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Updating the description and taxonomic status of *Brachionus sessilis* Varga, 1951 (Rotifera: Brachionidae) based on detailed morphological analysis and molecular data

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Abstract

Brachionus sessilis Varga, 1951 is an epizoic rotifer living exclusively on cladocerans of the genus *Diaphanosoma*. Current taxonomic knowledge relies solely on limited morphological information, whereas there is no type material. Here, we aim to resolve issues concerning its morphology and taxonomy using both morphological and genetic characters on material sampled from Lake Balaton (Hungary), as well as Lake Doirani (Greece) that was selected for comparison purposes. Biometrical analysis was based on extensive lorica measurements. Phylogenetic reconstruction was based on DNA sequence information of the mitochondrial cytochrome c oxidase subunit I (COI) and 16S rRNA gene regions as well as of the nuclear internal transcribed spacer 1 (ITS1). Well-supported evidence for substantial differentiation of *B. sessilis* from its closest phylogenetic relatives supports its species-rank status. Our phylogenetic analysis suggests a highly supported clade encompassing *B. sessilis* and another epizoic rotifer, namely *B. rubens*.

Key words: taxonomy, species delimitation, DNA barcoding, biometry, *Brachionus,* epizoism, phylogenetic trait conservatism

Introduction

Rotifera is a phylum of microscopic organisms commonly found in freshwater environments throughout the world (Segers 2007). *Brachionus* Pallas, 1766 is one of the most speciose genera of the phylum (Ahlstrom 1940), largely known for its use in aquaculture as food to fish larvae (Lubzens 1987). Recognizing species boundaries in *Brachionus* rotifers has proven to be a challenging task even in routine microscopic observations. On the one hand, *Brachionus* species are renowned for their great phenotypic variability which has been partly attributed to a high degree of plasticity (Segers 2007). As a consequence, markedly different morphological variants can be found within the same species (Ahlstrom 1940). On the other hand, remarkable interspecific similarity also exists as in the case of the *B. plicatilis* complex of cryptic species in which several morphologically similar albeit phylogenetically distinct lineages have been identified (Gómez *et al.* 2002; Suatoni *et al.* 2006). These characteristics, along with other taxonomic difficulties typical of the phylum, such as the dearth of taxonomically important morphological characters (Ahlstrom 1940), the deficiency of comprehensive descriptions including analysis of biometry or geometric morphometrics (Koste & Shiel 1989; Adams *et al.* 2004), the improper use of infraspecific rank names and a long list of synonyms (Harring 1913; Segers 2007) pose major taxonomic impediments to the taxonomists dealing with the systematics of *Brachionus* rotifers.

Accurate species delineation is fundamental in order to explain patterns of biological diversity, understand population genetic processes, detect ecological divergence and ultimately assess the ways in which ecosystems function. Traditional species delimitation has been based on morphological comparisons in which phenotypically

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distinguishable clusters are assigned to different species, a fundamental assumption being that morphological discontinuities reflect independent evolution. Modern taxonomy however, is now best implemented through a multidisciplinary framework that combines morphological with genetic and ecological data (DeSalle *et al.* 2005; McManus & Katz 2009; Padial *et al.* 2010). Under this approach, phylogenetics is often a key component to taxonomic delineations. Over the past few years, DNA sequencing analysis and DNA barcoding have revolutionized species identification and have allowed biologists to discriminate inter- from intraspecific variation even for morphologically similar species (Bickford *et al.* 2007; Hajibabaei *et al.* 2007). Several novel approaches have been introduced to quantify the amount of variation present and quantitatively define barcode gaps (e.g. Generalized Mixed Yule Coalescent method in Pons *et al.* 2006, 4x rule in Birky *et al.* 2010, Automated Barcode Gap Discovery in Puillandre *et al.* 2012). At the same time, advances in molecular ecology have refined our understanding of important ecological processes, elucidating the interplay between ecology, microevolution and diversification (Bickford *et al.* 2007; Montero-Pau *et al.* 2011; Xiang *et al.* 2011; Papakostas *et al.* 2013). Consequently, the taxonomic status of many species as well as the number of species in many taxa has been re-evaluated (Ciros-Pérez *et al.* 2001; Fontaneto *et al.* 2007a) and this has also contributed to a better understanding of the taxonomic value of several phenotypic characters.

In this study, we revise the taxonomic status of *Brachionus sessilis* in light of both morphological and genetic data. This small (*ca.* 100 μm in length) rotifer is an exemplary case of how insufficient taxonomic data can hinder correct identification and obstruct reporting of a taxon. *Brachionus sessilis* was first described by Varga (1951) in Lake Balaton (Hungary). It is a thermophilic epizoic rotifer (Varga 1951) living exclusively on individuals of the cladoceran genus *Diaphanosoma* (Chengalath *et al.* 1973). Ever since the description of *B. sessilis*, the schematic drawings that have been published were all based on major lorica measurements, i.e. length, width, thickness and foot aperture (Varga 1951; Sudzuki 1964; Chengalath *et al.* 1973). To establish its systematic position and relationships, Varga (1951) compared it with *B. rubens*, *B. orientalis* [currently *species inquirenda* (Jersabek *et al.* 2012)], *B. quadridentatus*, and members of the present-day *B. plicatilis* (including its varieties). Its closest resemblance was then attributed to *B. rotundiformis* (at that time, i.e. in 1951, *B. rotundiformis* was considered to be as of infrasubspecific rank, as *B. plicatilis* var. *rotundiformis*). Koste (1978) and Fernando & Zankai (1981) classified and treated it as a subspecies, namely *B. urceolaris sessilis.* Recently however, Segers (2007) and Jersabek *et al.* (2012) restored the species-group rank of the taxon *B. sessilis*. The full record of the nomenclatorial use of *B. sessilis* in the past can be seen in Table 1. Notably, all these rearrangements were based solely on morphological comparisons while, to our knowledge, there exists no type specimen (Jersabek *et al.* 2012). Although widely distributed, the existing records of its presence do not exceed a total of 47 (Table 1).

TABLE 1. Records of *Brachionus sessilis* with notes on the name used in the source reference.

Country	Source reference	Reference used	Name used in the reference used
Argentina	De Paggi 1990	De Paggi 1990	*B. sessilis*
Argentina	De Paggi 1993	De Paggi 1993	*B. sessilis*
Australia NT	Koste 1981	Koste 1981	*B. sessilis*
Australia NT	Koste & Shiel 1980	Koste & Shiel 1980	*B. urceolaris sessilis*
Australia NT	Koste & Shiel 1987	Koste & Shiel 1987	*B. sessilis*
Australia NT	Tait *et al.* 1984	Tait *et al.* 1984	*B. urceolaris sessilis*
Australia undefined	Dumont 1983	Dumont 1983	*B. sessilis*
Australia Victoria	Koste & Shiel 1980	Shiel 1983	*B. urceolaris sessilis*
Australia Western	Koste 1981	Koste 1981	*B. sessilis*
Austria	Koste 1978	Koste 1978	*B. urceolaris sessilis*
Austria	Jersabek & Leitner 2013	Jersabek & Leitner 2013	*B. sessilis*
China	Shao *et al.* 2001	Shao *et al.* 2001	*B. urceolaris* f. *sessilis*
Ethiopia	Green & Mengestou 1991	Green & Mengestou 1991	*B. sessilis*
France	Lair & Sargos 1981	De Ridder & Segers 1997	*B. sessilis*

.....continued on the next page

TABLE 1. (Continued)

Country	Source reference	Reference used	Name used in the reference used
Greece	Danielidis *et al*. 1996	Danielidis et al. 1996	*B. sessilis*
Hungary	Varga 1951	Varga 1951	*B. sessilis*
Hungary	Berzins 1978	Berzins 1978	*B. sessilis*
Hungary	Zankai 1968	De Ridder & Segers 1997	*B. sessilis*
Hungary	Zankai 1989	Zankai 1989	*B. urceolaris sessilis*
Hungary	Zankai & Kertesz 1967	Zankai & Kertesz 1967	*B. sessilis*
Hungary	Zankai & Ponyi 1970	De Ridder & Segers 1997	*B. sessilis*
Hungary	Zankai & Ponyi 1971	De Ridder & Segers 1997	*B. sessilis*
India	Sharma 1983	Sharma 1983	*B. sessilis*
India	Sharma & Michael 1980	Sharma & Michael 1980	*B. sessilis*
India	Sudzuki 1989	Sudzuki 1989	*B. urceolaris sessilis*
Japan	Sudzuki 1964	Sudzuki 1964	*B. sessilis*
Former Yugoslav Republic of Macedonia	Popovska-Stankovic 1990	De Ridder & Segers 1997	*B. sessilis*
Malaysia	Fernando & Zankai 1981	Fernando & Zankai 1981	*B. urceolaris* var. *sessilis*
Malaysia	Sudzuki 1989	Sudzuki 1989	*B. urceolaris sessilis*
Myanmar	Koste & Tobias 1990	Koste & Tobias 1990	*B. sessilis*
Senegal	De Ridder 1983	De Ridder 1983	*B. urceolaris* f. *sessilis*
Singapore	Fernando & Zankai 1981	Fernando & Zankai 1981	*B. urceolaris* var. *sessilis*
Spain	Velasco 1990	Velasco 1990	*B. urceolaris sessilis*
Sri Lanka	Chengalath *et al.* 1973	Chengalath *et al.* 1973	*B. sessilis*
Sri Lanka	Fernando 1980	Fernando 1980a	*B. sessilis*
Sri Lanka	Sudzuki 1989	Sudzuki 1989	*B. urceolaris sessilis*
Taiwan	Sudzuki 1988	De Ridder & Segers 1997	*B. sessilis*
Taiwan	Sudzuki 1989	Sudzuki 1989	*B. urceolaris sessilis*
Thailand	Sanoamuang *et al*. 1995	Sanoamuang *et al*. 1995	*B. sessilis*
Turkey	Ustaoglu 2004	Ustaoglu 2004	*B. sessilis*
Venezuela	Vasquez 1984	Vasquez 1984	*B. urceolaris sessilis*
Zaire	De Ridder 1981	De Ridder 1981	*B. sessilis* as synonym of *B. urceolaris*
Zambia	De Ridder 1981	De Ridder 1981	*B. sessilis* as synonym of *B. urceolaris*
Zambia	Thomasson 1966	De Ridder 1981	*B. urceolaris sessilis*
Zimbabwe	Green 1985	Green 1985	*B. sessilis*
Zimbabwe	Thomasson 1965	Thomasson 1965	*B. sessilis*
Zimbabwe	Thomasson 1980	Thomasson 1980	*B. sessilis*

To clarify the uncertainties over the taxonomic status of *B. sessilis* and provide a modern taxonomic description of the species we performed a) biometrical analysis based on phenotypic characters, here in particular extensive lorica measurements (e.g. anterior-dorsal spine lengths, head aperture), b) DNA sequencing on three genetic markers (parts of the mitochondrial COI and 16S rRNA gene regions and of the nuclear ITS1), and c) phylogenetic analysis by incorporating a large amount of *Brachionus* sequence data available in GenBank. To examine levels of inter-population diversity, samples from Lake Balaton, Hungary were compared with *B. sessilis* rotifers from Lake Doirani, Greece.

