Zoological Studies
a SpringerOpen Journal

RESEARCH Open Access

A new intertidal *Brachionus* and intrageneric phylogenetic relationships among *Brachionus* as revealed by allometry and *CO1-ITS1* gene analysis

Dae-Sik Hwang[1], Hans-Uwe Dahms[2], Heum Gi Park[3] and Jae-Seong Lee[1,4*]

Abstract

Background: The rotifer genus *Brachionus* is distributed worldwide along a gradient from freshwater to seawater habitats. This genus is recognized as a suitable organism for testing environmental and evolutionary hypotheses. Here we present the description of a new species and the phylogenetic relationship within the genus *Brachionus* as revealed by morphometric allometry of two representatives and *CO1-ITS1* gene analysis of five representatives belonging to *Brachionus* distributed in the fresh and seawaters of Korea, Japan, and China.

Results: Similarities of populations were studied using nuclear rDNA *ITS1* sequences from *Brachionus* spp. collected from different geographical areas of Far East Asia. The phylogeographic analysis of nuclear DNA *ITS1* and mitochondrial *CO1* sequences showed that *Brachionus* from South Korea formed five distinct clades according to their geographic origin. Interspecific differences suggest that *Brachionus* species established on conventional morphological characters also forms five separate clades. When *ITS1-CO1* of the Asian specimens was compared with representatives of *Brachionus* worldwide, a high genotypic similarity was found. However, they slightly differed between localities.

Conclusions: This study sets a first step for an integrative morphological and molecular characterization of the diversity contained within the ecologically and economically important rotifer genus *Brachionus*.

Keywords: Rotifera; *Brachionus*; rDNA ITS1; *CO1*; Morphometry; Phylogenetics

Background

Rotifers comprise several groups such as the marine Seisonida, Monogononta, and parthenogenetic Bdelloidea. Among them, the monogonont genus *Brachionus* spp. is widely distributed in marine and freshwaters worldwide (Hagiwara et al. 1995; Gómez and Snell 1996). Rotifers are considered as model species in diverse research areas such as aquaculture, ecophysiology, and ecotoxicology (Snell and Janssen 1995; Lubzens et al. 2001; Dahms et al. 2011).

The *Brachionus* species described formally here as *Brachionus koreanus* sp. nov. was studied before as a *nomen nudum*. Hwang et al. (2013) reported the complete mitochondrial DNA from *Brachionus plicatilis* and a Korean population here described as *B. koreanus* sp. nov. Concerning genetic approaches, Suga et al. (2008) described the mitogenome of *B. plicatilis* from Japan. The mitochondrial DNA of both species consisted of two circular mitochondrial genomes with a rearrangement of *tRNA-Cys* between *tRNA-Arg* and *tRNA-Ile*, whereas other protein-coding genes, tRNAs and rDNAs, were conserved in their placement between the two species. Min and Park (2009) reported the complete mitochondrial genome sequence of *Rotaria rotatoria* (Bdelloidea: Rotifera: Syndermata), having a single circular mitochondrial genome. Thus, the structure of the complete mitochondrial genome and the gene order is not conserved among different rotifer taxa, as shown in *Brachionus* and the bdelloid *R. rotatoria*. As for the closest taxon to the Rotifera, the Acanthocephala, Steinauer et al. (2007) showed a difference of the mitogenome of the acanthocephalan *Leptorhynchoides thecatus.*

* Correspondence: jslee2@hanyang.ac.kr
[1]Department of Molecular and Environmental Bioscience, Graduate School, Hanyang University, Seoul 133-791, South Korea
[4]Department of Chemistry, College of Natural Sciences, Hanyang University, Seoul 133-791, South Korea
Full list of author information is available at the end of the article

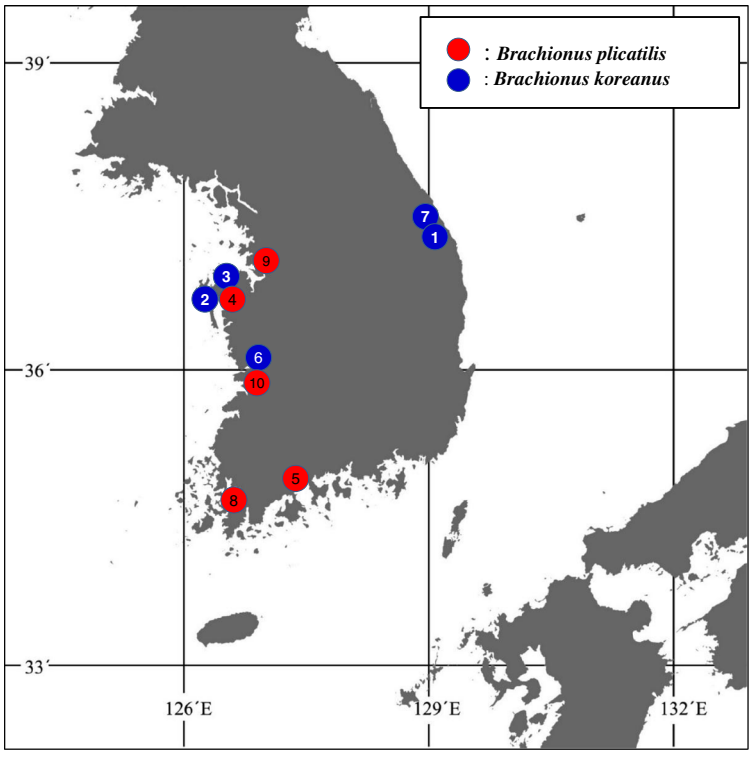

Figure 1 Geographic distribution of sampling sites of *B. koreanus* and *B. plicatilis*. The figure shows the geographic distribution in the Far East western Asian study area.

