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Molecular phylogeny of the rotifers with two Indonesian *Brachionus* lineages

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Abstract—The rotifer *Brachionus plicatilis* is an ecologically and commercially important species, and has been studied in various fields such as population dynamics, ecotoxicology and aging. However, recent studies have revealed that the *B. plicatilis* lineages involve an unknown number of cryptic species, and the group has been regarded as the *Brachionus* complex. One cause of this complicated taxonomy is the lack of surveys in the tropical zone, which is characterized by enormous species-richness. Accordingly, in this study we collected two *Brachionus* rotifers from the Sumatra and Sulawesi Islands, Indonesia, and determined their partial nucleotide sequences of mitochondrial DNA cytochrome *c* oxidase subunit I gene. Subsequently, we constructed molecular phylogenetic trees with fourteen species/lineages from four genera including the two Indonesian rotifers. The two Indonesian *Brachionus* rotifers were respectively found to be phylogenetically close to *B. ibericus* and *B. rotundiformis*. On the other hand, Japanese *B. plicatilis* was suggested to be phylogenetically closer to *B.* Manjavacas, which is proposed to be a new species, than to Spanish *B. plicatilis*. These results imply that the current taxonomy of the *Brachionus* is problematic, and a major revision is necessary to establish a reliable taxonomy of this group.

Key words: *Brachionus*, cytochrome *c* oxidase subunit I, mitochondrial DNA, phylogeny, rotifera, taxonomy

Introduction

Rotifers often dominate in aquatic ecosystems and play an important role in the nutrient cycle (Pennak 1989). The euryhaline species *Brachionus plicatilis* has been investigated in various fields such as population dynamics (Yoshinaga et al. 1999, 2000, 2001a, b, 2002, 2003a, b), population genetics (Gómez et al. 1995, 2000, 2002, Gómez and Snell 1996, Derry et al. 2003), aging (Kaneko et al. 2002a, b, in press, Yoshinaga et al. in press), ecotoxicology (Snell and Persoone 1989, Moffat and Snell 1995) and highly-sensitive trace elements imaging (Ezoe et al. 2002). In addition, *B. plicatilis* and its allied species, *B. rotundiformis*, are commercially important organisms as live food at finfish hatcheries (Lubzens 1987, Hagiwara et al. 2001).

However, a taxonomic problem exists in the genus *Brachionus*. When *B. plicatilis* was introduced into Japanese hatcheries in the 1970s, the rotifer was observed to change its body size seasonally (Fukusho and Hirayama 1989). It was once supposed that a single species had a morphological variation (cyclomorphosis), but later proposed that two lineages with different thermal preferences coexisted in the culture, and dominated alternatively with seasonal succession (Fukusho and Hirayama 1989). The two lineages were different in lorica length, and were categorized as large (L) and small (S) strains. Subsequent allozyme analysis of sixty-seven aquaculture strains showed that they could be divided into two genetically distinct groups, each corresponding to the L and S strains, respectively (Fu et al. 1991). Accordingly, Segers (1995) proposed that the L and S strains should be different species, and should be re-described as *B. plicatilis* O. F. Müller, 1786, and *B. rotundiformis* Tschugunoff, 1921, respectively. Besides these two species, the ultra-minute *Brachionus* strains (SS) have also been found in subtropical and tropical waters (Hagiwara et al. 1995). However, the taxonomic relationship of the SS strains with the other two *Brachionus* species is still controversial (Serra et al. 1998). Moreover, Gómez and Snell (1996) and Serra et al. (1998) pointed out that there could be at least three cryptic species among the sympatric *Brachionus* rotifers in a small salt pond in Spain.

The type specimen of *B. plicatilis* is not available, and the type locality is not specified by the nomenclator, O. F. Müller (Ciros-Pérez et al. 2001). Recently, Ciros-Pérez et al. (2001) reexamined the euryhaline *Brachionus* rotifers by mor-

Table 1. Rotifers examined in this study.

Species/lineage	Strain	Locality	Habitat	Lorical length (μm)	Gen Bank accesion number	Reference
Brachionus plicatilis	ISKW	Japan	SW	247.6	n.a.	this study
Indonesia-1	LMPG	Indonesia	SW	190.5	n.a.	this study
Indonesia-2	MNBO	Indonesia	SW	142.9	n.a.	this study
B. plicatilis sensu stricto	6TOS-L4**	Spain	SW	299.0	AF266860	Ciros-Pétez et al. 2001
B. ibericus	6TON-SM6**	Spain	SW	193.4	AF387270	Ciros-Pétez et al. 2001
B. rotundiformis	6HON-SS**	Spain	SW	148.7	AF387293	Ciros-Pétez et al. 2001
B. Manjavacas	Russia**	Russia	SW	260	AF387250	Gómez and Snell 1996
B. calyciflorus	n.a.	U.S.A.	FW	n.a.	AF499053	Derry et al. 2003
*B. urceolaris**	n.a.	Canada	FW	n.a.	AF499070	Derry et al. 2003
Keratella cochlearis	n.a.	Canada	FW	n.a.	AF499073	Derry et al. 2003
K. quadrata	n.a.	Canada	FW	n.a.	AF499084	Derry et al. 2003
K. hiemalis	n.a.	Canada	FW	n.a.	AF499077	Derry et al. 2003
Synchaeta pectinata	n.a.	Canada	FW	n.a.	AF499088	Derry et al. 2003
Asplanchna sp.	n.a.	Canada	FW	n.a.	AF499052	Derry et al. 2003

*, Also known as *B. sericus*; **, according to Gómez et al. (2002); n.a., not available; SW, sea water; FW, fresh water.

phometry analysis, re-described *B. plicatilis* and *B. rotundiformis*, and described a new species of *B. ibericus*. However, the existence of additional new species of the euryhaline *Brachionus* has been suggested based on allozyme analysis (Ortells et al. 2000). For example, *B.* Manjavacas, which has morphological characteristics similar to *B. plicatilis* (Gómez and Snell 1996; Table 1), has been proposed to be a new species (Gómez et al. 2002; notice that Manjavacas is not a specific epithet). *B. plicatilis* and *B.* Manjavacas were highly genetically divergent, and no evidence of hybrids was found in the pond where the two lineages coexisted (Gómez et al. 2002). Consequently, the genus *Brachionus* has been considered to involve an unknown number of cryptic species, and is regarded as the *Brachionus* complex (Gómez et al. 2002).

The current taxonomy of the genus *Brachionus* is thus likely far beyond the valid systematics, and requires a breakthrough. *Brachionus* rotifers have been recognized as a cosmopolitan found from the tropical to subarctic zones (Nogrady et al. 1993). However, substantially no survey of the rotifers has been carried out in the tropical zone, despite the numerous studies done in the temperate and subarctic zones (Gómez et al. 2002, Derry et al. 2003). The tropical zone is generally characterized by its extraordinary species-richness for various taxa of organisms (Roberts et al. 2002), and the majority of the genetic variation can occur there. The lack of information on such a distinctive area may explain why the current taxonomy of the genus *Brachionus* has problems. Accordingly, in this study we collected two *Brachionus* rotifers from the Sumatra and Sulawesi Islands, Indonesia. Subsequently, we determined their partial nucleotide sequences of mitochondrial DNA (mtDNA) cytochrome *c* oxidase subunit I (COI) gene, and constructed molecular phylogenetic tree with a total of fourteen species/lineages from four genera including the above two Indonesian rotifers. The tree not only showed the phylogenetic positions of the tropical rotifers among the genus *Brachionus*, but also suggested a major problem in the current taxonomy of the well-studied temperate species. To our knowledge, this is the first report to present genetic information for tropical rotifers, and can be a starting point for the major revision of the taxonomy of this group.