Materials and methods

Sample collection. Samplings were conducted in Lake Balaton, Hungary in August 2009 and Lake Doirani, Greece during 2007 and 2008, between July and September each year. Samples were collected with vertical and horizontal hauls from the pelagic area of the two lakes using plankton nets (50 and 100 μm mesh size). The samples were inspected the same day and *B. sessilis* egg-bearing females were detached from live *Diaphanosoma* individuals under a stereo-microscope. In order to obtain a more representative dataset for phylogenetic analysis, additional *Brachionus* species (i.e. *B. angularis*, *B. calyciflorus*, *B. dimidiatus*, *B. ibericus* and *B. urceolaris*) sampled from Lake Koronia, Greece, during the years 2003–2007 following the same sampling protocol were included. Rotifers were preserved in 4% formalin for morphological and biometric analyses and in absolute ethanol for genetic analyses.

Morphology and biometry. For morphological inspection, formalin-fixed individuals were examined with a LeitzLaborLux S optical microscope. Microphotographs were taken under a Nikon TE2000-S inverted microscope. For biometry, landmarks were digitized using ImageJ (http://rsbweb.nih.gov/ij/) and a total of 20 dimensions were measured on the basis of Ciros-Pérez *et al.* (2001) and Fu *et al.* (1991) (Fig. 1 & 2, Table 2).

TABLE 2. Linear measurements chosen on the basis of Ciros-Pérez *et al.* (2001) and Fu *et al.* (1991).

$\frac{h1+h2}{2}$	Mean medial edge length of the 3rd dorsal anterior spine
$\frac{l1+l2}{2}$	Mean lateral edge length of the 2nd dorsal anterior spine
$\frac{g1+g2}{2}$	Mean medial edge length of the 2nd dorsal anterior spine
$\frac{k1+k2}{2}$	Mean lateral edge length of the 1st dorsal anterior spine
$\frac{j1+j2}{2}$	Mean medial edge length of the 1st dorsal anterior spine
$\frac{n1+n2}{2}$	Mean base length of the 2nd dorsal anterior spine
$\frac{o1+o2}{2}$	Mean base length of the 1st dorsal anterior spine
$\frac{f1+f2}{2}$	Distance between the tips of the 1st and the 2nd dorsal anterior spine
e	Depth of the anterior medial sinus between the 1st dorsal spines
d	Length between the anterior tips of the 1st dorsal spines
m	Length between the anterior tips of the 2nd dorsal spines
b	Length between the anterior tips of the 3rd dorsal spines
c	Width
i	Head aperture
a	Length
z	Foot aperture
$\frac{p1+p2}{2}$	Mean base length of the lateral anterior ventral margin
$\frac{q1+q2}{2}$	Mean base length of the medial anterior ventral margin
r	Distance between the tips of the medial ventral lobules
w	Thickness (or Height)

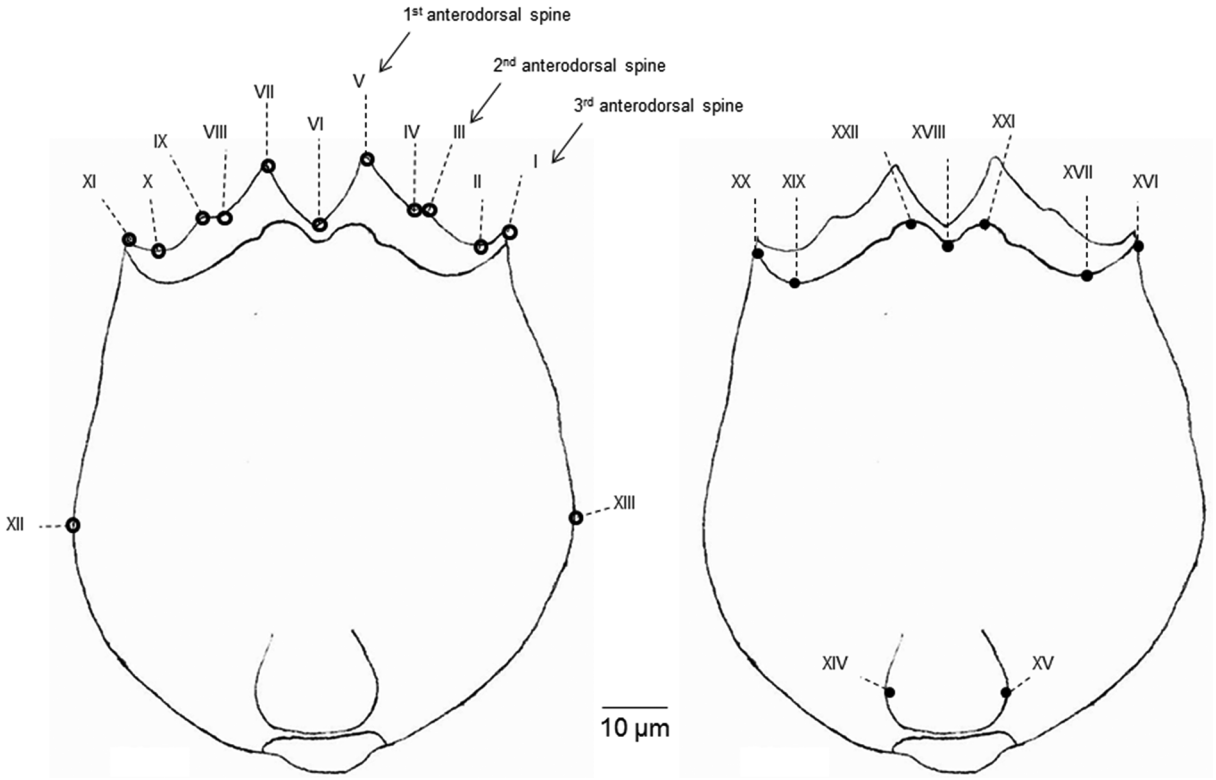

FIGURE 1. Schematic drawings of *B. sessilis* showing the landmarks that were taken on ImageJ. Open (dorsal side) and closed (ventral side) dots indicate the landmark points that were digitized on ImageJ and subsequently used for biometrical measurements.

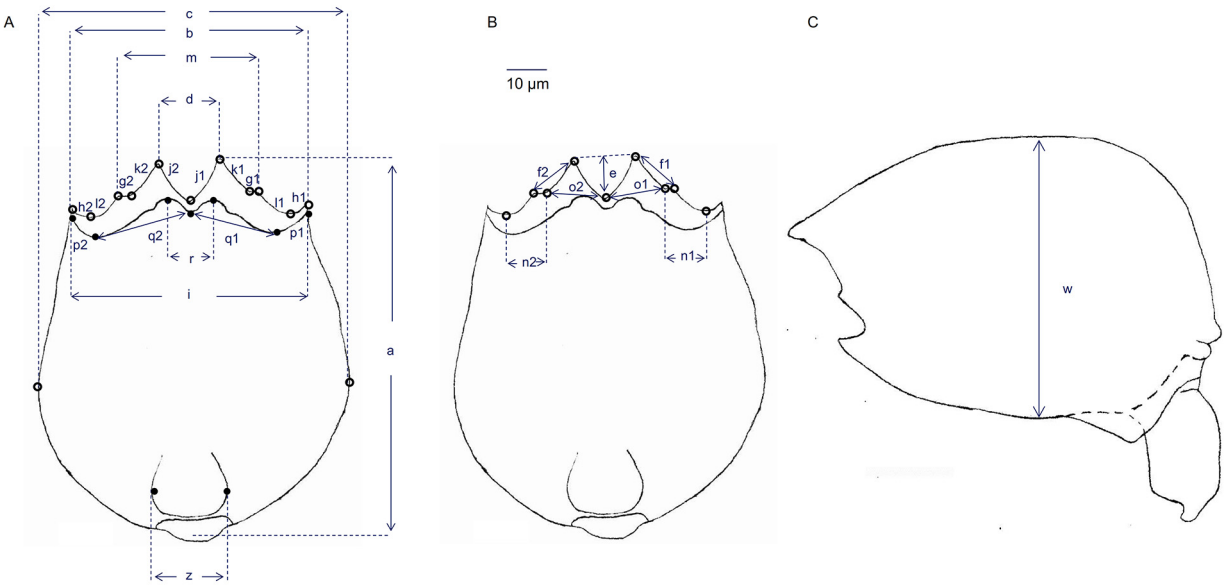

FIGURE 2. Schematic drawings of *B. sessilis* showing the measurements that were taken on ImageJ. **A, B)** Biometric dimensions measured on the dorsal and ventral side of the lorica (open dots: dorsal side; closed dots: ventral side); **C)** Biometric dimensions measured on a lateral view of the lorica.

To test whether mean rotifer dimensions differ significantly between the two *B. sessilis* populations, in lakes Balaton and Doirani, a two sample t-test assuming non-equality of variances (Welch version) was performed in R ver. 2.13.0 (R Development Core Team 2011). A Bonferroni adjustment was performed to correct for multiple comparison tests on the same two populations.

DNA extraction, amplification and sequencing. The DNA extraction from ethanol-preserved single

individuals was performed using Chelex100 resin (BioRad, Hemel Hempstead, UK), as described in Papakostas *et al.* (2005).