Morphological similarity (i.e., cryptic or sibling species) is recognized in several organisms (Serra et al. 1998; Chullasorn et al. 2009). This holds also for rotifers where several species are genetically distinct but reveal only little morphological dissimilarity (Walker 1981). Regarding the morphological taxonomy of *Brachionus*, there is little information available, so misidentification might occur due to morphological ambiguity. In the case of *Brachionus*, no geophylogenetic analysis of representatives of *Brachionus* has been performed using mitochondrial and rDNA sequences. In this study, we analyzed the molecular phylogenetic relationships of rotifers, including five representatives of *Brachionus* (i.e., *B. plicatilis*, *Brachionus manjavacas*, *Brachionus rotundiformis*, *B. koreanus*, and *Brachionus*

Table 1 Morphometric characteristics of two *Brachionus* species

Strain		Mean of body length (μm)								
		a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
B. koreanus	Uljin-1 (S)	196.07	106.46	151.09	17.53	21.59	22.91	12.40	15.16	108.15
	Daesan	192.26	90.61	139.13	17.56	18.50	20.49	11.02	13.72	91.93
	Okgu	194.59	97.14	146.31	18.39	19.25	21.93	11.83	14.37	99.53
	Uljin-2 (L)	213.40	95.25	156.35	17.56	20.09	20.32	11.90	11.77	101.96
	Taean	212.46	99.45	162.46	20.17	20.25	19.62	12.57	13.67	102.34
B. plicatilis	China	276.22	127.55	210.38	29.90	26.41	26.17	14.83	16.67	139.65
	Hwasung	306.13	130.89	239.03	30.45	26.13	28.55	14.03	13.17	148.90
	Mokpo	277.25	119.98	202.01	28.53	23.78	27.83	13.38	15.98	129.33
	Buan	303.54	136.50	229.79	28.62	29.17	29.38	15.30	16.14	146.22
	Seosan	329.32	123.29	249.24	22.29	25.19	26.41	12.79	13.88	147.21
	Suncheon	300.24	122.26	218.95	30.46	25.62	26.38	14.16	14.43	133.31
	Japan	256.14	123.14	189.32	27.53	23.36	24.79	12.77	16.32	134.17

ibericus) that were inferred from rDNA *ITS1* and mitochondrial *CO1* sequences. Finally, we investigated the biogeography of Asian marine *Brachionus* spp. (*B. plicatilis* and *B. koreanus* sp. nov.) using morphometric allometry. These findings were compared with those from ITS1 and mitochondrial sequences.

Methods

Sample collection

The organisms used in this study were sampled from different locations as summarized in Figure 1. After sampling, rotifers were concentrated through filtration onto a 100-μm pore-sized mesh and preserved in 70% EtOH. The identities of species were initially checked by stereomicroscopy and further verified by genomic tools used herein (mt *COI* and rDNA *ITS1* sequence comparison). Samples were stored at 4°C until used. A total of 12 clones from the two sibling species of the genus *Brachionus* were investigated. The clones were obtained from the rotifer culture collection at the Hanyang University in Seoul. They consisted of parthenogenetically cultured strains based on the isolation of single amictic females. They were collected from several localities (Table 1). All the specimens used for the morphological description and morphometric analysis came from stock cultures that were also used for the morphometric and genomic analysis.

Culture conditions

Stock cultures were maintained at 25°C under a light–dark 12:12 h photoperiod, and salinity was 15 PSU. Animals were fed with the green algae *Chlorella* sp. (approximately 6×10^4 cells/mL) and maintained at constant light conditions, and the medium was renewed weekly for at least 1 month prior to experimentation. Saline water was made with commercial sea salt (TetraMarine Salt Pro, Tetra™, Columbus, OH, USA).

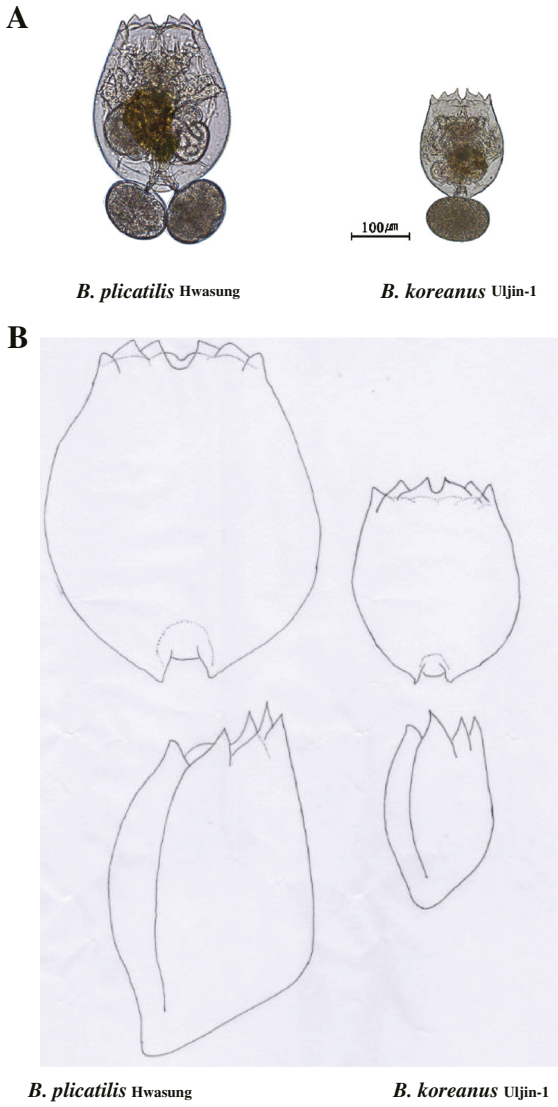

Figure 2 Characteristics and measurements of the lorica of *Brachionus* spp. (A) Photomicrography. **(B)** Drawing. Characters follow Fu et al. (1991).