Materials and Methods

Samples

Three euryhaline *Brachionus* rotifers were examined in this study (Table 1). *B. plicatilis* Ishikawa (abbreviated as ISKW; Fig. 1A) was originally isolated from a Japanese eel culture pond several decades ago (Prof. Hino, The University of Tokyo, personal communication), and has been cultured in our laboratory (Yoshinaga et al. 1999). One Indonesian rotifer was collected from a culture tank at a private hatchery in Bandar Lampung, Sumatra (LMPG; Fig. 1B). Another Indonesian rotifer was collected from a brackish water pond in Manembo-Nembo Bitung, North Sulawesi (MNBO; Fig. 1C). The two Indonesian rotifers had been morphologically identified as belonging to the genus *Brachionus* and kept by I. F. M R., and were introduced into the laboratory culture in Japan. Prior to the experiments, a genetically cloned population was established from a single amictic female. No male was observed in the cultures.

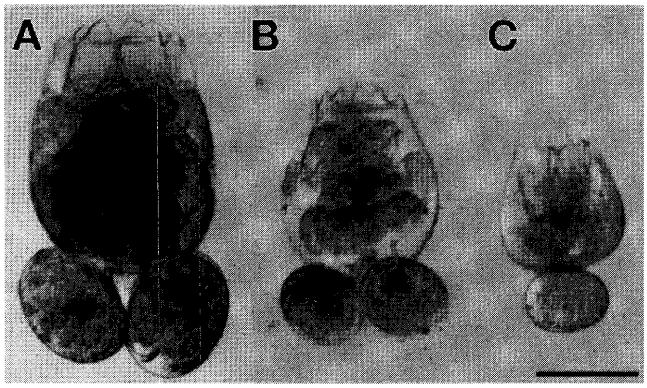

Fig. 1. Three euryhaline *Brachionus* rotifers examined in this study. (A) Ishikawa (ISKW) was collected from Japan, and (B) Lampung (LMPG) and (C) Manembo-Nembo (MNBO) were from the Sumatra and Sulawesi Islands, Indonesia, respectively. Scale bar, 100 μm.

PCR and DNA sequencing

Genomic and mitochondrial DNAs were extracted from the above Brachionus rotifers. Twenty individuals were rinsed twice with sterilized artificial seawater (Yoshinaga et al. 1999) and placed in a sterilized microtube with c.a. 10 ml of seawater. Subsequently, the rotifers were crushed in 500 ml of 5% Chelex 100 resin (Bio-Rad). The tubes were then incubated at 95°C for 15 minutes, and centrifuged at 15000 g for 15 minutes. Supernatants were used for subsequent polymerase chain reactions (PCRs).

A partial fragment of the COI gene was amplified as follows. In the reaction, the total volume of 20 μl contained 5 μl of template DNA, 2 μl of Ex*Taq* buffer (Takara), 0.2 mM each of the dNTPs, 0.5 μm each of the forward and reverse primers (Folmer et al. 1994), LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′), and 1U Ex*Taq* polymerase (Takara). PCR was carried out for 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by 72°C for 7 minutes with a GenAmp PCR System 9700 (Applied Biosystems). Amplification was confirmed by resolving the PCR resultant on an agarose gel. Because the amount of the PCR resultant produced by a single PCR amplification was not enough for subsequent direct sequencing, five μl of the 1/100 first PCR resultant was used for the second PCR under the above-mentioned conditions.

To remove the excess primers and dNTPs, the double-stranded PCR product was purified using an ExoSap-IT (USB). All sequencing procedures were performed for both the 5′ and 3′ strands of the purified PCR products according to the manufacturer's protocol (Applied Biosystems) with the same primers as those of the PCR. The labeled fragments were analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequence homology was examined using a BLAST search.

Phylogenetic analyses

To construct molecular phylogenetic trees of the rotifers, partial COI gene nucleotide sequences of the following eleven species/lineages were obtained from the database: *Asplanchna* sp., *Brachionus calyciflorus*, *B. ibericus*, *B.* Manjavacas (undescribed lineage), *B. plicatilis* sensu stricto, *B. rotundiformis*, *B. urceolaris*, *Keratella cochlearis*, *K. hiemalis*. *K. quadrata* and *Synchaeta pectinata*. The GenBank accession numbers are summarized in Table 1, and the details of each species/lineage are described in Gómez et al. (2002) and Derry et al. (2003). All the species examined in this study belong to the phylum Rotifera, class Monogononta, order Ploima (Integrated Taxonomic Integrated System). *Asplanchna* sp. was assigned as an outgroup in the molecular phylogenetic trees.

The nucleotide and deduced amino acid sequences of the COI gene from the total fourteen species/lineages were manually aligned using DNASIS-Mac v3.7 (Hitachi Software Engineering). To examine the saturation in the nucleotide substitutions, the number of transitions (TS) and transversions (TV) were calculated using PAUP* 4.0b5 (Swofford 2001), and were plotted against the gamma-corrected genetic distance (the HKY85 model by Hasegawa et al. 1985) with treepuzzle-50 (Schmidt 2000) from the COI gene nucleotide sequences, except for the sites at the third codon position.

The neighbor-joining (NJ) tree (Saitou and Nei 1987) was constructed using PHYLIP 3.57c (Felsenstein 1995) based on the above-mentioned gamma-corrected genetic distance.

Heuristic maximum-parsimony (MP) analysis with TBR (tree bisection-reconnection) branch swapping and 2000 random addition sequences was conducted using PAUP* 4.0b5 (Swofford 2001). Only the TS sites at the third codon position were excluded from the data set (see RESULTS). All phylogenetically uninformative sites were ignored.

Heuristic maximum-likelihood (ML) analysis with TBR branch swapping was conducted using PAUP* 4.0b5 (Swofford 2001). In the ML analysis, the data set contained the sites at the first and second codon positions only. The GTR+I+Γ model (Yang 1994) was selected as the best-fit one by Modeltest 3.06 (Posada and Crandall 1998). The ML parameters were optimized on the MP tree topology by the above-mentioned method, except for the data set of the sites at the first and second codon positions.