Polymerase Chain Reaction (PCR) was performed in 25 µl total reaction volumes using an Eppendorf Mastercycler thermocycler. For the 16S rRNA, a 378 bp fragment was amplified using the primers Br16SL and Br16SH (Papakostas *et al.* 2005). The PCR mix contained 4 µl of DNA template, 1.25 µl of $MgCl_2$ (25 mM) and 2 U of Taq DNA polymerase (Expand High FidelityPLUS PCR System, Roche). PCR conditions were: 2 min at 94ºC, 35 cycles of 20 s at 94ºC, 30 s at 60ºC, 40 s at 72ºC and a final extension of 3 min at 72ºC. For the COI, a 641 bp fragment was amplified using the universal primer LCO1490 (Folmer *et al.* 1994) and the primer HCO667 (5' CAAAGAANGADGTRTTAAAATTACG 3') that was designed using *Brachionus* sequences available in GenBank. The PCR mix contained 5 µl of DNA template, 2 µl of $MgCl_2$ (25 mM) and 2 U of Taq DNA polymerase. Cycling conditions were: 4 min at 93 ºC, 40 cycles of 40 s at 94ºC, 40 s at 50ºC, 50 s at 72ºC and a final extension of 3 min at 72ºC. For the ITS1, a 314 bp fragment was amplified using the primers VIII (Palumbi 1996) and R58 (Baxevanis *et al.* 2006). The PCR mix contained 3 µl of DNA template, 1 µl of $MgCl_2$ (25 mM) and 1.5 U of Taq DNA polymerase. Cycling conditions were: 5 min at 94ºC, 40 cycles of 50 s at 94ºC, 50 s at 60ºC, 60 s at 72ºC and a final extension of 5 min at 72ºC. DNA purification and sequencing were carried out by Macrogen Inc. (Seoul, Korea). All three markers were sequenced on both DNA strands. All sequences were deposited in GenBank (Accession numbers: for COI KM051929–KM051945; for ITS1 KM051946–KM051961; for 16S KM051962–KM051975).

Data assembly and phylogenetic analysis. For each of the three markers, the *B. sessilis* sequences were analyzed together with a total of 155 *Brachionus* sequences retrieved from GenBank (Suppl. Table 1) in order to include most of the known genetic polymorphism. These sequences belong to at least 20 previously characterized *Brachionus* species or biotypes (Suppl. Table 1). Using samples from Lake Koronia, we increased the amount of inter- and intraspecific variation to be included in our analyses. All in all, 16/20/18 species or biotypes and 65/50/55 sequences were used for the COI/ITS1/16S analyses, respectively (Suppl. Table 1). A combined gene analysis including COI+ITS1+16S genetic data was also performed by including the taxa for which COI, ITS1 and 16S sequence data were available (18 species/biotypes, 39 sequences) (Suppl. Table 1). In this case, an Incongruence Length Difference (ILD) test (Farris *et al.* 1994, 1995) with 100 replicates was performed in PAUP* 4.0b10 (Swofford 2003) prior to phylogenetic analysis to test the congruence of the evolutionary rates between the three genes. Outgroups were selected on the basis of Reyna-Fabián *et al.* (2010) so that at least one outgroup sequence in each dataset belonged to the family Brachionidae. More specifically, sequences of *Plationus patulus* (Rotifera: Monogononta: Ploima: Brachionidae) (COI: AF416995, ITS1: DQ834368, 16S: FJ426639) were used to root phylogenies in all cases. The sequences DQ089731 and EU202669, both belonging to *Lecane bulla* (Rotifera: Monogononta: Ploima: Lecanidae), were incorporated as additional outgroups for COI and ITS1, respectively, and the sequence AF499043 from *Keratella cochlearis* (Rotifera: Monogononta: Ploima: Brachionidae) was added to the 16S phylogeny.

For alignment, different parameter combinations of gap-opening and gap-extension penalties were tested in ClustalX 2 (Thompson *et al.* 1997). Alignments were also performed using the MUSCLE algorithm (Edgar 2004) applying default parameters and in MAFFT (Katoh *et al.* 2002) applying the Q-INS-i method. Alignments were then compared using the MEGA4 software (Tamura *et al.* 2007) under the maximum parsimony criterion. Best solution was considered the one with the minimum tree length and the maximum proportion of parsimony informative sites (Wheeler & Gladstein 1994; Simmons 2004). The best alignment for COI was generated by ClustalX (slow accurate mode, gap opening = 15, gap extension = 6, delay divergent sequence = 30% in both cases) and was also checked for the presence of stop codons using the ORF Finder [National Center for Biotechnology Information (NCBI)]. For ITS1 the best alignment was obtained by MUSCLE and for 16S by MAFFT.

The presence of phylogenetic signal was evaluated by inspecting for substitution saturation using transitions/transversions vs. genetic distance (estimated with the F84 model) plots in DAMBE 5.1.1 (Xia & Xie 2001), having first removed the gaps from the alignments to exclude regions of uncertain homology (Olsen & Woese 1993; Castresana 2000). Phylogenetic signal was also checked with the evaluation of the g1 statistic for 10^5 randomly sampled trees as implemented in PAUP. According to this measure, a significantly different from bell-shaped (skewed) tree-length distribution is an indication that the data set is of phylogenetic value (Hillis & Huelsenbeck 1992).

Phylogenetic analysis was implemented in PAUP under Distance, Maximum Parsimony (MP) and Maximum Likelihood (ML) criteria and in MrBayes 3.2.2 (Ronquist & Huelsenbeck 2003; Ronquist *et al.* 2012) under Bayesian inference (BI) for the individual loci. For the combined dataset we performed ML and BI searches. Due to limited computer capacity analyses of the concatenated data were carried out using RAxML Black Box (Stamatakis 2014) and MrBayes 3.2.2 (Ronquist & Huelsenbeck 2003; Ronquist *et al.* 2012) on XSEDE utilities as implemented on CIPRES Web portal (Miller *et al.* 2010). In PAUP, heuristic searches were executed with the Tree-Bisection-Reconnection (TBR) branch swapping algorithm. Branches with zero maximum lengths were collapsed. Character optimization was assessed using the ACCTRAN algorithm (Agnarsson & Miller 2008). MP searches were conducted twice, once ignoring gaps and once considering them as a fifth state. Robustness of the generated trees was inferred using non-parametric bootstrap (Felsenstein 1985) with 1000 pseudoreplicates and applying a Nearest Neighbor Interchange (NNI) branch swapping. Bayesian-based phylogenetic inference was executed in two parallel searches, each using four Markov Monte Carlo chains (one cold and three heated) that were run for 10^7 generations and sampling every 1000^{th} generation. The first 7000 sampled trees were discarded as burn-in and a 50% majority rule consensus tree was produced with the remaining 3000 trees.

For each dataset, the best-fit model of nucleotide substitution was chosen by calculating the hierarchical Likelihood Ratio Test (hLRT) along with Akaike Information (AIC), Bayesian Information (BIC) and corrected Akaike Information (AICc) criteria, as implemented in Modeltest3.7 (Posada & Crandall 1998). In each dataset, the best-fit model was selected by comparing the models proposed by the different tests/criteria using a likelihood ratio test (Posada & Buckley 2004). The parameters of the best-fit model were subsequently parsed in PAUP and MrBayes 3.2.2.

Species delimitation. The consensus tree of the concatenated phylogeny was converted to an ultrametric tree using penalised likelihood as implemented in r8s 1.7 (Sanderson 2003). The smoothing parameter was set to -3.00 and was selected by cross-validation of values ranging from -4.00 to 8.00 in increments of 0.50. The root of the tree was set to 1. Species delimitation was then performed by applying the General Mixed Yule Coalescent (GMYC) model (Pons *et al.* 2006; Fujisawa & Barraclough 2013) on the ultrametric tree. Prior to the GMYC analysis, outgroups *Plationus patulus* and *Keratella cochlearis* were removed using the *drop.tip* function of the *ape* R package (Paradis *et al*. 2004).

Results

Morphology and biometry

Table 3 shows the results of the biometric analysis. The small number of specimens examined in some cases is due to the practical difficulties of placing them in the desired orientation. Overall, *B. sessilis* rotifers sampled from the two localities did not differ significantly although for nine out of the twenty dimensions measured the t test indicated significant non-congruence ($p<0.05$) (Table 3). Significance was reduced to four of these dimensions after the Bonferroni correction was applied. Two out of these four dimensions are placed on the anterior margin (h, n), and refer to the lateral spines that can be hard to quantify due to their dorsoventral curvature. The other two (w, z) concern the foot aperture and width which can be hard to quantify due to difficulties acquiring microphotographs at the desired orientation.

DNA sequence data and genetic distances

For the 16S rRNA region (55 sequences), alignment length was 287 bp, with 124 variable and 90 parsimony informative sites. Corrected interspecific pairwise distances (model GTR+I+G) ranged from 0.083 to 0.908 with an overall mean of 0.334. *B. sessilis* was least distant from *B. rubens* (0.214). The genetic distance between *B. sessilis* and the members of the *B. plicatilis* species complex was 0.418 and from *B. rotundiformis* alone 0.529 (Suppl. Table 2). The g1 statistic suggested the presence of phylogenetic signal ($g1=-0.441$, $p<0.05$). However, the transitions/transversions vs. genetic distance plot showed significant saturation.

TABLE 3. Mean, standard deviation (S.D), minimum and maximum values of the biometrical measurements of *B. sessilis* populations in Lake Balaton and Lake Doirani.