Morphology, morphometry, and statistical analyses

Morphometric values were compared using animals of the same age. For each clone, several amictic egg-bearing females were taken from exponentially growing stock cultures. For each clone, ten triplicates (a total of 30 individuals) of fixed amictic egg-bearing females (48 h old) were randomly chosen, and a photo was taken under a fluorescence microscope before measurements. Nine characters of the lorica were measured under a fluorescent microscope (×20) with the measurement software MetaMorph (version 7.6). Seven of the characters used in the analysis (Figure 2) were selected on the basis of Fu et al. (1991), and two others were used in addition as we considered them to be of taxonomic importance.

All statistical analyses were performed using the SPSS program (release 10. SPSS Inc., Chicago, IL, USA). Two stepwise discriminating analyses were performed to discriminate among strains on previously log-transformed (Ln) measurements. According to the method of Fu et al. (1991) (Figure 2), we analyzed nine characters and used six characters to distinguish two *Brachionus* species (12 clones) as shown before by Ciros-Pérez et al. (2001) (Figure 3). Six of the characters measured include lorica length (a), lorica shape (c/a and i/a), the relative length of dorsolateral spines 2 and 3 (g/h), and length of spine 3 in relation to lorica length (h/a) (Figure 2). After measurement, one-way ANOVA was conducted to compare morphological differences among two sibling species (*B. plicatilis* and

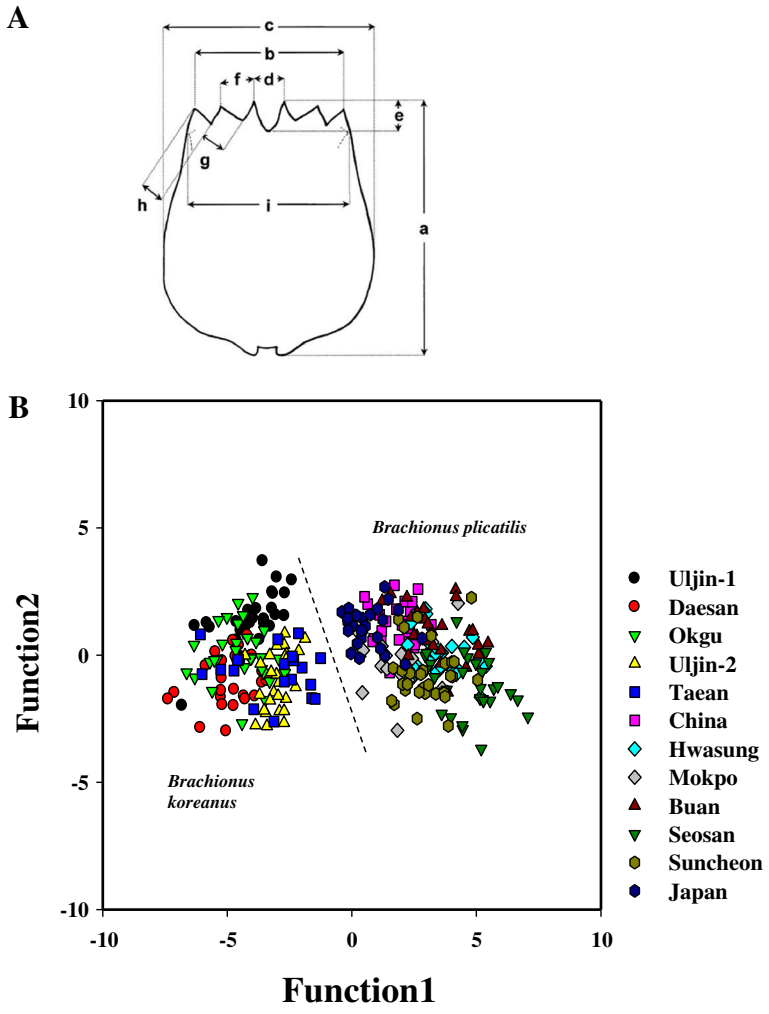

Figure 3 Scatterplot of the measured individuals in the space defined by two canonical discriminant functions. (A) Measurement of rotifer characterization. **(B)** Scatter-plot analysis of measured characters. The functions were retrieved from discriminant stepwise analyses of several lorica measurements of individuals belonging to two putative *Brachionus* spp. and 12 clones. We modified the method of Fu et al. (1991) according to Ciros-Pérez et al. (2001).

B. koreanus). When differences were found, post-hoc (multiple comparisons among means) Student-Newman-Keuls (Sokal and Rohlf 1969) tests were carried out.

CO1 and *ITS1 rDNA* sequences from geographic samples

For *CO1* and *ITS1* amplification from different geographical samples, we used pooled individuals of *Brachionus* specimens as DNA templates without genomic DNA extraction. *CO1* and *ITS1* rDNA was amplified by conventional polymerase chain reaction (PCR) protocols with its *CO1* and *ITS1* targeting primers (for *CO1* gene, Br-CO1-LCO1490, 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′, Br-CO1-HCO2198, 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′, 812 bp; for *ITS1*, Br-ITS1-III, 5′-CAC ACC GCC CGT CGC TAC TAC CGA TTG-3′ and Br-ITS1-VIII, 5′-GTG CGT TCG AAG TGT CGA TGA TCA A-3′, 596 bp). PCR thermocycling was as follows: 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 60 s, and a final extension at 72°C for 5 min. PCR products from 12 geographic samples were subcloned into a pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA, USA), and all the subcloned DNAs were sequenced according to the manufacturer's suggested protocol with commercial primers (e.g., T7 and M13R).