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                          10         20         30         40         50
B. plicatilis ISKW        GGTCTTATTG GCCTGAGTAT GAGATTTATC ATCCGATTAG AGTTAGGAGT
LMPG                      ........G. T.T.A..G.. A......T.A ..T..T.... ..C.T..T..
MNBO                      ...T.A.... .TT.A..A.. ...T...T.A ..T..T.... ..C.T.....
B. plicatilis sensu stricto ..G..C.... .T..T..G.. A......C.T ..T..TC.T. ..C....G..
B. rotundiformis          ...T.A.... .TT.A..... ...G...T.A ..T..T.... .AC.T..T..
B. ibericus               ..G....... .TT....A.. ...C...C.T .....T.... ..C.T..T..
B. Manjavacas             .......... .T..T..G.. A.....CC.T ..T..C.... .A........
B. calyciflorus           ...T.A.... .TT.A..A.. .......T.A ..T..T.... .......T..
B. urceolaris             ..CT.A..C. .GT.A..A.. A..G..CT.A ..T..CC... ..C.T..T..
S. pectinata              ...T.AG... .T..A..... A..T..AC.A ..T..T.... .A......A.
K. cochlearis             ...T....C. .T..A..... ...TC.CT.A ..T..TC... ..C.C..T..
K. quadrata               ...T.C..C. .T..A..A.. ....C..T.G .....T.... ..C....G..
K. hiemalis               ...T...... .T..T..... A..C..A..T ..T...C.T. .......T..
Asplanchna sp.            ...T.C.... .TT.A..A.. ...T..AT.A ..T..TC.T. .A.....T..

                          60         70         80         90         100
B. plicatilis ISKW        TGTAGGATCC TATCTAGGTG ATGAGCATCT TTACAATGTT CTAGTTACCG
LMPG                      ...C..T..T ...A.T.... ....A...T. A..T...... T.........
MNBO                      ...T..C..T ...T...... .C..A...T. A..T...... ........T.
B. plicatilis sensu stricto C.....C..T ..CT...... .C.....C.. ...T.....C T....G..T.
B. rotundiformis          ......T..T ..CT...... .C..A...T. A..T...... T.......T.
B. ibericus               A..T..T..T ...A.T.... .......... ...T...... A.T.......
B. Manjavacas             A..T..T..T ........G. ....A...T. A..T.....G .....C..A.
B. calyciflorus           A..T..T..T ...T....A. .......... ...T...... T....A..A.
B. urceolaris             ......T..G .....T.... .C..A..C.. .........A T.G.....T.
S. pectinata              ...T...C.T .T.T...... ....A...T. A..T...... T.......A.
K. cochlearis             ......TC.A .T.T.GTCA. .......... ...T.....C A.T..A..T.
K. quadrata               A..C..C..T ..CA.T..A. .C........ ...T..C... ..T.....A.
K. hiemalis               ......T... ...A.T.... ........A. ...T...... .....A..T.
Asplanchna sp.            ...T..TC.A .TCT...... .......CA. ...T...... ..T.....T.

                          110        120        130        140        150
B. plicatilis ISKW        CTCATGCATT TGTTATGATT TTCTTTATAG TTATGCCTGT GTCTATAGGA
LMPG                      .A.....G.. ......A... .......... .A..A..... T.........
MNBO                      ....C..T.. C......... ........G. .A.....C.. T.........
B. plicatilis sensu stricto .A..C..C.. ...A...... .....C.... .......... ...A..G...
B. rotundiformis          ....C..T.. ......A... .......... .......... C.........
B. ibericus               .C.....T.. C.....A... .......... ....A..A.. C.........
B. Manjavacas             .......T.. ...C...... .....C.... ....A..A.. T.........
B. calyciflorus           .......C.. ...A..A... .......... .......... C.........
B. urceolaris             .......... ...A...... .......... .......... T.....G..T
S. pectinata              .A.....T.. ...A..A... .......... ....A...A. T.........
K. cochlearis             .......C.. CA....A... ..T....... ........A. T........T
K. quadrata               .C.....T.. .A.C..A..C ..T....... .A..A..CA. T.........
K. hiemalis               .......... CA........ ..T....... ........A. C........T
Asplanchna sp.            .......T.. .A........ .......... ........A. C.....G..T

                          160        170        180        190        200
B. plicatilis ISKW        GGATTCGGTA ACTGATTAAT TCCTCTTATA TTAGGTGTTG CTGATATAGC
LMPG                      ..T..T.... .T.....G.. .........G ........A. .G........
MNBO                      ..G....... .T..G..G.. C...T.G... C....A.... ....C..G..
B. plicatilis sensu stricto .....T.... .T..G..... C.....G..G C......... ....C..G..
B. rotundiformis          ..G....... .T..G..... C.....A... .....A.... ..........
B. ibericus               ..T..T..A. .T..GC.T.. ....T.A..G .....A.... ..........
B. Manjavacas             ..G....... .....C.... .........G ..G..A..A. ..........
B. calyciflorus           ..T..T.... ....G..... .........G .....A..A. .A.....G..
B. urceolaris             ..T..T.... .T........ ....T.A... G......... .A........
S. pectinata              .....T.... .......... ....T.A..G C.......A. .A........
K. cochlearis             ..T..T.... .T..GC.T.. ...A..A..G .....G.... .......G..
K. quadrata               ..T..T..A. .T...C.C.. .........G C.T....... .A.....G..
K. hiemalis               ..T..T..G. ....GC.T.. ....T.G..G C.T....... .A........
Asplanchna sp.            ..G..T.... .T...C.T.. .........G ........C. ..........
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Fig. 2. Partial nucleotide sequences of mitochondrial DNA cytochrome *c* oxidase subunit I (mtDNA COI) gene of fourteen species/lineages of the rotifers. Dots indicate that the bases are similar with those of *B. plicatilis* ISKW. No gap was found in the aligned sequences.

Results

PCR and DNA sequencing

The PCR resulted in amplification of the fragment with approximately 700 base pairs (bp) that was consistent with the expected size (data not shown). Subsequently, the nucleotide sequences of the ISKW, LMPG and MNBO were determined (Fig. 2). The DNA extraction, PCR and DNA sequencing were repeated two to three times independently to confirm the accuracy of the results. The nucleotide sequences of the three

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                          210        220        230        240        250
B. plicatilis ISKW        CTTTCCACGC ATAAATAATC TATCCTTTTG ACTATTAGTA CCTGCTTTTA
LMPG                      T.....T..T .........T ....T..... .T....G..T ..........
MNBO                      T..C..T..T .......... .T..T..C.. GT.......G ..A..A....
B. plicatilis sensu stricto T..C..T..T .........CT .......... G..G.....G ..A..C....
B. rotundiformis          A.....T..T ..G....... .T..T..C.. .T.G.....T .....A....
B. ibericus               T..C..C..T .......... .G..A..C.. ....C.T... ..........
B. Manjavacas             T..C..C..T .........CT ....A..... ...G.....C ..A.....C.
B. calyciflorus           A.....T..A ..G......T ....T..... ...T.....T ..A.......
B. urceolaris             ...C..T..A ..G....... .T.....C.. .T.G.....T ..A......T
S. pectinata              T..C..T..T .........T ....T..C.. .T.....A.T .........T
K. cochlearis             T.....T..T .........T .G..A..C.. ...TC.TA.T ...T......
K. quadrata               T..C..T..T .......... .T..T..... ...TC.CT.T ...T.C....
K. hiemalis               T..C..C..T .....C.... .T..T..C.. G..TC.TC.C ...T......
Asplanchna sp.            T..C..T..T ..G.....CT ....T..... ...T.....T ...T......