Morphological dimension (see Fig. 1& 2, Table 2)	*Present study*												*Varga 1951*	
	Balaton					**Doirani**					**Inter-population comparisons**		**Balaton**	
	Mean	S.D.	Min.	Max.	n	Mean	S.D.	Min.	Max.	n	t-test	Bonferroni correction	Min.	Max.
h	3.71	1.26	1.63	7.48	18	7.07	1.15	5.20	8.61	29	<0.001	<0.001	-	-
l	11.48	1.55	9.46	14.94	18	10.21	1.35	7.76	13.90	29	0.008	0.156	-	-
g	2.68	0.54	1.69	3.35	18	2.40	0.45	1.25	3.14	29	0.087	1	-	-
k	11.90	0.99	9.41	13.92	18	12.01	0.99	9.84	13.89	29	0.621	1	-	-
j	11.84	1.47	8.69	14.67	18	12.95	1.23	9.51	15.28	29	0.012	0.237	-	-
f	13.59	1.32	9.81	15.67	17	13.46	1.21	11.56	16.34	29	0.446	1	-	-
n	13.22	1.58	9.57	16.28	17	11.65	1.42	8.44	14.60	28	<0.001	<0.001	-	-
o	17.30	2.28	9.60	20.00	17	16.72	0.85	14.98	18.48	29	0.057	1	-	-
d	16.01	2.36	11.63	19.65	18	18.29	1.42	15.48	20.99	29	0.001	0.022	-	-
m	39.94	2.76	34.13	45.78	18	37.78	1.93	33.84	42.38	28	0.010	0.202	-	-
e	8.63	1.61	5.62	12.33	18	9.15	1.41	5.13	11.28	29	0.278	1	-	-
b	63.26	3.10	58.52	69.51	18	63.58	3.23	57.24	70.06	28	0.718	1	-	-
p	9.64	1.77	6.96	13.74	15	9.67	1.51	7.08	13.44	26	0.948	1	-	-
q	25.22	1.94	22.45	30.35	15	25.65	2.13	18.73	27.93	26	0.388	1	-	-
z	18.73	2.26	14.95	22.45	9	23.07	7.27	15.70	40.95	27	<0.001	<0.001	17	20
r	11.98	2.79	8.97	19.88	15	11.86	0.92	10.57	13.68	26	0.066	1	-	-
i	63.74	3.40	56.61	71.43	15	65.65	2.60	61.47	69.88	26	0.019	0.394	-	-
a	93.32	5.49	84.14	100.82	17	101.02	4.34	94.06	107.79	28	0.617	1	95	115
c	83.62	5.04	73.93	94.55	17	87.88	4.40	77.45	94.59	29	0.934	1	90	98
w	72.81	5.90	60.60	77.58	7	71.13	5.08	60.79	80.31	13	<0.001	0.006	70	80

For the COI gene region (65 sequences), alignment length was 575 bp containing 269 variable, 247 parsimony informative sites and no stop codons. The transitions/transversions vs. genetic distance plot showed no signs of substitution saturation while the g1 statistic argued for the presence of phylogenetic signal (g1=-0.588, $p<0.05$). Corrected interspecific pairwise distances (model TIM+I+G) ranged from 0.146 to 1.098 with an overall mean of 0.686. The uncorrected intra-population genetic distance (p-distance) between *B. sessilis* from Doirani and Balaton was zero (0.008 after accounting for multiple substitutions per site using the TIM+I+G model). The lowest genetic distances regarding *B. sessilis* were observed with *B. rubens* (0.591) and *B. urceolaris* (0.589). Mean sequence divergence between *B. sessilis* and the members of the *B. plicatilis* species complex was 0.869. The average nucleotide divergence between *B. sessilis* and *B. rotundiformis* was 0.828 (Suppl. Table 3).

For the ITS1 region (50 sequences), alignment length was 428 bp, with 320 variable and 274 parsimony informative sites. Data appeared to be moderately saturated, whereas the estimated g1 value indicated the presence of phylogenetic signal (g1=-0.467, $p<0.05$). Interspecific pairwise distances based on the chosen evolutionary model (HKY+I+G) ranged from 0.018 to 1.679, with an overall mean of 0.650. The uncorrected p-distance between *B. sessilis* from Doirani and Balaton was zero (0.001 after applying the evolutionary model HKY+I+G). In concordance with the results from the COI gene, the lowest inter-specific nucleotide divergence regarding *B. sessilis* was found with *B. rubens* (0.171). The mean genetic distances for the comparisons *B.sessilis*-*B. plicatilis* species complex and *B. sessilis*-*B. rotundiformis* were 0.696 and 0.713, respectively (Suppl. Table 4).

For the combined gene dataset (COI+ITS1+16S, 39 sequences) the alignment length was 1301 bp (575 bp for COI, 438 bp for ITS1 and 288 bp for 16S). The transitions/transversions vs. genetic distance plot showed no substitution saturation while the g1 statistic indicated the presence of phylogenetic signal (g1=-0.622, $p<0.05$).

All alignment matrices were deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S16282?x-access-code=93d3b29fda697bedd273f8c11b37a4fd&format=html).

Phylogenetic reconstruction

The 16S sequence data failed to yield a resolved and well-supported phylogeny in any of the reconstruction methods (not shown). This is likely due to significant levels of substitution saturation. However, 16S data were incorporated in the concatenated dataset adopting a 'total evidence' approach (Huelsenbeck *et al.* 1996; Lecointre & Deleporte 2004).

For the other two separate datasets, COI and ITS1, ML and Bayesian phylogenies agreed on the phylogenetic position of *B. sessilis* closer to either *B. urceolaris* or *B. rubens*, respectively (Suppl. Fig. 1, 2, 3). Distance and MP methods provided in general less resolved phylogenies with low support values, especially in COI (not shown). In ITS1, MP analysis recovered two most parsimonious trees and provided similar phylogenetic relationships to ML and BI, but with lower support values (Suppl. Fig. 4).

For the joint analysis, the ILD test showed that the phylogenetic signal between the three genes was different ($p<0.05$). Therefore, the combined analysis was performed by employing a different evolutionary model to each gene partition (TIM+I+G for COI, HKY+I+G for ITS1 and GTR+I+G for 16S). Considering the support values of both ML and BI analyses, the combined dataset returned a phylogeny of even higher confidence (Fig. 3). In this combined analysis, which comprised our best phylogenetic hypothesis, *B. sessilis* grouped with *B. rubens* (posterior probability = 0.99, bootstrap = 55%) (Fig. 3). *Brachionus urceolaris* was placed as a sister clade to these species along with *B. quadridentatus* (posterior probability = 1, bootstrap = 99%).

All phylogenies were deposited in TreeBase and can be viewed at http://purl.org/phylo/treebase/phylows/study/TB2:S16282?x-access-code=93d3b29fda697bedd273f8c11b37a4fd&format=html .

Species delimitation

The GMYC model recovered 25 ML entities (confidence interval 16–29) (Table 4). The two populations of *B. sessilis,* Balaton and Doirani, were grouped together in a single cluster suggesting that the rotifers from these two locations belong to the same species. Sequences of *B. calyciflorus* and some members of the *B. plicatilis* complex, namely *B.* sp. Austria, *B.* sp. Cayman and *B. plicatilis* were found to comprise more than a single species in concordance with previous reports (e.g. Gómez *et al.* 2002; Suatoni *et al.* 2006).

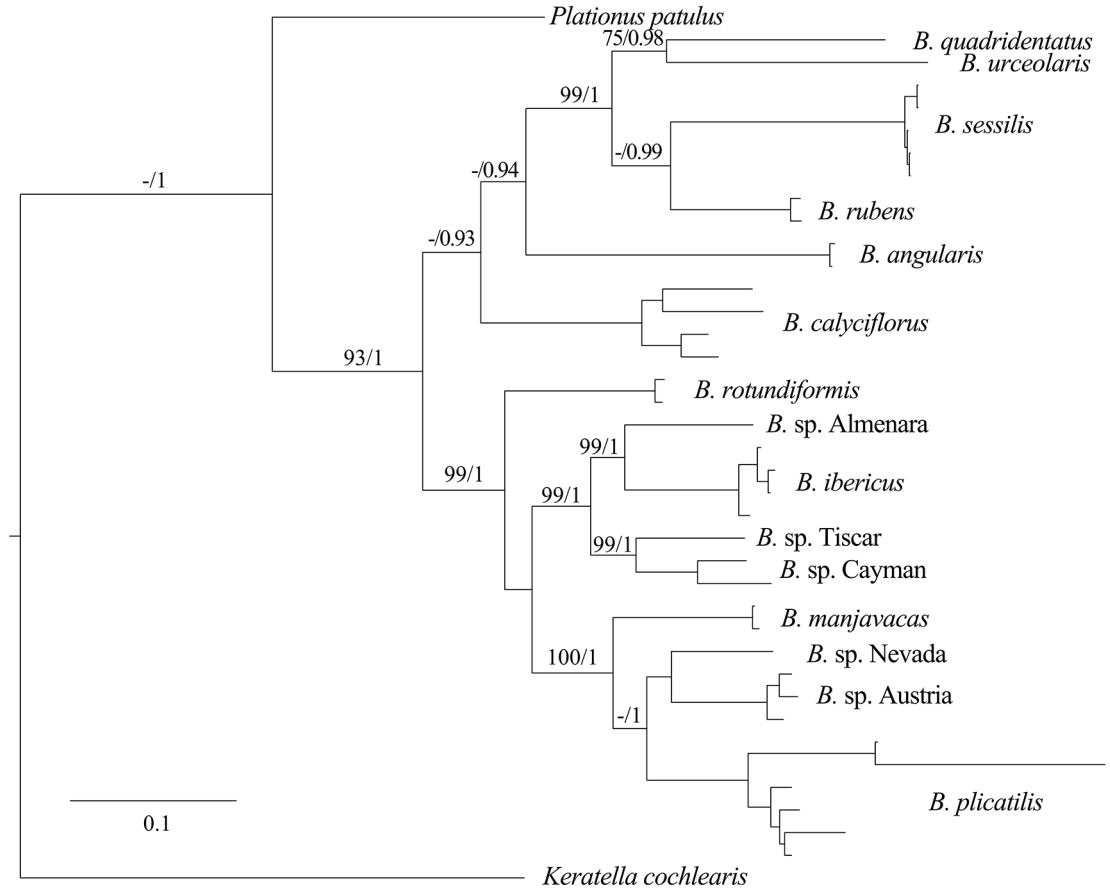

FIGURE 3. Phylogenetic analysis performed on the combined COI, ITS1 and 16S dataset. Parameters correspond to TIM+I+G HKY+I+G and GTR+I+G evolutionary models, respectively. Values above branches represent ML bootstrap values/BI posterior probabilities. Support values below 70/0.90 and for intra-species relationships are not shown. Scale bar indicates 0.1 nucleotide substitutions per site. Branch lengths are proportional to the scale bar. *Keratella cochlearis* and *Plationus patulus* were used as outgroups.

TABLE 4. Species delimitation performed on the concatenated sequence data COI+ITS1+16S using the General Mixed Yule Coalescent (GMYC) model on an ultrametric tree that was obtained from the consensus tree of the Bayesian analysis using penalised likelihood (see text for details). Outgroups *Plationus patulus* and *Keratella cochlearis* were excluded prior to the analysis. Confidence interval for ML entities was 16–29.