Editing and contig assembly of the rDNA sequences were carried out with Sequencher 4.1.4 (Gene Codes, Ann Arbor, MI, USA). All sequences determined here have been deposited at GenBank ([GenBank:GU987061-GU987072] for the *CO1* sequence and [GenBank:GU987073-GU987084] for the *ITS1* sequence).

Mitochondrial *CO1* sequence analyses

For genetic variation in five *Brachionus* spp., mitochondrial *CO1* sequences (see 12 isolates in Table 1) were subjected to a similarity analysis. The *ITS1* and *CO1* sequences were individually aligned with ClustalW ver.1.8 (Thompson et al. 1994). To distinguish five *Brachionus* species, we used six domains of the *CO1* gene as shown in Vasileiadou et al. (2009).

Phylogeography of *Brachionus* spp. inferred from *CO1* and *ITS1* rDNA

The phylogeography of Asian *Brachionus* spp. was studied with mitochondrial *CO1* (812 bp) and nuclear *ITS1* rDNA (596 bp) sequences. To place the identified *CO1-ITS1* rDNA (1,408 bp) in the phylogenetic tree, we aligned them with those of other species at nucleotide level by ClustalX 1.83. Gaps and missing data matrix were excluded from the analysis. The generated data matrix (932 bp) was converted to Nexus format, and the data matrix was analyzed with the Mr. Bayes v3.1.2 program (Huelsenbeck and Ronquist 2001) using the general time-reversible model. A total of 1 million generations with the Markov chain Monte Carlo process were conducted, and the sampling frequency was assigned as every 100 generations. After analysis, the first 10,000 generations were deleted as the burn-in process, and a consensus tree was constructed.

Results

Parthenogenetic female of *B. koreanus* sp. nov

Type locality. The parthogenetic female of *B. koreanus* sp. nov was collected from Uljin-1 (S), South Korea.

Holotype. A parthenogenetic female was deposited at the Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, D.C. with the following catalogue number: USNM 1115523. About 100 allotypes were deposited at the same institution in a vial preserved in 70% ethanol/glycerin (USNM 1115524). The lorica was smooth and pear shaped (Figure 2) with ventral and dorsal plates fused dorsally and laterally. The U-like sinus carries three pairs of spines at each side laterally. All spines are triangular and dissimilar in length; the outer and inner spines are longer than the middle one. The outer spine has a sigmoid outer margin. The anterior ventral margin of the lorica with two pairs of rounded lobules was located at both sides of the slender sinus. Inner lobes show a narrower base than the outer one. The lateral antennae are pointing medially. The foot opening is located subterminally on the ventral plate. The trophi are symmetrical and lobular. The fulcrum is hollow, short, and truncated in shape. The rami are rectangular with a ventral flat surface. The unci are plate-like and carry six solid ridges.

Table 2 Stepwise discriminant analyses of *Brachionus* body measurements

	Function 1		Function 2	
	Coefficient	Correlation	Coefficient	Correlation
Ln(*a*)	0.946	0.971[a]	−0.557	−0.155
Ln(*b*)	-	-	-	-
Ln(*c*)	−0.084	0.834[a]	−0.213	0.045
Ln(*d*)	-	-	-	-
Ln(*e*)	0.071	0.330	0.360	0.465
Ln(*f*)	-	-	-	-
Ln(*g*)	−0.138	0.148	0.250	0.305
Ln(*h*)	0.042	0.044	0.335	0.469
Ln(*i*)	0.236	0.621[a]	0.883	0.592
Eigenvalue	12.642		0.870	
% variance	88.9		6.1	

Only values for the first two canonical functions (Functions 1 and 2) of each discriminant analysis are shown. [a]Statistically significant based on morphological characters of the lorica between *B. koreanus* and *B. plicatilis* to separate two different species under morphometrical discriminant analysis.

Differential diagnosis. B. koreanus sp. nov. differs from its sibling *B. plicatilis* Müeller in the following: The three pairs of anterodorsal spines are different in length; the outer and inner spines are longer than the middle one. All spines are triangular in shape, but the outer one has a sigmoid outer margin. Resting eggs are ovoid, showing a smooth surface but carry many more pores all over

Table 3 Mean (SE) of body length and four body ratios for the two siblings *B. plicatilis* and *B. koreanus*

	Morphometric variable				
	a	***c/a***	***i/a***	***h/a***	***g/h***
B. koreanus					
AVE	201.04	0.7481	0.5021[a]	0.0690[a]	0.8873[a]
Uljin-1 (S)	196.07	0.7706	0.5516	0.0773	0.8178
Daesan	192.26	0.7235	0.4783	0.0715	0.8235
Okgu	194.59	0.7515	0.5118	0.0742	0.8280
Uljin-2 (L)	213.40	0.7331	0.4783	0.0552	1.0190
Taean	212.46	0.7649	0.4828	0.0652	0.9523
B. plicatilis					
AVE	293.89	0.7514	0.4797[a]	0.0524[a]	0.9516[a]
China	276.22	0.7618	0.5059	0.0605	0.9165
Hwasung	306.13	0.7808	0.4873	0.0431	1.0904
Mokpo	277.25	0.7281	0.4667	0.0580	0.8537
Buan	303.54	0.7570	0.4829	0.0534	0.9762
Seosan	329.32	0.7573	0.4486	0.0422	0.9614
Suncheon	300.24	0.7289	0.4445	0.0483	1.0192
Japan	256.14	0.7400	0.5246	0.0638	0.8082

[a]Statistically significant based on morphological characters of the lorica between *B. koreanus* and *B. plicatilis* to separate two different species under morphometrical discriminant analysis.

their surface than in *B. plicatilis*. Body size is substantially smaller than in *B. plicatilis* (see morphometry below).