                          260        270        280        290        300
B. plicatilis ISKW        TATTTCTTTT ACTGTCATCG GCTATCGATG CAGGTGCAGG AACTGGTTGA
LMPG                      .T..A...C. .T.A..T..A .....T.... .T....TT.. T.........
MNBO                      .....T.GC. T..T..T..C .....T..C. .T..A.TT.. T.........
B. plicatilis sensu stricto .G......C. GT....C..C .....T.... ....G..C.. T..C......
B. rotundiformis          .G.....AC. TT.A..T..T .....T.... .T..C.TT.. T.........
B. ibericus               .GC..T.A.. GT.A..C..T .....T.... .T..A..T.. T..A......
B. Manjavacas             .G......C. T..T..T..T .....T..C. ......TT.. ...A......
B. calyciflorus           .G...T.AC. T..T..C..A ..A..T.... ......TT.. C..A......
B. urceolaris             .C...T.AC. TT.A..T..T AT.T.A.... .....T... T.........
S. pectinata              ....A..... .T....TAGT AT.T.A.... ....A.T... ...A......
K. cochlearis             C...C...C. T..C..T..T AT.T.A...T ....G.TT.. ...C......
K. quadrata               CT........ ...C..T..T AT.T.A.... ......TT.. T.....A..G
K. hiemalis               CT......C. TT.A..T..T .T.C.T.... .T..A.TC.. T..A..A...
Asplanchna sp.            .G.....AC. CT.A..C..T AT.T.A.... .T....TT.. T.........

                          310        320        330        340        350
B. plicatilis ISKW        ACTGTTTACC CTCCTTTATC TGACTCTACA TATCATGCAG GTGTTTCAGT
LMPG                      .......... .......... A........T ........T. .......G..
MNBO                      .....C..T. .....C.C.. ...T.....T ........T. ..........
B. plicatilis sensu stricto ..C..G.... .A........ G..T.....T .....C.... .......T..
B. rotundiformis          .......... .A..CC.... A..T.....T ........T. .A........
B. ibericus               ..A....... ....AC.T.. A..T.....T ..C.....T. .......T..
B. Manjavacas             ..A.....T. .....C.T.. .........G .....C.... .A..A..T..
B. calyciflorus           ........T. .....C.... ...T..C.G. ..C...AG.. ..A.......
B. urceolaris             ..A..C.... .......... ...T.....T ......AG.. .G.....T..
S. pectinata              ...A....T. .......... ...T....A. .T....T.T. ..A....T..
K. cochlearis             .......... .C..CC.C.. ...T....AG ..C...T.C. ..A.......
K. quadrata               .......... .......... C..T....A. .TC..CAG.. .......T..
K. hiemalis               ..G.....T. .C...C.T.. ...T....A. .T....AG.. .GA....T..
Asplanchna sp.            .....G..T. .......... .......... ......T.T. ..A.C..T..

                          360        370        380        390        400
B. plicatilis ISKW        AGATTTAGCT ATTTTTAGTT TACATCTATC AGGTATTTCT TCTATCCTAG
LMPG                      T......... .........C .T..C..T.. T..G...... .....T..T.
MNBO                      T......... .......... .....T.... T......... ....TT.G.
B. plicatilis sensu stricto T........G .........CC ....C..... T.....C... .....T....
B. rotundiformis          T......... .......... .....T.... G........A .....T..T.
B. ibericus               G.....G... .......... .....T.... T......... ......T...
B. Manjavacas             ....C.G... .........AC ....CT.... ...C...... .....T..T.
B. calyciflorus           .......... ........GC .T...T.... T......... .....TT...
B. urceolaris             T...C..... .......... .....G.T.. T...G..... .....TT...
S. pectinata              T........A .......... .....T.... T..A...... .....TT...
K. cochlearis             T...C.T... .........C .C......G. ...A...... ..G..T..T.
K. quadrata               .........A ..C.....CC .C..C...G. T........C .....TT...
K. hiemalis               ....C.T... ..C.....CC .T...T..G. T......... .....TT...
Asplanchna sp.            T......... .........C .....T..G. T...G..... ......T...
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Fig. 2. Continued.

rotifers showed more than 95% similarity with those of the COI gene of the other rotifers in the database. Thus, we could successfully determine the nucleotide sequences of the COI gene for the ISKW, LMPG and MNBO.

Phylogenetic analyses

Five-hundred ninety-seven bp of the COI gene nucleotide sequences and 199 residues of the deduced amino acid sequences of the ISKW, LMPG and MNBO were aligned with those of eleven species/lineages from four genera (Figs. 2 and 3). No insertion or deletion was found in either the nucleotide

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                          410        420        430        440        450
B. plicatilis ISKW        GAAGAATTAA CTTTTTAACT ACTATTATTT GCTCTCGTAC TACGAAAAGA
LMPG                      .T........ T..C...... .......... .T........ ...T......
MNBO                      .T........ T......... .....C.... .T........ ...A..G...
B. plicatilis sensu stricto .T.....C.. ....C.T... .......... .T........ ...A..G..G
B. rotundiformis          .T........ T...C..... .......... .T........ ...T..G...
B. ibericus               .T........ ...CC.T... ..G.....C. .T.....A.. ...T.....T
B. Manjavacas             .T..G..... ...CC..... .......... ....C..... ...A..G..G
B. calyciflorus           .T........ T......... ..A.....C. .T........ A..A..G...
B. urceolaris             .T........ .......... .......... .......... A..T......
S. pectinata              ....T..... T...A..... .......... .T........ ...T..G.T.
K. cochlearis             .C........ T...C.T... ..C...G... .T........ ...T...GTT
K. quadrata               .......C.. ......G..A .....C.... ....A..... .G.C...GCT
K. hiemalis               .T.....C.. ...CC.T... ...G...... .T.....A.. AG.....GC.
Asplanchna sp.            .G........ ....C....A .......... .T........ ...T..GCTT

                          460        470        480        490        500
B. plicatilis ISKW        GTCTCTCTAG ATCGATTACC TTTAATGCTT TGAGCTATTG CTGTGACTGC
LMPG                      ..T...T.G. ....T..... .......... .......... .A..T.....
MNBO                      ..T...T... ....T..... .........A .....A.... .A..T.....
B. plicatilis sensu stricto ......T... .C..TC.G.. C......... .......... ....T..G..
B. rotundiformis          ..G...T... ....T..... .......... .....A..C. .G..A.....
B. ibericus               ..T...T... ....CC.T.. .......T.A ..G..A..C. ....T.....
B. Manjavacas             ..T...T... ....T..G.. AC.......A .....A.... .......A..
B. calyciflorus           A.T...T... ....T..G.. ....T.CT.A ......G... ....A..A..
B. urceolaris             A.....T... ....CA.G.. .......T.G ........C. ....T..A..
S. pectinata              A.TAG.A... ....T..... .......T.A ...T.AG.A. G...T.....
K. cochlearis             A.T..C..T. ....TA.G.. CC.T..A... ........CT .A..T.....
K. quadrata               A.T...T... .C...A.G.. .C.T...... ......C..T ..A.T..A..
K. hiemalis               A.T.....C. .....A.G.. .C....A..A ......T.AT ..A.T.....
Asplanchna sp.            A.T.....T. ....T..... .C.GT.T..A .......... ....T.....