Accession number	Species	GMYC ML entity
KM051934	*B. sessilis* (Balaton)	1
KM051935	*B. sessilis* (Balaton)	1
KM051933	*B. sessilis* (Doirani)	1
KM051930	*B. sessilis* (Doirani)	1
KM051931	*B. sessilis* (Doirani)	1
KM051939	*B. rubens*	2
KM051938	*B. rubens*	2
KM051944	*B. angularis*	3
KM051945	*B. angularis*	3
AF387293	*B. rotundiformis*	4
AY785224	*B. rotundiformis*	4

.....continued on the next page

TABLE 4. (Continued)

Accession number	Species	GMYC ML entity
AF387273	*B. ibericus*	5
AY785220	*B. ibericus*	5
KM051941	*B. ibericus*	5
KM051942	*B. ibericus*	5
AF387252	*B. manjavacas*	6
AF387254	*B. manjavacas*	6
AF387247	*B*. sp. Austria	7
AF387248	*B*. sp. Austria	7
AF387249	*B*. sp. Austria	8
AF387295	*B. quadridentatus*	9
KM051937	*B. urceolaris*	10
DQ071673	*B. calyciflorus*	11
DQ071674	*B. calyciflorus*	12
FJ826917	*B. calyciflorus*	13
FJ826918	*B. calyciflorus*	14
AF387265	*B*. sp. Almenara	15
AF387282	*B*. sp. Tiscar	16
AF387285	*B*. sp. Cayman	17
AF387286	*B*. sp. Cayman	18
AF387246	*B*. sp. Nevada	19
KM051943	*B. dimidiatus*	20
AY785192	*B. plicatilis*	21
AY785178	*B. plicatilis*	22
AF266914	*B. plicatilis*	23
AF266853	*B. plicatilis*	24
AY785188	*B. plicatilis*	25

Topotypic material

Permanent glycerin glass slide mounts were prepared according to Jersabek *et al.* (2010), and deposited in the Frank J. Myers collection at the Academy of Natural Sciences in Philadelphia (ANSP) with catalogue numbers ANSP 2081-2086. Each contains a single specimen, placed in different positions focusing on different characteristics.

Amictic female description

Lorica soft, ovoid shaped. Anterior dorsal margin with six roughly pointed, wide-based, triangular spines, three on each side of a V-shaped sinus. The inner spines are the most prominent, each formed like an equilateral triangle (i.e., spines 1 in Fig. 4A); the external ones are shorter (i.e., spines 3 in Fig. 4A). The median spines seem to be the least developed with a wave-like shape, slightly bending towards the ventral plate (i.e., spines 2 in Fig. 4A). From a lateral view, they appear sharper and longer (Fig. 4C, D). Anterior ventral margin curved-shaped, with one pair of lobules placed under the dorsal sinus (Fig. 4B).

When placed laterally, a notch appears at the final posterior margin of the body, conceivably separating the dorsal from the ventral 'plate' (Fig. 4E, dashed line). This notch lies just above the region where the cloaca must be

placed (although under the optical microscope this was not clear).The posterior edge of the dorsal plate exceeds that of the ventral plate (Fig. 4E, dashed line arrow). Foot aperture is placed on the ventral plate right under the notch, posteriorly [Fig. 4E, Fig. 4F (right), dashed line].

Foot retractile. When the foot is extended, the foot aperture is no longer visible in both horizontal (dorsal, ventral) and vertical (lateral) views; this is due to a hump that juts out as a projection of the lorica close to the base of the foot and hinders sight of the region (Fig. 4F, arrow). When the foot retracts into the lorica, foot aperture —in horizontal view—is barely discernible, as a U-shaped structure, over the final posterior margin [Fig. 4F (left)]. In this case, the rear region appears more transparent than the rest of the body and the notch separating the dorsal from the ventral 'plate' is formed like an ellipse [Fig. 4A, B, F (left)].

Schematic drawings of *B. sessilis* as illustrated by Varga (1951) and Chengalath *et al.* (1973) are given in Fig. 4G for comparison.

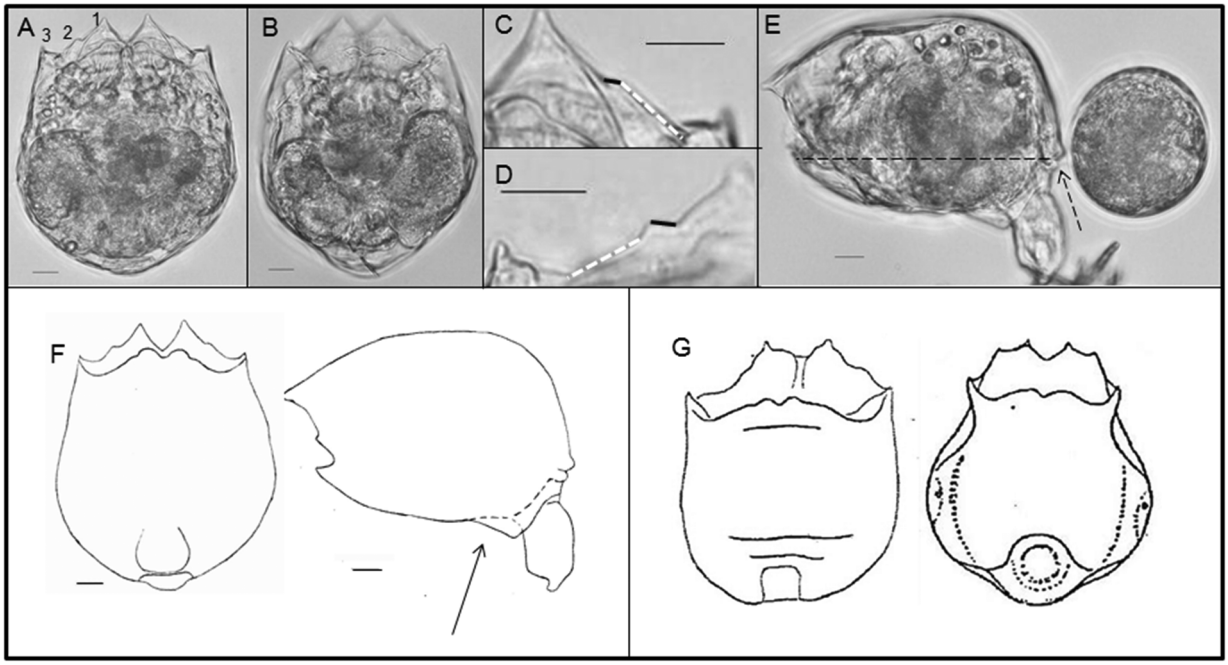

FIGURE 4. *B. sessilis* microphotographs. **A)** Dorsal view, numbers indicate pairs of spines; **B)** ventral view; **C, D)** anterior dorsal margin showing the difference between distances l (white dashed line) and g (black solid line) of Fig. 2, when viewed from different angles (for details see Description); **E)** Lateral view. Dashed line indicates the conceivable limits between the dorsal and the ventral plate, dashed arrow indicates the notch (see text for details); **F)** Schematic drawings of *B. sessilis*, horizontal (left) and vertical (right) view; **G)** Schematic drawings from Varga (1951) and Chengalath *et al.* (1973) (right). (Scale bars: 10 μm).

Discussion

Previous studies in rotifers have shown how detailed traditional biometry can give valuable insights in discriminating subtle differences even between members of species complexes. Such studies have commonly examined measurements of the lorica (e.g. Fu *et al.* 1991; Ciros-Pérez *et al.* 2001; Campillo *et al.* 2005; Fontaneto *et al.* 2007b), and less commonly measurements of resting eggs (Munuswamy *et al.* 1996; Ciros-Pérez *et al.* 2001) and trophi (Fontaneto & Melone 2005; Fontaneto *et al.* 2007b) as potential taxonomic classifiers.

Here, we used 20 linear measurements of the dorsal, ventral and lateral aspects of the lorica of *B. sessilis*. Early works (Harring 1913; Ahlstrom 1940) and several modern studies have used subsets of these measurements in taxonomic delineations demonstrating their value in determining species boundaries in *Brachionus* rotifers (e.g. Fu *et al.* 1991; Ciros-Pérez *et al.* 2001; Campillo *et al.* 2005). We use these studies as a basis to deliver a formal and comprehensive update of the morphology of *B. sessilis*. Although aware of the taxonomic relevance of other structures, e.g. resting eggs or the trophi, we did not attempt to include these descriptors since our aim was to provide a simple set of morphological traits that are handy in routine sample identifications. By providing

topotypical material and a formal description we also offer a basis on which the recognition and report of *B. sessilis* is facilitated.

Complementary to the biometrical measurements, this work uses molecular data (for the first time) to clarify previous taxonomic ambiguities regarding the taxonomic status of *B. sessilis* in relation to the *B. plicatilis* group and *B. urceolaris*. Strong support is obtained for substantial phylogenetic divergence between *B. sessilis* and *B. rotundiformis*, which once was considered as its closest relative (Varga 1951). In fact, *B. sessilis* clusters with high confidence outside the *B. plicatilis* complex of cryptic species (Fig. 3). Our results firmly indicate that *B. sessilis* is certainly not a subspecies of *B. urceolaris* (Fig. 3, Suppl. Figure 1, 2, 3, 4) but is rather to be treated as a distinct species, in concordance with the latest morphology-based taxonomic re-assessments (Segers 2007; Jersabek *et al.* 2012).

Our molecular data reveal *B. rubens*, also an epizoic rotifer (May 1989; Vanjare *et al.* 2010) as the closest relative of *B. sessilis*. Previously, *B. rubens* had also been classified as a variety *B. urceolaris* var. *rubens* (Koste 1978). However, the results of our study (Fig. 3, Suppl. Figures, Suppl. Tables 2, 3, 4) support that *B. sessilis* and *B. rubens* are strongly differentiated from each other as well as from their next closest relatives, *B. urceolaris* and *B. quadridentatus*. Consequently, we suggest that *B. sessilis* and *B. rubens,* should be treated as species-level taxa and not at sub- or infrasubspecific levels.