Morphometric measurements

Morphological characters of the lorica (Figure 2) were chosen for the analyses. Characteristics and measurements of the lorica characters of *Brachionus* spp. follow Fu et al. (1991). Mean body length and four body ratios for different populations of *B. koreanus* sp. nov. and *B. plicatilis* are provided in Tables 1, 2, and 3. Morphometrical discriminant analysis was following the method of Ciros-Pérez et al. (2001) to clearly distinguish the morphometry as shown in Figure 3. Function 1 is largely correlated not only with the two major body measurements (*a* and *c*; r = 0.971 and 0.834, respectively) but also with the head aperture (i.e., *i*, r = 0.621). Function 2 is correlated with traits associated with spines and represented by variables *e* (dorsal sinus length; r = 0.465) and *h* (spine 3 length; r = 0.469).

Comparison of mitochondrial *CO1* sequences between five *Brachionus* species

To analyze the mitochondrial *CO1* gene from five *Brachionus* species, we amplified partial *CO1* sequence (812 bp) using conserved primers and compared six specific regions that are able to distinguish six species belonging to *Brachionus*. As shown in Figure 4, we found several alleles from five rotifer species. Of them, *B. koreanus* sp. nov. was clearly isolated from others.

Phylogenetic relationships among *Brachionus* spp. as inferred from *CO1* and *ITS1* rDNA

Mitochondrial *CO1* and nuclear rDNA sequences are useful in molecular systematics to estimate genetic diversity and phylogenetic relationships between taxa (Kellogg et al. 1996). Of these, nuclear rDNAs were particularly applied for delineating higher taxon levels, e.g., families and orders due to the high level of conservation among these sequences. In contrast, *ITS* regions are considered to be useful in defining interspecific differences, as they are less subject to functional constraints and would evolve more rapidly. Therefore, we investigated rotifer phylogeny using *ITS1* along with *CO1* gene from the rotifer genus *Brachionus*. Using *CO1* and *ITS1*, we reconstructed a phylogenetic tree of six species belonging to the genus *Brachionus* (Figure 5).

To get a cue about large-scale biogeographic intraspecific variation, we investigated *CO1-ITS1* sequences of 45 *Brachionus* populations collected from geographical areas as disjunct as Korea, China, and Japan as well as *CO1-ITS1* sequences from GenBank (Figure 1, Table 4).

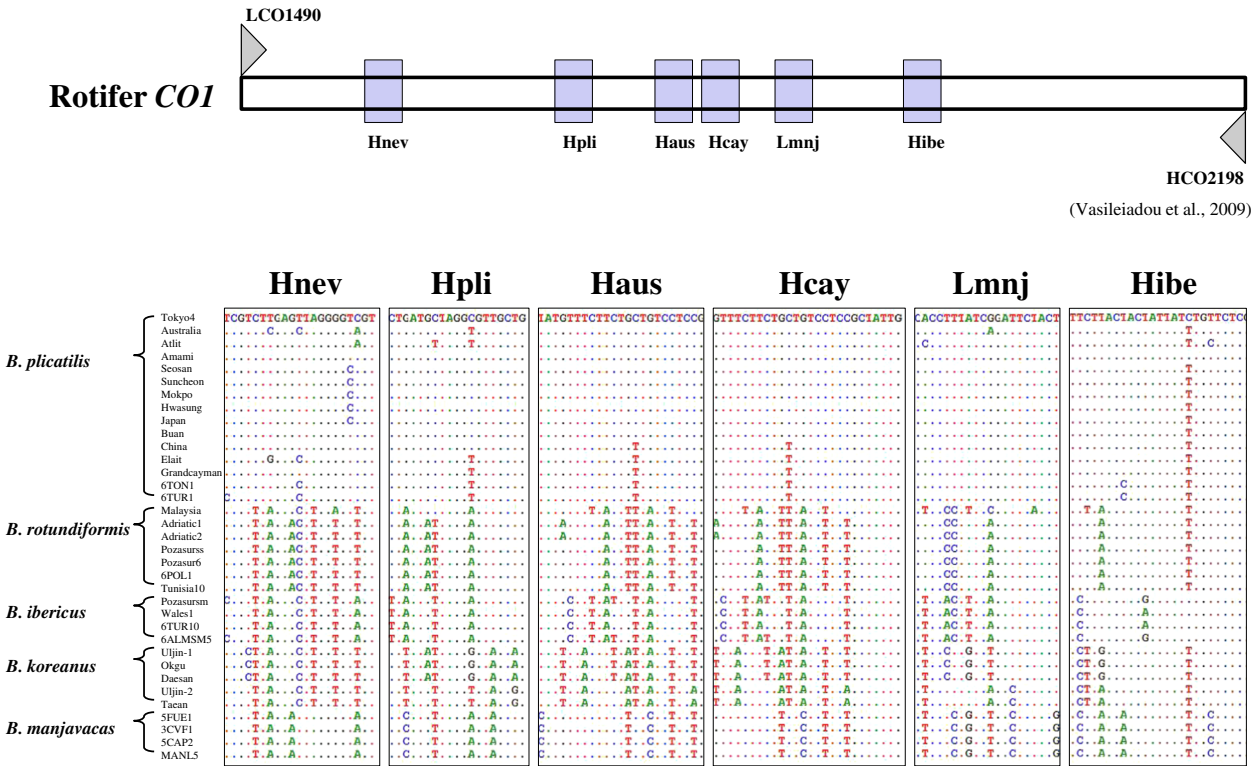

Figure 4 Mitochondrial *CO1* variations of five species belonging to 35 clones of the genus *Brachionus*. Hnev, *Brachionus* sp. *Nevada*; Hpli, *B. plicatilis* s.s.; Haus, *Brachionus* sp. *Austria*; Hcay, *Brachionus* sp. *Cayman*; Lmnj, *Brachionus* sp. *manjavacas*; Hibe, *B. ibericus*.