                          510        520        530        540        550
B. plicatilis ISKW        AGTTCTTTTA ATTACTAGTT TGCCTGTTTT AGCGGGAGCT ATTACTATGT
LMPG                      ...CT.AC.. ..C.....A. .A........ ...T..C... ........AC
MNBO                      .......... ..C.....AC .T.....CC. T..A..T... .........C
B. plicatilis sensu stricto T.....CC.. ........AC .T..A..GC. ...T..G... ..C..A...C
B. rotundiformis          ....T.A... ........AC .T......C. T..A..T... .....A...C
B. ibericus               ...A...C.T ........A. .A.....GC. T..C..T... ..C.......
B. Manjavacas             T..CT.A... ..C.....GC .T......C. ...A...... ........AC
B. calyciflorus           .A..T.A... ........GC .T..A..A.. ...T..T... .....A...C
B. urceolaris             TA.....C.. ........GC .T........ ...T..T... .........C
S. pectinata              TT.CT.A... T.A....C.. .A........ ...T..T... .....A..A.
K. cochlearis             T........G ........AC .......... G..T..T..C ..C..C...C
K. quadrata               T......... .........C .A..C..C.. .......... ..C......C
K. hiemalis               T..CT.A... .........C .T.....C.. ...A..T... ..C..A..AC
Asplanchna sp.            .......C.T ......TC.C .T......C. ...T..T... ..........

                          560        570        580        590      597
B. plicatilis ISKW        TACTTACTGA CCGTAATTTC AACACCTCTT TTTTTGACCC TGCTGGT
LMPG                      .T.....A.. T.........T ..T.....C. .......T.. ...A...
MNBO                      .T..A..... T.........T .....T.... .......T.. ...G...
B. plicatilis sensu stricto .CT.A..A.. T.........T ..T..T..C. .C.....T.. ...C...
B. rotundiformis          .TT.A..A.. T.........T .....T..G. .C.....T.. ...A...
B. ibericus               .......... .......... ..T..T.... .C.....T.. C..A...
B. Manjavacas             .......A.. T.........T .......... .C.....T.. A..A..C
B. calyciflorus           .TT.A..... T.........T ..T..T.... .C.....T.. ...A...
B. urceolaris             .TT.A..... T..C.....T ..T..T..A. .C.....T.. .......
S. pectinata              ..T.A..... T.........T ..T..A..A. .C.....T.. AT....A
K. cochlearis             .TT.A..C.. T......C.T ..T..T..C. .C........ .......
K. quadrata               .T.....A.. T.....C..T ..T..T..C. ....C..T.. A..A..A
K. hiemalis               .T.....A.. T..A.....T ..T..T.... .C..C..T.. G..A..A
Asplanchna sp.            ..T.A..... T.........T ..T..... .C.....T.. .T....G
```

Fig. 2. Continued.

or amino acid sequences.

The number of TS plotted against the gamma-corrected genetic distance suggested that the nucleotide substitutions at the sites of the third codon position of the rotifer's COI gene were saturated (Fig. 4A). Further analysis revealed that at the third codon position, the TV have not yet been saturated (Fig. 4B).

The NJ tree showed the monophyly of the two genera, *Brachionus* and *Keratella* (Fig. 5). Within the genus *Brachionus*, one freshwater species, *B. urceolaris*, was the most basal species, and subsequently another freshwater species, *B. calyciflorus*, and other seawater species/lineages, *B. plicatilis*,

```
                          10         20         30         40         50
B. plicatilis ISKW        GLIGLSMSFI IRLELGVVGS YLGDEHLYNV LVTAHAFVMI FFMVMPVSMG
LMPG                      ..MV.....L .......... .I........ .......... ..........
MNBO                      .........L .......... .......... .......... ..........
B. plicatilis sensu stricto .........L .......... .......... .......... ..........
B. rotundiformis          .........L .......... .......... .......... ..........
B. ibericus               .........L .......... .I........ I......... ..........
B. Manjavacas             .........L .......... .......... .......... ..........
B. calyciflorus           .........L .......... .......... .......... ..........
B. urceolaris             .........L .......... .......... .......... ..........
S. pectinata              ..V.....LL ......I..P F......... .......... ......I...
K. cochlearis             .F......LL .........P F.S....... I......I.. ......I...
K. quadrata               .F......LL .......... .I........ .......I.. ......I...
K. hiemalis               .F......L. .......... .I....I... .......I.. ......I...
Asplanchna sp             .F......LL .........P F.....I... .......I.. ......I...

                          60         70         80         90         100
B. plicatilis ISKW        GFGNWLIPLM LGVADMAFPR MNNLSFWLLV PAFMFLLLSS AIDAGAGTGW
LMPG                      .......... .......... .......... ...IL..... .....V....
MNBO                      .......... .......... .......... .......... .....V....
B. plicatilis sensu stricto .......... .......... .......... .......... ..........
B. rotundiformis          .......... .......... .......... .......... .....V....
B. ibericus               .......... .......... .......... ....L..... ..........
B. Manjavacas             .......... .......... .......... .......... .....V....
B. calyciflorus           .......... .......... .......... .......... .....V....
B. urceolaris             .......... V......... .......... ...F...... IL...V....
S. pectinata              .......... .......... .........I ...LL..... IL...V....
K. cochlearis             .......... .......... .........I .S.T...... IL.S.V....
K. quadrata               .......... .......... .........F .S.T...... IL...V....
K. hiemalis               .......... .......... .........L .S.T...... VL...V....
Asplanchna sp             .......... .......... .......... .S........ IL...V....

                          110        120        130        140        150
B. plicatilis ISKW        TVYPPLSDST YHAGVSVDLA IFSLHLSGIS SILGSINFLT TIICSRTTKS
LMPG                      .......... .......... .......... .......... ..........
MNBO                      .......... .......... .......... .......... ..........
B. plicatilis sensu stricto .......... .......... .......... .......... ..........
B. rotundiformis          .......... .......... .......... .......... ..........
B. ibericus               .......... .......... .......... .......... ..........
B. Manjavacas             .......... .......... .......... .......... ..........
B. calyciflorus           .........S ..S.I..... .......... .......... ..........
B. urceolaris             .......... ..S....... .....V..V. .......... ..........
S. pectinata              .I.......K F.S.I..... .......... ........M. .........M
K. cochlearis             .........K ..S.I..... ......A... .......... ..V......V
K. quadrata               .........K F.S....... ......A... .......... .......A.A
K. hiemalis               .........K F.S.I..... ......A... .......... .V.....A.A
Asplanchna sp             .......... ..S.I..... ......A.V. .......... .........L

