P. marinus* and *P. nihonkaiensis*, is not evident (Fig. 2). This clade is retained in the ME and MP trees, but is only marginally supported (53%) in the 50% majority-rule consensus tree of the ME bootstrap analysis and disappears in the MP bootstrap tree. The MP bootstrap tree places the two species in a polytomy with the outgroup species, *M. gerlachei*. The second and

third most parsimonious trees, which have only slightly more steps than the MP tree (599.5 and 600.5 steps vs 599 steps), do not make a separate cluster of the Ramosus-group species at all, nor does the ML tree show the Ramosus-group cluster. The ML tree places *P. marinus* in the basal position next to the outgroup species, which is supported by about a 70% bootstrap value. When the cluster was enforced in the ML analysis, the resulting tree became significantly less likely than the ML tree (–lnL=2679.3109 vs 2676.3255).

In fact, traditional taxonomy based on morphological characters also identified a distant relationship between the two species: it assigned *P. marinus* to the *hickmani* subgroup and *P. nihonkaiensis* to the *serricaudatus* subgroup of the Ramosus group. The former subgroup is widely distributed in the Indo-West Pacific while the latter is mainly restricted to the western Pacific (Walter, 1986b). However, species of these two subgroups occurring in northeastern Asia are restricted in distribution to the western Pacific (Walter *et al*., 2002). These facts support that the speciation of these subgroups in East Asia could have occurred when ancient East Asia existed as a huge gulf during the Pleistocene, as hypothesized for speciation in a *Tortanus* subgenus, *Eutortanus* (Ohtsuka *et al*., 1992; Ohtsuka and Reid, 1998), and the Lobus species group of *Pseudodiaptomus* (Walter *et al*., 2002).

The results of this study show that the recently identified species *Pseudodiaptomus* sp. is a separate, valid species and that the phylogenetic relationship among the five Korean pseudodiaptomid species are ((*P. inopinus*, (*Pseudodiaptomus* sp.*, P. poplesia*)), (*P. marinus, P. nihonkaiensis*)) in which the clade containing the Lobus-group species and *Pseudodiaptomus* sp. is robust, suggesting its valid assignment as a species group. This study also reveals that *P.* sp. is more closely related to *P. poplesia* than to *P. inopinus*. The former two species are restricted to brackish waters of northeastern Asia, while the last one shows a wider distribution extending to the fresh or brackish waters of East Asia (Mizuno, 1984; Soh *et al*., 2001). These facts indicate that the *P.* sp.-*P. poplesia* clade might have originated after the speciation of *P. inopinus* caused by the repeated transgressions and regressions in East Asia during the Pleistocene. In contrast, the clade of the Ramosus-group species is not well supported in the mtCOI molecular phylogenies, indicating the possibility of its classification being invalid and the necessity of further evaluation. *P. marinus* and *P. nihonkaiensis* of the Ramosus group are distributed in coastal or neritic waters of the western Pacific (Hirakawa, 1984; Walter, 1986a; Soh *et al*., 2001). On the other hand, Walter *et al*. (2002) suggested the possibility that vicariance between the Indian Ocean and western Pacific occurred among species of both the Lobus and Ramosus groups, because these species groups both contain species restricted to the Indian Ocean.

Molecular analyses of *Pseudodiaptomus* species, such as our mtCOI sequence analysis, can help define species, reveal cryptic species, identify morphologically ambiguous taxa, and elucidate evolutionary relationships. Also, molecular approaches have been useful in detecting the introduction of Asian copepods, including *Pseudodiaptomus* species, to the United States by aquaculture projects or in the ballast water of ships (*e.g*., Fleminger and Kramer, 1988; Orsi and Walter, 1991; Cordell *et al*., 1992; Cordell and Morrison, 1996).

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