Interestingly, the closest relatives *B. sessilis* and *B. rubens* are both epizoic unlike their sister clade *B. urceolaris/B. quadridentatus* (Fig. 3). *B. rubens* is often found on the carapace of *Daphnia* species (May 1989). Other *Brachionus* rotifers are also known to be epizoic, for instance *B. caudatus* (Chandra & Kameswara 1976; Sharma 1979), *B. variabilis* (Ahlstrom 1940) and *B. novaezealandiae* (May 1989). These other taxa could not be incorporated into the combined dataset due to the lack of sequence data for the markers used. Regardless, the case of *B. sessilis* and *B. rubens* can be an example of phylogenetic trait conservatism – the tendency of closely related clades to preserve similar ecological traits (Cooper *et al.* 2010; Wiens *et al.* 2010) – with regard to epizoism. A recent study pointed at the eco-evolutionary implications of host-epibiont interactions showing that epizoism of *B. rubens* on *D. magna* can have considerable effects on life history traits of the host, promoting micro-evolutionary responses against rotifer infections (Pauwels *et al.* 2014). Under this perspective, phylogenetic trait conservatism offers a promising prism under which the addition of more taxa to the current dataset could provide valuable insights as to the ecological implications and the mode of evolution of epizoic life in *Brachionus* rotifers.

Conclusions

Our study resolves taxonomic uncertainties surrounding the species-rank status of *B. sessilis*. Sequence analysis of *B. sessilis* rotifers (for the first time) and other relatives provides evidence that *B. rubens*, also epizoic, is its closest phylogenetic relative, whereas *B. urceolaris* is placed as a sister clade to these two species. We present a more detailed description of the species using samples from Lake Balaton (Hungary) where it was originally described, and provide topotypic material to conclude the species description. The current work provides a robust scheme for species delineation and fills a critical gap in rotifer systematics.

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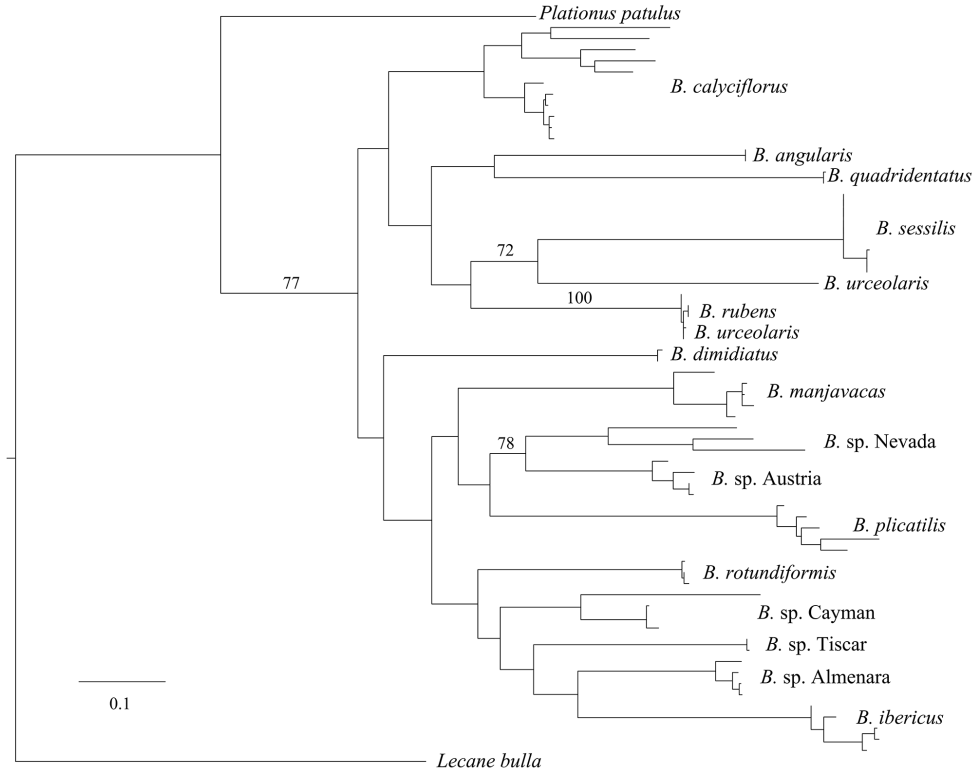
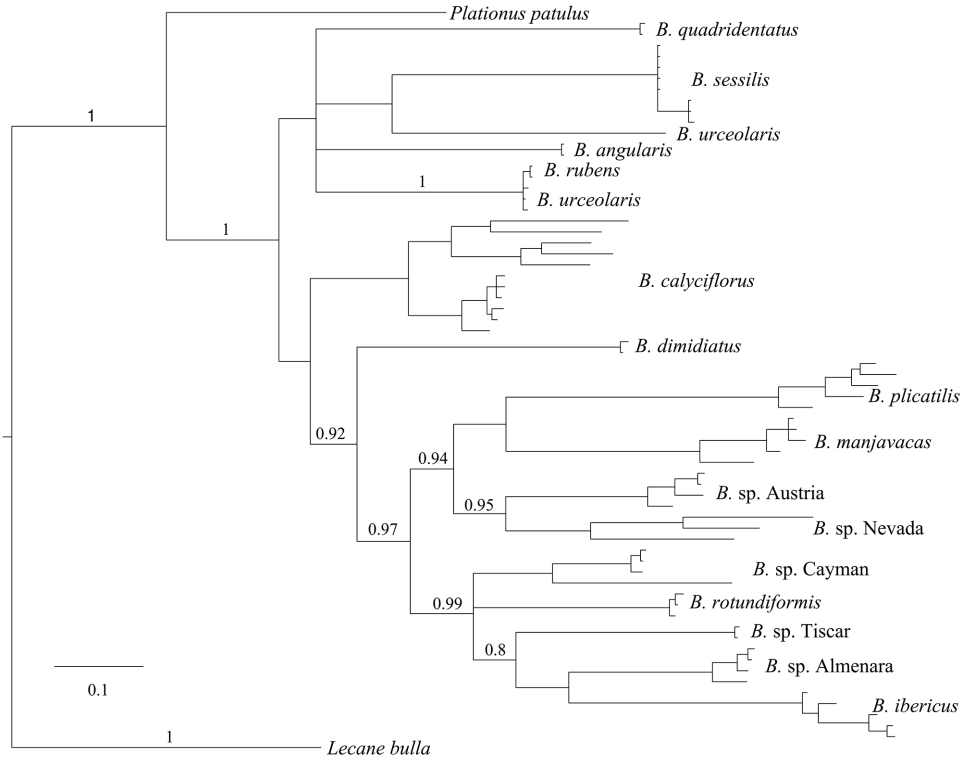
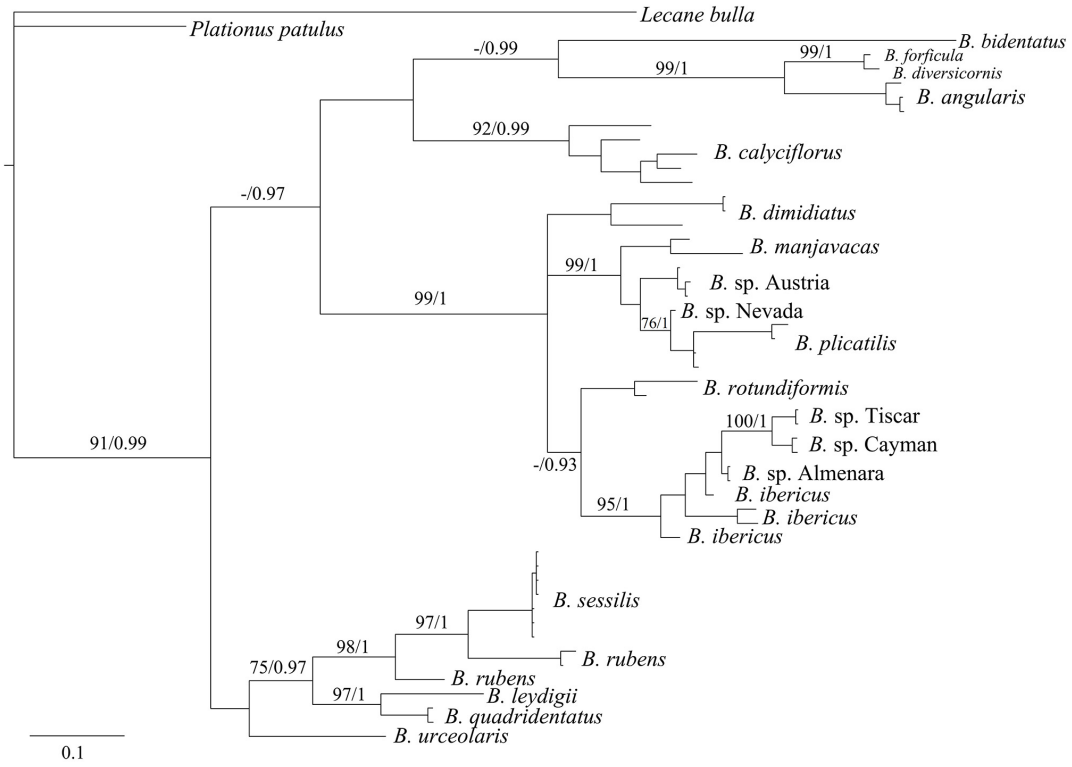
SUPPLEMENTARY MATERIAL