                          160        170        180        190        199
B. plicatilis ISKW        VSLDRLPLML WAIAVTAVLL ITSLPVLAGA ITMLLTDRNF NTSFFDPAG
LMPG                      .......... .......... .......... .......... .........
MNBO                      .......... .......... .......... .......... .........
B. plicatilis sensu stricto .......... .......... .......... .......... .........
B. rotundiformis          .......... .......... .......... .......... .........
B. ibericus               .......... .......... .......... .......... .........
B. Manjavacas             .......... .......... .......... .......... .........
B. calyciflorus           I.......F. ..V....I.. .......... .......... .........
B. urceolaris             I....M.... .......I.. .......... .......... .........
S. pectinata              I.M....... .SVG...F.. L.T....... .......... .......S.
K. cochlearis             I....M.... ...S...... .......... .........L .........
K. quadrata               I....M.... ..LSI..... .......... .......... .........
K. hiemalis               I....M.... ..LSI..... .......... .......... .........
Asplanchna sp             I.......F. .......... .......... .......... .......S.
```

Fig. 3. Deduced amino acid sequences of the partial mtDNA COI gene of the rotifers. Dots indicate that the residues are similar to those of *B. plicatilis* ISKW.

B. Manjavacas, *B. ibericus*, *B. rotundiformis* and the two Indonesian rotifers, were derived. Thus, the euryhaline *Brachionus* rotifers formed a monophyleic group, and were further separated into three groups: the *B. plicatilis* plus *B.* Manjavacas group, the *B. ibericus* group and the *B. rotundiformis* group. The two Indonesian rotifers, LMPG and MNBO, formed sister relationships with *B. ibericus* and *B. rotundiformis*, respectively (Fig. 5). Among the three groups, each species/lineage shared approximate lorica lengths with its sister species/lineage (Table 1).

The topology of the MP tree was almost the same as that of the NJ tree with one exception (Fig. 6). The monophyly of the two genera, *Brachionus* and *Keratella*, was consistent with that of the NJ tree (Fig. 5). In the genus *Brachionus*, the fresh-

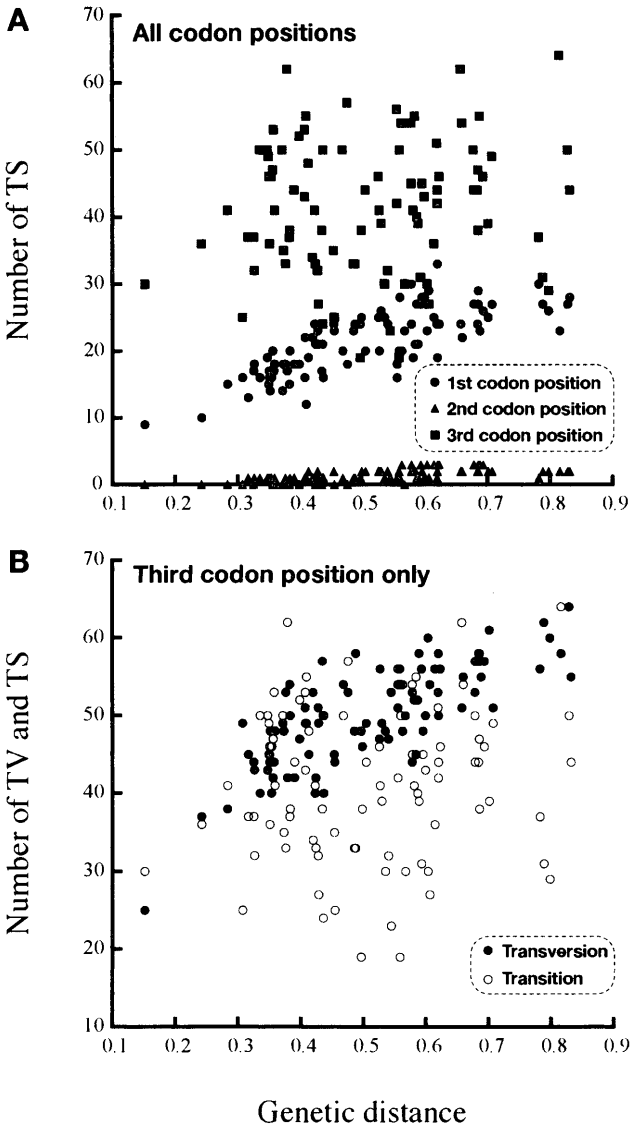

Fig. 4. (A) Number of transitions (TS) at each codon position, and (B) number of TS and transversions (TV) at the third codon position, plotted against the gamma-corrected genetic distance (HKY85 by Hasegawa et al. 1985).

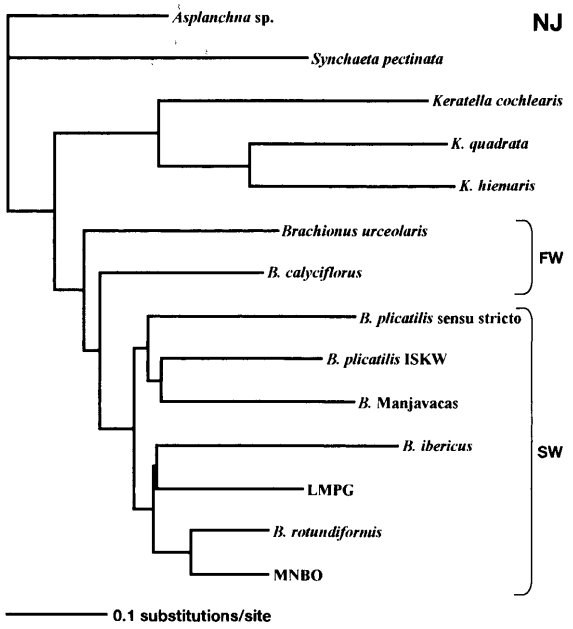

Fig. 5. Neighbor-joining (NJ) tree of the fourteen species of the rotifers using the partial COI gene nucleotide sequences. The sites at the third codon position were excluded from the analysis. FW and SW indicate freshwater and seawater habitats, respectively.

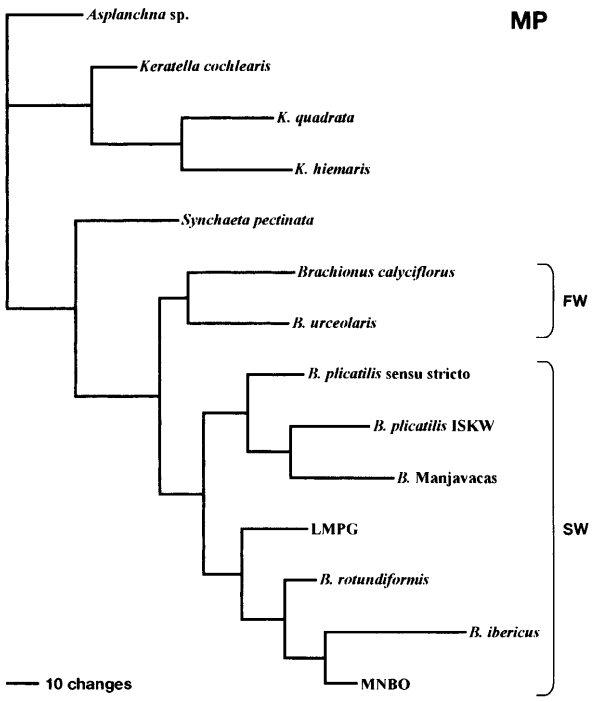

Fig. 6. Maximum-parsimonious (MP) tree of the fourteen species of the rotifers using the partial COI gene nucleotide sequences (length, 970 steps; consistency index, 0.457; retention index, 0.292; rescaled consistency index, 0.133). The sites of transition (TS) at the third codon position were excluded from the analysis. FW and SW indicate freshwater and seawater habitats, respectively.

water and seawater groups formed the monophyleic clade. The difference between the NJ and MP tree topologies was found in the clade of the euryhaline *Brachionus*. The monophyly of the *B. plicatilis* plus *B.* Manjavacas group was observed, whereas the *B. ibericus* and *B. rotundiformis* groups were not separated (Fig. 6).The two Indonesian rotifers were involved in the *B. ibericus* plus *B. rotundiformis* group.

The ML tree showed a significantly different topology than the NJ and MP trees (Fig. 7). Even the monophyly of the two genera, *Brachionus* and *Keratella*, was consistently observed among the three trees (Figs. 5–7); two major differences were found in the *Brachionus* clade of the ML tree: (1) in the ML tree, the seawater species were derived first, fol-

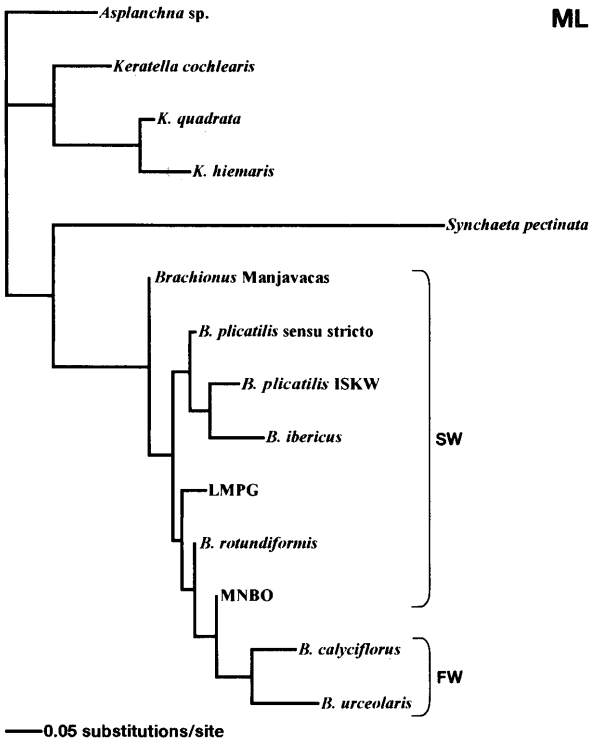

Fig. 7. Maximum-likelihood (ML) tree of the fourteen species of the rotifers using the partial COI gene nucleotide sequences (−ln likelihood, 1437.8). All sites at the third codon position were excluded from the analysis. The GTR+I+Γ model by Yang (1994) was used as a molecular evolutionary model. SW and FW indicate seawater and freshwater habitats, respectively.

lowed by a divergence of the freshwater species, which was the converse of the order in the case of the NJ tree (Fig. 5); and (2) among the euryhaline *Brachionus*, each species/lineage tended to be derived separately, and therefore significant clades were not observed (Fig. 7).

In this study, we adopted the NJ and MP trees and rejected the ML tree according to the ecological and morphological information (see DISCUSSION).

Discussion

Indonesian rotifers

To our knowledge, this is the first report presenting the DNA sequences of the Indonesian rotifers. The past rotifer samplings have been biased, and currently available information about the rotifer's biogeography substantially reflects the distribution of rotifer research mostly in the temperate and subarctic zones (Nogrady et al. 1993). The biogeography of the tropical zone has been studied a great deal because of its extraordinary species richness (Roberts et al. 2002), and therefore we believe that the present study would stimulate future investigations of the rotifers in the tropical zone. So far, no extensive field survey has been carried out in the tropical zone, but it is known that *Brachionus* rotifers commonly inhabit Indonesian waters with various thermal and saline conditions (I. F. M. R., personal observation). The rotifers from such different environments are important not only for taxonomic and ecological studies, but also as a potential live food with unique features.