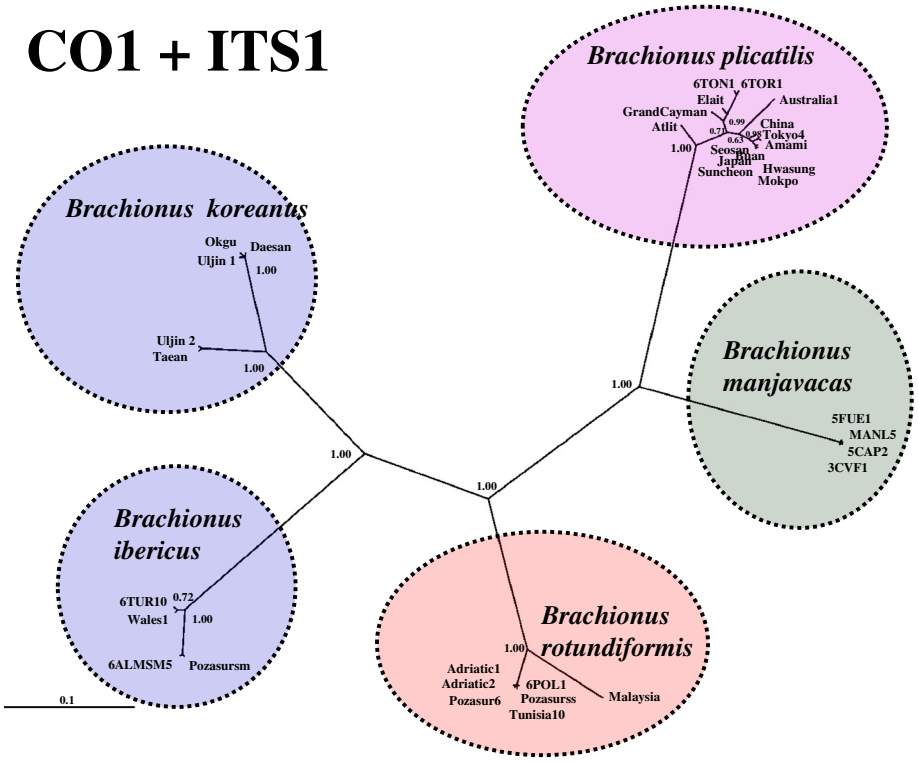

Figure 5 Molecular phylogenetic tree of *Brachionus* spp. inferred from *CO1-ITS1* sequences using Bayesian method.

Molecular analyses of the geographic samples showed that *CO1* sequences were quite different between these populations (Figure 4).

Comparison of complete mitochondrial DNA between *B. plicatilis* and *B. koreanus*

We sequenced the complete mitochondrial genome from the rotifer *B. koreanus*, which consisted of two circular forms of mitochondrial DNA as shown in Figure 6A, and compared these with those of its supposedly closest related species, *B. plicatilis* (Figure 6B). The organization of both mitochondrial genomes was almost identical, but there was one rearrangement of *tRNA-Cys* between *tRNA-arg* and *tRNA-ile*.

Discussion

Our morphometrical discriminant analysis following the method of Ciros-Pérez et al. (2001) clearly distinguished the morphometries of the different species. As Ciros-Pérez et al. (2001) mentioned, this analysis was much more efficient in separating species groups of rotifers. Hence, we could separate three groups, each corresponding to one of the two sibling species. The most reliable measurements to differentiate between species are those regarding body size and general body shape, and the relative length of the spines.

A major problem with the systematics of the genus *Brachionus* seems to be the unresolved status of several sibling species clusters (Gómez and Snell 1996; Serra et al. 1998). The species involved might have a more restricted distribution than previously thought (Sudzuki 1987). Several similar strains were classified although no type material is available for most of the species belonging to *Brachionus*. The present study employing both morphological and molecular data is meant as a reference base for future comparative studies. As for the *B. plicatilis* species complex, Segers (1995) proposed the use of *B. plicatilis* Müller (1976) and *B. rotundiformis* Tschugunoff (1921) as the correct names for the formerly designated S- and L-morphotypes, respectively. Although the distinction of S- vs. L-type might be of sound systematic importance, this does not mean that only two species belong to this species complex as emphasized by Ciros-Pérez et al. (2001). In this sense, the analysis based on populations (several strains belonging to each population) rather than on single strains (each belonging to a different population) will provide more information for the delineation of species within this genus.

As for the mitochondrial *CO1* gene from five *Brachionus* species, we found several alleles from five rotifer species (Figure 4). Of them, *B. koreanus* sp. nov. was clearly isolated from others. Vasileiadou et al. (2009)

Table 4 Characteristics of *Brachionus* species, including other Rotifera, used in this study and DNA sequence accession numbers