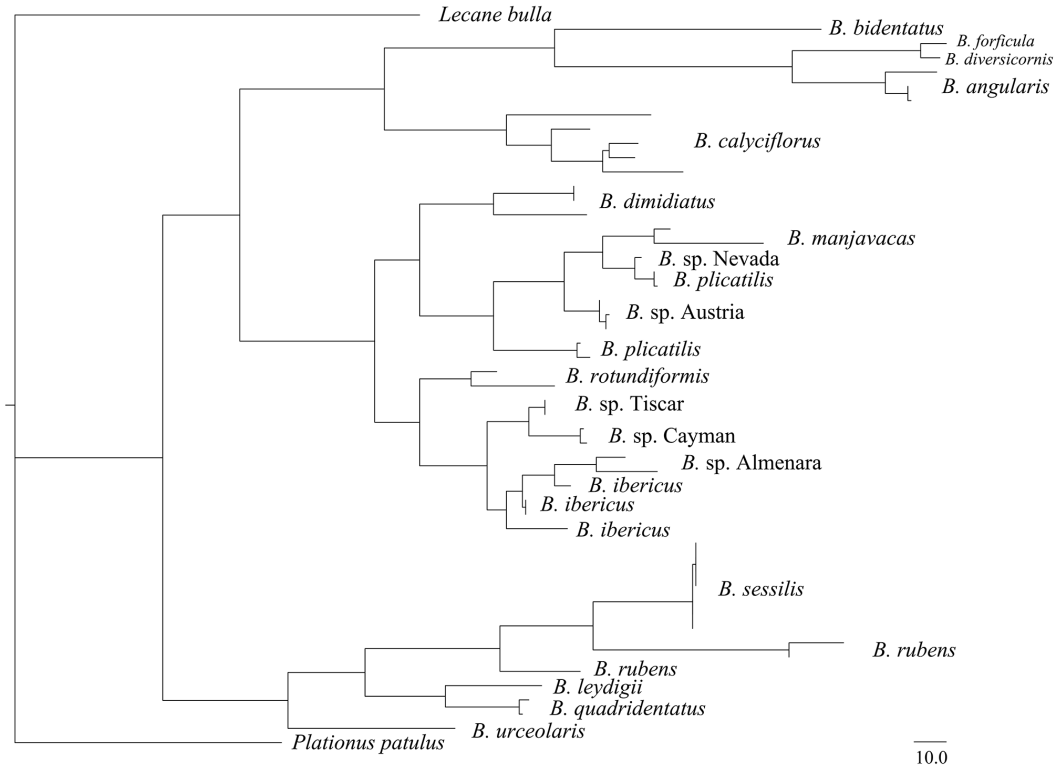
Suppl. FIGURE. 1. Maximum Likelihood phylogram of COI sequence data. Parameters correspond to TIM+I+G evolutionary model. Values above branches represent bootstrap support values. Values below 70% and for intra-species relationships are not shown. Scale bar indicates 0.1 nucleotide substitutions per site. Branch lengths are proportional to the scale bar. *Lecane bulla* and *Plationus patulus* were used as outgroups.



Suppl. FIGURE. 2. Bayesian phylogram of COI sequence data. Parameters correspond to TIM+I+G evolutionary model. Values above branches represent posterior probabilities. Posterior probabilities below 0.90 and for intra-species relationships are not shown. . Scale bar indicates 0.1 nucleotide substitutions per site. Branch lengths are proportional to the scale bar. *Lecane bulla* and *Plationus patulus* were used as outgroups.



Suppl. FIGURE. 3. Phylogram of ITS1 sequence data. Parameters correspond to HKY+I+G evolutionary model. Values above branches represent ML bootstrap values/BI posterior probabilities. Support values below 70/0.90 and for intra-species relationships are not shown. Scale bar indicates 0.1 nucleotide substitutions per site. Branch lengths are proportional to the scale bar. *Lecane bulla* and *Plationus patulus* were used as outgroups.



Suppl. FIGURE. 4. One of the two most parsimonious phylograms of ITS1 sequence data. The two trees showed a minor difference in one terminal node inside the *B. calyciflorus* species complex. Values above branches represent bootstrap support values. Values below 70% and for intra-species relationships are not shown. Scale bar indicates 10 nucleotide substitutions. Branch lengths are proportional to the scale bar. *Lecane bulla* and *Plationus patulus* were used as outgroups.

SUPPL. TABLE 1. Species list and GenBank accession numbers used in the present study. Sequences KM051929-KM051975 come from the present study.

Species/Biotype	Accession number					
	COI	ITS1	16S	COI+ITS1		
				COI	ITS1	16S
B. angularis	KM051944	DQ834362[9]	KM051965	KM051944	KM051958	KM051966
	KM051945	KM051958	KM051966	KM051945	KM051957	FJ426630
		KM051957	FJ426630[11]			
B. calyciflorus	KM051940	DQ071671[1]	EU719112[12]	KM051940	KM051956	
	DQ071674[1]	AF387243[5]	AF499037[6]	DQ071674	DQ071671	GQ203201[19]
	FJ826917[2]	EU978876[10]	KM051969	FJ826917	AF387243	AF499037
	FJ826918[2]	DQ834364[9]		FJ826918	EU978876	EU719112
	FJ826919[2]	KM051956		DQ071673	DQ834364	KM051969
	FJ826932[2]					
	FJ826926[2]					
	FJ826934[2]					
	DQ071673[1]					
	FJ826981[2]					
	FJ827018[2]					
B. dimidiatus	EU046249[3]	KM051961	KM051964	KM051943	KM051961	KM051964
	KM051943		EU046264[3]			
B. ibericus	KM051941	AF387228[5]	KM051963	KM051941	KM051959	KM051963
	AY785220[4]	KM051960	AY647205[13]	AY785220	AY772142	AY647205
	AF387273[5]	KM051959	KM051962	AF387273	AF387228	AM180761[14]
	KM051942	AY772142[4]		KM051942	KM051960	KM051962
	AF387274[5]					
B. plicatilis	AF266914[5]	AY772160[4]	AM180757[14]	AF266914	AF387206	AF499040
	AY785188[4]	AY772159[4]	AM180756[14]	AY785188	AF387198	AY647200
	AF266853[5]	AF387206[5]	AM180760[14]	AF266853	AF387200	AM180760
	AY785178[4]	AF387198[5]	AM180759[14]	AY785178	AF387205	AY647198
	AY785192[4]	AF387200[5]	AM040260[14]	AY785192	AY772160	AJ748702
		AF387205[5]	AM040261[14]			
			AY647200[13]			
			AJ748704[14]			
			AM040258[14]			
			AY647198[13]			
			AM180758[14]			
			AM180755[14]			
			AF499040[6]			

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SUPPL. TABLE 1. (Continued)

Species/Biotype	Accession number					
	COI	ITS1	16S	COI+ITS1		
				COI	ITS1	16S
			AJ748702[14]			
			AF499039[6]			
			AJ748699[14]			
			AF364507[15]			
B. quadridentatus	AF387295[5]	AF387242[5]	FJ426635[11]	AF387295	AF387242	FJ426635
	AF387294[5]	AF387241[5]		AF387294	AF387241	
B. rotundiformis	AF387293[5]	AF387238[5]	AM180762[14]	AF387293	AF387238	AM180762
	AF387288[5]	AY772147[4]	AM292539[16]	AY785224	AY772147	AM292539
	AY785224[4]					
B. rubens	KM051939	KM051953	KM051967	KM051939	KM051953	KM051967
	KM051938	KM051954	KM051968	KM051938	KM051954	FJ426636
		DQ834369[9]	FJ426636[11]			
B. sessilis	KM051933	KM051947	KM051970	KM051933	KM051950	KM051970
	KM051930	KM051948	KM051971	KM051930	KM051947	KM051971
	KM051932	KM051946	KM051972	KM051932	KM051948	
	KM051929	KM051949	KM051973	KM051931	KM051949	KM051972
	KM051931	KM051950	KM051974	KM051929	KM051946	
	KM051934	KM051951		KM051934	KM051951	KM051973
	KM051935	KM051952		KM051935	KM051952	KM051974
B. sp. Almenara	AF387265[5]	AF387221[5]	AM292538[16]	AF387265	AF387221	AM292538
	AF387264[5]	AF387220[5]		AF387264	AF387220	
	AF387268[5]					
	AF387269[5]					
B. sp. Austria	AF387248[5]	AF387210[5]	EU046262[3]	AF387248	AF387210	EU046262
	AY785200[4]	AF387209[5]	EU046263[3]	AF387247	AF387209	EU046263
	AF387247[5]	AF387208[5]	AY647202[13]	AF387249	AF387208	AY647202
	AF387249[5]					
B. sp. Cayman	AF387285[5]	AF387230[5]	AY647204[13]	AF387285	AF387230	AY647204
	AY785203[4]	AF387229[5]		AF387286	AF387229	EU046255
	AY785207[4]					
	AF387286[5]					
B. manjavacas	AF387254[5]	AF387213[5]	AY647201[13]	AF387254	AF387213	AY647201
	AF387252[5]	AF387212[5]		AF387252	AF387212	EU046254[3]
	AF387250[5]					
	AF387256[5]					
	AF387259[5]					

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SUPPL. TABLE 1. (Continued)

Species/Biotype	Accession number					
	COI	ITS1	16S	COI+ITS1		
				COI	ITS1	16S
B. sp. Nevada	AF387246[5]	AF387207[5]	AY647203[13]	AF387246	AF387207	AY647203
	AY785198[4]					
	AY785197[4]					
B. sp. Tiscar	AF387282[5]	AF387235[5]	AM292537[16]	AF387282	AF387235	AM292537
	AF387281[5]	AF387234[5]		AF387281	AF387234	
B. urceolaris	KM051937	KM051955	FJ426637[11]	KM051937	KM051955	KM051975
	AF499070[6]		KM051975			
	AF499071[6]		AF499041[6]			
	AF499072[6]					
Lecane bulla	EU188933[7]	EU202669[7]		EU188933	EU202669	
Plationus patulus	AF416995[8]	DQ834368[9]	AF325132[8]	AF416995	DQ834368	AF325132
			FJ426639[11]			
Keratella cochlearis			AF499043[6]	KC618856[17]	JN574771[18]	AF499043
B. bidentatus		DQ834363[9]				
B. diversicornis		DQ834365[9]	FJ426633[11]			
B. forficula		DQ834366[9]	FJ426640[11]			
B. leydigii		DQ834367[9]				

[1]Gilbert & Walsh (2005); [2]Xiang *et al.* (2010); [3]Papakostas *et al.* (unpublished data); [4]Suatoni *et al.* (2006); [5] Gómez *et al.* (2000); [6]Derry *et al.* (2003); [7]Walsh *et al.* (2009); [8]Garcia-Varela *et al.* (unpublished data); [9]Xiang *et al.* (2005); [10]Zhang *et al.* (unpublished data); [11]Cheng & Xi (unpublished data); [12]Li & Yang (unpublished data); [13]Papakostas *et al.* (2005); [14]Dooms *et al.* (2007); [15]Dornhoff & Ciufo (unpublished data); [16]Dooms *et al.* (unpublished data); [19]Xiang & Xi (unpublished data);