In this study, we determined the partial COI gene nucleotide sequences of the two Indonesian *Brachionus* rotifers in order to examine their phylogenetic positions within the genus *Brachionus*. In the NJ and MP trees, the LMPG and MNBO were found to be phylogenetically close to small species of the genus *Brachionus* (Figs. 5 and 6). The LMPG and MNBO each formed sister relationships with *B. ibericus* and *B. rotundiformis*, respectively, in the NJ tree, whereas the MP tree did not support these relationships. The LMPG and MNBO have approximately the same lorica length as their sister species in the NJ tree, suggesting that these two Indonesian rotifers are likely to be kin to *B. ibericus* and *B. rotundiformis*, respectively (Table 1). There has been a debate about the taxonomy of small *Brachionus* species (see below), and further investigations such as studies of the morphometry and mating behavior of the Indonesian rotifers are neccesary to elucidate the taxonomy of these rotifers.

Taxonomy of the genus *Brachionus*

The euryhaline *Brachionus* is proposed to involve at least three species, *B. plicatilis* sensu stricto, *B. ibericus* and *B. rotundiformis* (Ciros-Pérez et al. 2001). These three species have different lorica lengths of approximately 300, 200 and 150 μm, respectively (Table 1). Another classification by Hagiwara et al. (1995) divides the euryhaline *Brachionus* into three groups, the L strain with 130–340 μm, the S strain with 100–210 μm and the SS strain with 90–150 μm, respectively. In addition to the lorica length, these three strains can be discriminated by their corona shape, because the smaller lineages have more acute spines (Hagiwara et al. 1995). Although a comparative study between these two classifications has not yet been performed, *B. plicatilis*, *B. ibericus* and *B. rotundiformis* in Ciros-Pérez et al. (2001) may correspond to the L, S, and SS strains in Hagiwara et al. (1995), respectively (notice that the S and SS strains are the SM and S strains, respectively, in Ciros-Pérez et al. 2001).

Assuming that the three species/strains correspond between the two classifications, a difference can be found in the smallest group. Ciros-Pérez et al. (2001) recognized that *B. plicatilis*, *B. ibericus* and *B. rotundiformis* are all different species. Hagiwara et al. (1995) also proposed that the L (*B. plicatilis*) and S (*B. rotundiformis*) strains should be different

species, but they concluded that the SS strain could be included in the S strain based on the following observation. Copulation occurred between the S and SS strains, whereas reproductive isolation existed between the L and either the S or SS strains (Hagiwara et al. 1995). In the NJ tree of this study, the euryhaline *Brachionus* was divided into three groups, the *B. plicatilis* plus *B.* Manjavacas group, the *B. ibericus* group and the *B. rotundiformis* group (Fig. 5). Moreover, the lorica lengths of the species/lineages in each group were found to be close to each other (Table 1). The results from the NJ tree were thus consistent with the classification by Ciros-Pérez et al. (2001). On the other hand, in the MP tree, the euryhaline *Brachionus* were divided into two groups, the *B. plicatilis* plus *B.* Manjavacas group and the group involving *B. ibericus* and *B. rotundiformis* (Fig. 6), which may support the observation of Hagiwara et al. (1995). Accordingly, our results seem to be insufficient to provide further information to resolve the disagreement between the two classifications of the euryhaline *Brachionus*.

The genetic distance is another measure of the species definition because it reflects the degree of gene flow among the populations. In this study, the pairwise comparison of the genetic distance showed a high divergence in the genus *Brachionus*, even between closely related species/lineages (Table 2). The genetic distance between *B. plicatilis* ISKW and *B. plicatilis* sensu stricto was 0.3791, and it was larger than the distance between *B. plicatilis* ISKW and *B. rotundiformis*, which was 0.3590 (Table 2). Moreover, the distance of 0.3515 between *B. plicatilis* sensu stricto and *B. rotundiformis* was also smaller than the distance between the two lineages of *B. plicatilis*, which was 0.3791 (Table 2). Even in *B. plicatilis* and *B. rotundiformis*, which are widely accepted as different species (Hagiwara et al. 1995, Segers 1995, Ciros-Pérez et al. 2001), the intraspecific variations are larger than the interspecific one. The COI gene has been analyzed in molecular phylogenetic studies of the genus *Brachionus* (Gómez et al. 1995, 2000, 2002, Derry et al. 2003); however, our results suggest that the locus is unlikely to be informative for the purpose of taxonomic diagnosis because the variation does not provide a threshold between intra and interspecies difference. Two protein coding genes, *hsp82* and *tbp*, and two ribosomal genes in *B. plicatilis* have unique base compositions (high GC content), and these may be due to the unusual evolution of this species (Welch 2001). If the same evolution had occurred in the COI gene of *B. plicatilis*, its nucleotide substitution may be inadequate for comparison with those of the other species in the genus *Brachionus*.