Genus	Species	Strain	Accession number	
			CO1	*ITS1*
Brachionus	*B. plicatilis*	Tokyo4	[GenBank:AY785177.1]	[GenBank:AY772097.1]
		Australia1	[GenBank:AF387244.1]	[GenBank:AF387206.1]
		Caperomaine2	[GenBank:AY785233.1]	[GenBank:AY772155.1]
		Atlit	[GenBank:AY785190.1]	[GenBank:AY772110.1]
		Amami	[GenBank:AY785174.1]	[GenBank:AY772094.1]
		Austria4	[GenBank:AY785200.1]	[GenBank:AY772120.1]
		Elait	[GenBank:AY785188.1]	[GenBank:AY772108.1]
		GrandCayman2	[GenBank:AY785189.1]	[GenBank:AY772109.1]
		6TON1	[GenBank:AF266858.1]	[GenBank:AF387199.1]
		6TUR1	[GenBank:AF266859.1]	[GenBank:AF387197.1]
		Seaazov	[GenBank:AY785194.1]	[GenBank:AY772114.1]
		Mortlock1	[GenBank:AY785227.1]	[GenBank:AY772149.1]
		Seosan	[GenBank:GU987064]	[GenBank:GU987076]
		Suncheon	[GenBank:GU987065]	[GenBank:GU987077]
		China	[GenBank:GU987066]	[GenBank:GU987078]
		Mokpo	[GenBank:GU987069]	[GenBank:GU987081]
		Hwasung	[GenBank:GU987070]	[GenBank:GU987082]
		Japan	[GenBank:GU987071]	[GenBank:GU987083]
		Buan	[GenBank:GU987072]	[GenBank:GU987084]
	B. rotundiformis	Malaysia	[GenBank:AY785225.1]	[GenBank:AY772147.1]
		Adriatic1	[GenBank:AY785223.1]	[GenBank:AY772145.1]
		Adriatic2	[GenBank:AY785224.1]	[GenBank:AY772146.1]
		Pozasurss	[GenBank:AY785222.1]	[GenBank:AY772144.1]
		Pozasur6	[GenBank:AY785221.1]	[GenBank:AY772143.1]
		6POL1	[GenBank:AF387289.1]	[GenBank:AF387239.1]
		Tunisia10	[GenBank:AF387288.1]	[GenBank:AF387240.1]
	B. calyciflorus	AHNU-Rotifer-T8	[GenBank:FJ826939.1]	[GenBank:GU321460.1]
		AHNU-Rotifer-T5	[GenBank:FJ826938.1]	[GenBank:GU321457]
		AHNU-Rotifer-W21	[GenBank:FJ827006.1]	[GenBank:FJ937603]
		Florida	[GenBank:DQ071673.1]	[GenBank:DQ071669.1]
		Australia	[GenBank:DQ071675.1]	[GenBank:DQ071671.1]
		Texas	[GenBank:DQ071674.1]	[GenBank:DQ071670.1]
	B. ibericus	Pozasursm	[GenBank:AY785220.1]	[GenBank:AY772142.1]
		Wales1	[GenBank:AF387275.1]	[GenBank:AF387228.1]
		6TUR10	[GenBank:AF387274.1]	[GenBank:AF387227.1]
		6ALMSM5	[GenBank:AF387271.1]	[GenBank:AF387224.1]
	B. manjavacas	5FUE1	[GenBank:AF387261.2]	[GenBank:AF387216.1]
		3CVF1	[GenBank:AF387260.2]	[GenBank:AF387214.1]
		5CAP2	[GenBank:AF387259.2]	[GenBank:AF387217.1]
		3MANL5	[GenBank:AF387257.2]	[GenBank:AF387213.1]

Table 4 Characteristics of *Brachionus* species, including other Rotifera, used in this study and DNA sequence accession numbers *(Continued)*

B. koreanus	Uljin1	[GenBank:GU987061]	[GenBank:GU987073]
	Taean	[GenBank:GU987062]	[GenBank:GU987074]
	Daesan	[GenBank:GU987063]	[GenBank:GU987075]
	Okgu	[GenBank:GU987067]	[GenBank:GU987079]
	Uljin2	[GenBank:GU987068]	[GenBank:GU987080]

used these six specific regions for rapid identification of *Brachionus* rotifers before and successfully accomplished an unambiguous species identification. Thus, a multiplex PCR method would be a proper congruent for species with ambiguous morphological characteristics.

Mitochondrial *CO1* and nuclear rDNA sequences are useful in molecular systematics to estimate genetic diversity and phylogenetic relationships between taxa (Kellogg et al. 1996). Nuclear rDNAs were delineating higher taxon levels, e.g., families and orders due to the high level of conservation among these sequences. In contrast, *ITS* regions are considered to be useful in defining interspecific differences, as they are less subject to functional constraints and would evolve more rapidly. Therefore, we investigated rotifer phylogeny using *ITS1* along with the *CO1* gene from the rotifer genus *Brachionus*. Using *CO1* and *ITS1*, we reconstructed a phylogenetic tree of six species belonging to the genus *Brachionus*.

The mitochondrial *CO1* and rDNA *ITS* have only a few evolutionary constraints, and they are expected to evolve at or near a neutral level (Bakker et al. 1995). Molecular