SUPPL. TABLE 2. Inter- and intraspecific genetic distances for 16S sequence data calculated under GTR+I+G evolutionary model (below diagonal) and uncorrected p-distance (above diagonal). **CAL**: *B. calyciflorus*; **RUB**: *B. rubens*; **URC:** *B. urceolaris*; **QUA**: *B. quadridentatus*; **SES**: *B. sessilis*; **PAT**: *Plationus patulus*; **ANG**: *B. angularis*; **FOR**: *B. forficula*; **DIV**: *B. diversicornis*; **L group**: mean of *B. plicatilis*, *B.* sp. Nevada, *B.* sp. Austria; **SM group**: mean of *B. ibericus*, *B.* sp. Almenara, *B.* sp. Tiscar & *B.* sp. Cayman; **ROT**: *B. rotundiformis*; **MNJ**: *B. manjavacas*; **DIM**: *B. dimidiatus*; **KER**: *Keratella cochlearis.*

	CAL	RUB	URC	QUA	SES	PAT	ANG	FOR	DIV	L GROUP	SM GROUP	ROT	MNJ	DIM	KER
CAL	0.051\0.040	0.061	0.094	0.073	0.075	0.115	0.094	0.124	0.129	0.094	0.090	0.118	0.100	0.100	0.184
RUB	0.134	0.042\0.029	0.067	0.051	0.067	0.123	0.097	0.122	0.129	0.091	0.087	0.115	0.094	0.107	0.192
URC	0.190	0.158	0.228\0.105	0.077	0.098	0.123	0.120	0.129	0.134	0.106	0.105	0.124	0.100	0.115	0.199
QUA	0.196	0.155	0.196	0\0	0.087	0.120	0.093	0.109	0.120	0.091	0.093	0.115	0.098	0.104	0.191
SES	0.218	0.214	0.287	0.261	0\0	0.107	0.086	0.120	0.148	0.108	0.101	0.137	0.093	0.082	0.186
PAT	0.302	0.408	0.362	0.393	0.252	0.227\0.227	0.128	0.128	0.161	0.118	0.114	0.148	0.112	0.126	0.175
ANG	0.244	0.242	0.310	0.295	0.305	0.414	0.005\0.003	0.086	0.146	0.115	0.119	0.137	0.118	0.115	0.173
FOR	0.369	0.361	0.347	0.270	0.376	0.391	0.225	0\0	0.126	0.115	0.119	0.131	0.109	0.131	0.175
DIV	0.311	0.304	0.319	0.379	0.388	0.421	0.393	0.313	0\0	0.134	0.141	0.148	0.126	0.169	0.213
L GROUP	0.301	0.226	0.315	0.279	0.352	0.395	0.280	0.328	0.396	0.089\0.053	0.082	0.100	0.058	0.098	0.192
SM GROUP	0.250	0.293	0.297	0.290	0.372	0.381	0.355	0.360	0.439	0.217	0.181\0.063	0.112	0.078	0.093	0.133
ROT	0.367	0.369	0.353	0.393	0.529	0.614	0.411	0.457	0.452	0.290	0.308	0.009\0.009	0.098	0.109	0.224
MNJ	0.312	0.267	0.291	0.299	0.295	0.368	0.272	0.279	0.383	0.083	0.199	0.252	0\0	0.071	0.191
DIM	0.342	0.317	0.393	0.439	0.425	0.483	0.333	0.519	0.723	0.287	0.351	0.381	0.197	0\0	0.208
KER	0.753	0.796	0.760	0.809	0.635	0.509	0.539	0.594	0.816	0.599	0.377	0.908	0.564	0.736	0\0

SUPPL. TABLE 3. Inter- and intraspecific genetic distances for COI sequence data calculated under TIM+I+G evolutionary model (below diagonal) and uncorrected p-distance (above diagonal). **QUA**: *B. quadridentatus*; **SES**: *B.sessilis*; **ANG**: *B. angularis*; **RUB**: *B. rubens*; **URC:** *B. urceolaris*; **LEC**: *Lecane bulla*; **PAT**: *Plationus patulus*; **CAL**: *B. calyciflorus*; **DIM**: *B. dimidiatus*; **SM group**: mean of *B. ibericus*, *B.* sp. Almenara, *B.* sp. Tiscar & *B.* sp. Cayman; **ROT**: *B. rotundiformis*; **L group**: mean of *B. plicatilis*, *B.* sp. Nevada, *B.* sp. Austria; **MNJ**: *B. manjavacas*.

	QUA	SES	ANG	RUB	URC	LEC	PAT	CAL	DIM	SM GROUP	ROT	L GROUP	MNJ
QUA	0.001\0.001	0.209	0.208	0.207	0.211	0.231	0.242	0.199	0.212	0.222	0.214	0.232	0.230
SES	0.645	0\0.008	0.203	0.180	0.183	0.190	0.228	0.185	0.215	0.212	0.199	0.230	0.219
ANG	0.600	0.951	0\0	0.165	0.173	0.183	0.209	0.172	0.188	0.196	0.179	0.210	0.210
RUB	0.622	0.591	0.435	0\0	0.053	0.188	0.204	0.165	0.186	0.199	0.170	0.195	0.229
URC	0.655	0.592	0.488	0.146	0.286\0.099	0.198	0.211	0.172	0.190	0.204	0.181	0.204	0.227
LEC	0.810	0.940	0.588	0.745	0.795	0\0	0.218	0.183	0.211	0.209	0.194	0.241	0.226
PAT	0.899	1.098	0.744	0.733	0.750	0.835	0\0	0.200	0.220	0.214	0.210	0.236	0.223
CAL	0.572	0.638	0.491	0.475	0.502	0.687	0.646	0.143\0.085	0.171	0.190	0.178	0.199	0.196
DIM	0.788	0.868	0.704	0.575	0.578	0.853	0.753	0.492	0.003\0	0.194	0.183	0.192	0.211
SM GROUP	0.760	0.839	0.662	0.662	0.640	0.864	0.745	0.627	0.620	0.300\0.140	0.164	0.196	0.191
ROT	0.751	0.828	0.592	0.516	0.552	0.778	0.871	0.585	0.621	0.469	0.004\0.004	0.188	0.188
L GROUP	0.879	0.915	0.665	0.530	0.681	0.990	0.816	0.571	0.432	0.608	0.531	0.254\0.142	0.195
MNJ	0.963	0.862	0.736	0.823	0.814	0.866	0.871	0.570	0.647	0.576	0.541	0.550	0.040\0.132

SUPPL. TABLE 4. Inter- and intraspecific genetic distances for ITS1 sequence data calculated under HKY+I+G evolutionary model (below diagonal) and uncorrected p-distance (above diagonal). **LEC**: *Lecane bulla* **BID**: *B. bidentatus*; **FOR**: *B. forficula*; **DIV**: *B. diversicornis*; **ANG**: *B. angularis*; **PAT**: *Plationus patulus*; **DIM**: *B. dimidiatus*; **MNJ**: *B. manjavacas*; **L group**: mean of *B. plicatilis*, *B.* sp. Nevada, *B.* sp. Austria; **ROT**: *B. rotundiformis*; **SM group**: mean of *B. ibericus*, *B.* sp. Almenara, *B.* sp. Tiscar & *B.* sp. Cayman; **CAL**: *B. calyciflorus*; **SES**: *B. sessilis*; **RUB**: *B. rubens*; **URC:** *B. urceolaris*; **LEY**: *B. leydigii*; **QUA**: *B. quadridentatus.*

	LEC	BID	FOR	DIV	ANG	PAT	DIM	MNJ	L GROUP	ROT	SM GROUP	CAL	SES	RUB	URC	LEY	QUA
LEC	0\0	0.394	0.378	0.370	0.378	0.315	0.346	0.370	0.375	0.354	0.318	0.329	0.323	0.331	0.339	0.331	0.323
BID	0.438	0\0	0.228	0.236	0.244	0.354	0.312	0.307	0.316	0.287	0.269	0.276	0.268	0.265	0.268	0.260	0.260
FOR	0.445	0.340	0\0	0.008	0.037	0.299	0.265	0.291	0.291	0.268	0.246	0.170	0.197	0.205	0.197	0.205	0.181
DIV	0.451	0.340	0.019	0\0	0.045	0.291	0.273	0.299	0.299	0.276	0.254	0.178	0.205	0.213	0.205	0.213	0.189
ANG	0.473	0.365	0.158	0.159	0.018\0.010	0.299	0.262	0.308	0.304	0.281	0.258	0.183	0.210	0.218	0.210	0.218	0.197
PAT	0.386	0.396	0.376	0.374	0.392	0\0	0.289	0.335	0.329	0.283	0.287	0.294	0.252	0.268	0.228	0.252	0.252
DIM	0.452	0.376	0.358	0.359	0.367	0.360	0.100\0.041	0.184	0.157	0.133	0.140	0.233	0.207	0.195	0.199	0.205	0.184
MNJ	0.439	0.368	0.387	0.390	0.389	0.398	0.236	0.081\0.031	0.083	0.124	0.149	0.233	0.205	0.213	0.228	0.244	0.220
L GROUP	0.450	0.349	0.387	0.384	0.397	0.405	0.213	0.127	0.215\0.039	0.111	0.141	0.245	0.209	0.201	0.218	0.245	0.219
ROT	0.443	0.362	0.395	0.401	0.380	0.358	0.205	0.190	0.182	0.070\0.031	0.112	0.214	0.165	0.171	0.165	0.189	0.173
SM GROUP	0.429	0.354	0.383	0.395	0.392	0.356	0.196	0.206	0.196	0.144	0.056\0.056	0.191	0.169	0.174	0.187	0.183	0.161
CAL	0.411	0.752	0.321	0.329	0.328	0.363	0.352	0.327	0.334	0.312	0.313	0.105\0.052	0.121	0.133	0.121	0.157	0.151
SES	0.435	0.365	0.366	0.366	0.353	0.349	0.353	0.353	0.351	0.352	0.337	0.294	0.001\0	0.034	0.071	0.071	0.063
RUB	0.422	0.368	0.348	0.346	0.341	0.353	0.355	0.354	0.355	0.349	0.344	0.321	0.141	0.119\0.026	0.079	0.079	0.055
URC	0.452	0.362	0.345	0.338	0.319	0.298	0.353	0.353	0.351	0.323	0.354	0.275	0.242	0.222	0\0	0.102	0.087
LEY	0.424	0.354	0.353	0.355	0.341	0.315	0.382	0.396	0.394	0.339	0.371	0.334	0.250	0.219	0.235	0\0	0.039
QUA	0.403	0.362	0.330	0.332	0.328	0.300	0.342	0.361	0.370	0.337	0.361	0.322	0.234	0.202	0.196	0.133	0.006\0