Presumably, given species names may also cause the confusing results. In this study, *B. plicatilis* ISKW was phylogenetically closer to *B.* Manjavacas than to *B. plicatilis* sensu stricto (Figs. 5 and 6). The genetic distance of 0.3492 between *B. plicatilis* ISKW and *B.* Manjavacas was also smaller than the distances between these two lineages and *B. plicatilis* sensu stricto, which were 0.3791 and 0.4073, respectively (Table 2). Consistent with these results, the lorica length of *B. plicatilis* ISKW (247.6 μm) is closer to that of *B.* Manjavacas (260 μm) than to that of *B. plicatilis* sensu stricto (299.0 μm) (Table 1). Thus, *B. plicatilis* ISKW is both genetically and morphologically closer to *B.* Manjavacas than to another lineage of the same species, *B. plicatilis* sensu stricto (Fig. 5; Table 1). On the other hand, Gómez et al. (2002) proposed that *B.* Manjavacas is an undescribed new species based on the large genetic differences in both the mitochondrial COI and nuclear ITS1 (ribosomal internal transcribed spacer 1) loci between *B. plicatilis* and *B.* Manjavacas. There are two possible causes of the incongruent consequence: (1) the three lineages are the same species, and the genetic distances observed among the three lineages are within the range of the intraspecific variation (a case of synonym); or (2) *B. plicatilis* ISKW and *B. plicatilis* sensu stricto are different species, and the former is the same species as or a species allied with *B.* Manjavacas (a case of homonym). It should be noted that the

Table 2. Pairwise genetic distance (HKY85 model by Hasegawa et al. 1985; low) and total number of nucleotide substitutions (upper) among *Brachionus* as revealed by partical mtDNA COI gene nucleotide sequences.

	plicatilis ISKW	LMPG	MNBO	*plicatilis* sensu stricto	*rotundiformis*	*ibericus*	Manjavacas	*calyciflorus*	*urceolaris*
plicatilis ISKW	—	99	110	122	109	115	111	114	115
LMPG	.3168	—	96	119	85	111	108	106	113
MNBO	.3504	.2837	—	113	64	109	114	101	109
plicatilis sensu stricto	.3791	.4093	.3474	—	109	130	121	118	120
rotundiformis	.3590	.2423	.1515	.3515	—	107	113	93	100
ibericus	.4345	.3834	.3353	.4756	.3762	—	127	121	127
Manjavacas	.3492	.3828	.3576	.4073	.3715	.4681	—	112	139
calyciflorus	.4196	.3730	.3253	.4077	.3076	.4878	.3559	—	104
urceolaris	.4279	.3904	.3538	.4129	.3266	.5272	.5600	.3508	—

taxonomic confusion can occur even in the lineages of *B. plicatilis* that have been described in numerous articles.

Future perspective for taxonomy and phylogeny of the rotifers

The taxonomic studies of the euryhaline *Brachionus* have been carried out using various techniques such as morphometry, cross breeding, bioassay with a mate-recognition pheromone, and genetic distances revealed by allozyme pattern and nucleotide sequences (Fu et al. 1991, Hagiwara et al. 1995, Ortells et al. 2000, Ciros-Pérez et al. 2001, Gómez et al. 2002). However, as mentioned above, the taxonomic problem still exists, mainly for the following two reasons. First, *a priori* presumed species names have been carelessly given to various lineages from distinct localities, and far less effort has been spent on the taxonomic examination than the collection of a huge number of specimens. Second, no study has ever covered a wide enough range of the habitat of the euryhaline *Brachionus*, and therefore estimation of the potential numbers of species and lineages is not available.

To solve these problems, first, we have to know the extent of genetic variation in the genus; otherwise, nomenclature mistakes such as synonyms and homonyms inevitably occur. The species name should not be given until after further examination, and should be tentatively described with a specific strain name. In addition, prior to calculation of the genetic variations, the lineages should be collected from virtually all habitats to avoid misestimation of the gene pool. The new information on the Indonesian rotifers provided by this study will contribute to more accurately estimating the genetic variation of the euryhaline *Brachionus* rotifers.

With the properly determined genetic variation of the group, the monogonont rotifers are an ideal organism for determining the threshold for species definition, because the cross mating test has the ability to prove the existence of the reproductive barrier. Further, the mate recognition pheromone may be useful for detecting the reproductive isolation among sympatric species (Snell 1998). Much attention has been paid to the genetic variation in efforts to classify closely related species by a unique DNA-barcode for each species (Hebert et al. 2003). However, the variations in the DNA sequences themselves are not the key element of the species definition, and a simple sequence comparison for homogeneity might lead to a meaningless conclusion in terms of biological significance. Accordingly, the combinational use of the cross-mating test and the DNA-barcode is one potential approach, and it will be important for elucidating the *Brachionus* complex.

In this study, we constructed molecular phylogenetic trees by the NJ, MP and ML methods. Even though the NJ and MP trees showed an almost identical topology (Figs. 5 and 6), the ML tree topology was significantly different from the other two tree topologies (Fig. 7). Based on the fact that most species of the phylum Rotifera inhabit freshwater (Nogrady et al. 1993), and on the consistency of the topology with the classification based on the lorica length (Table 1), we adopted the NJ and MP trees as the molecular phylogenetic tree of the rotifers as revealed by the partial DNA sequences of the COI gene. In the future, increasing the data set with the other loci of both the mtDNA and nuclear DNA will be essential for obtaining a more rigorous molecular phylogenetic tree that is independent of inherently variable morphological and ecological characteristics.

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