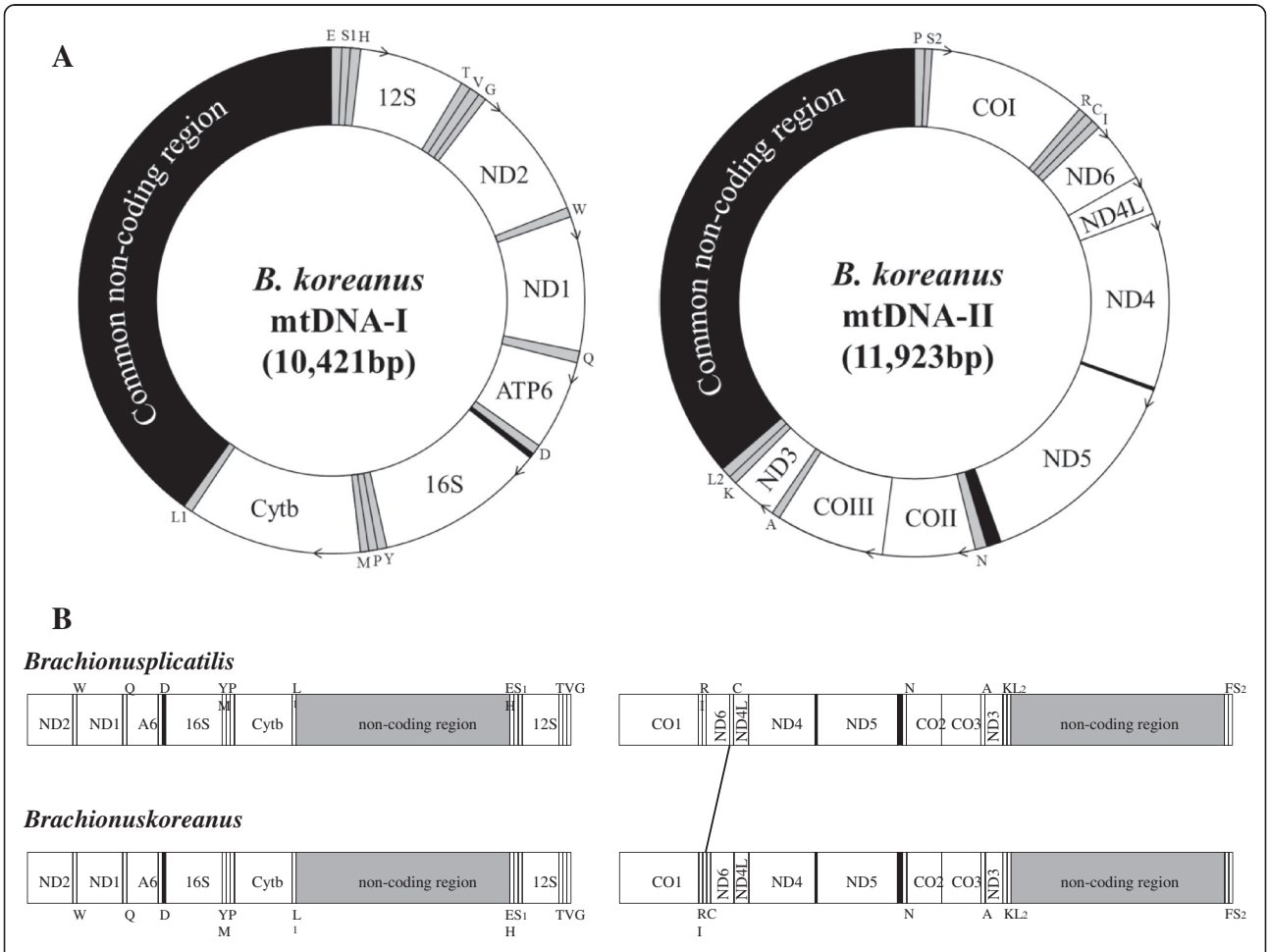

Figure 6 Gene organization of two mitochondrial genomes of the rotifers *B. plicatilis* and *B. koreanus* sp. nov. (A) Two circular mitochondrial genomes from *B. koreanus* sp. nov. One-letter abbreviations of amino acids are used to label the corresponding tRNA genes. **(B)** The thick line between pairs of mitogenomes between *B. plicatilis* and *B. koreanus* sp. nov. represents translocation. Modified from Hwang et al. (2013).

analyses revealed that individuals of *Brachionus* populations originating from the same localities had nearly identical genotypes. To get a cue about large-scale biogeographic intraspecific variation, we investigated *CO1-ITS1* sequences of 45 *Brachionus* populations collected from geographically areas as disjunct as Korea, China, and Japan as well as *CO1-ITS1* sequences from GenBank (Figure 1). Molecular analyses of the geographic samples showed that *CO1* sequences were quite different between these populations (Figure 4).

We sequenced the complete mitochondrial genome from the rotifer *B. koreanus*, which consisted of two circular forms of mitochondrial DNA (Figure 6A). When comparing the mitochondrial DNA of *B. koreanus* with those of its supposedly closest related species, *B. plicatilis* (Figure 6B), the mitochondrial organization was almost identical. The single rearrangement of *tRNA-Cys* between *tRNA-arg* and *tRNA-ile* indicated that the mitogenome rearrangement occurred in the closest related congeneric species with concerted evolution. We assume that among cryptic species, such as rotifers, the analysis of the complete mitochondrial genome will provide a suitable instrument for the characterization of population differences.

Conclusions

Care must be taken in the choice of characters since the discriminating power of a morphometric approach sensitively depends on the measured characters. Data obtained from clones cultivated at laboratory conditions need to be corroborated with populations from the field as well. As there are other and new species within *Brachionus* awaiting description, detailed comparisons are possible for meaningful systematic research within this morphologically variable genus. By distinguishing and characterizing different clones within five conspecifics within the *Brachionus* complex, our analysis bridged the gap between the classical taxonomy of rotifers and modern approaches. Our results show that morphometry can provide a powerful tool to differentiate similar species in addition to molecular, ecological, and physiological data.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DSH carried out allometry and molecular genetic studies and initially drafted the manuscript. HUD carried out the drawing of a rotifer and participated in the making of the draft. HGP participated in the collection of rotifers and initially maintained them in the aquarium. JSL conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by a grant from the National Research Foundation (2012R1A1A2000970) funded to Jae-Seong Lee and also was supported by a grant from NRF (2012-R1A2A2A02012617).

Author details

[1]Department of Molecular and Environmental Bioscience, Graduate School, Hanyang University, Seoul 133-791, South Korea. [2]Department of Life Science, College of Natural Sciences, Sangmyung University, Seoul 110-743, South Korea. [3]Faculty of Marine Bioscience and Technology, College of Life Sciences, Kangnung-Wonju National University, Gangneung 210-702, South Korea. [4]Department of Chemistry, College of Natural Sciences, Hanyang University, Seoul 133-791, South Korea.

Received: 10 June 2013 Accepted: 5 September 2013
Published: 23 September 2013

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doi:10.1186/1810-522X-52-13
Cite this article as: Hwang *et al.*: A new intertidal *Brachionus* and intrageneric phylogenetic relationships among *Brachionus* as revealed by allometry and *CO1-ITS1* gene analysis. *Zoological Studies* 2013 **52**:13.