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Diversity of the rotifer *Brachionus plicatilis* species complex (Rotifera: Monogononta) in Iran through integrative taxonomy

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Faunistic survey using a DNA taxonomy approach may provide different results from morphological methods, especially for small and understudied animals. In this study, we report the results from morphometric analyses (linear measurements of the lorica) and DNA taxonomy (generalized mixed Yule coalescent model on the barcoding mtDNA locus cytochrome *c* oxidase subunit I) performed on 15 clonal lineages of the rotifer *Brachionus plicatilis* species complex from six Iranian inland saltwaters. The DNA taxonomy approach found more units of diversity (four) than the morphometric approach (two) in the studied rotifers. Three of the taxa identified in this study are already known as described valid species or as-yet unnamed lineages, but a new, additional lineage is also identified from Iran.

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ADDITIONAL KEYWORDS: COI gene – DNA taxonomy – morphology – rotifers.

INTRODUCTION

Faunistic surveys are the basis for obtaining reliable species lists to be used for macroecological analyses, pivotal to understanding the correlates and determinants of global biodiversity (Orme *et al.*, 2005; Tittensor *et al.*, 2010). Extensive faunistic data sets exist only for well-studied taxa, which typically means only for large animals such as vertebrates and some arthropods (e.g. Kalkman *et al.,* 2008; Constable *et al.,* 2010). When dealing with understudied animals, faunistic studies and species lists are not adequately comprehensive and useful, so that any inference based on those records may be biased by our inability to correctly describe biological diversity (Barbosa *et al.*, 2010; Fontaneto *et al.*, 2012a). Moreover, the reliability of these data sets may be even lower for cases in which taxonomy is not completely resolved and complexes of cryptic species are present.

One of the animal phyla for which the morphological approach has failed to lump distinct entities under a single name is the Rotifera (Gómez *et al.*, 2002b; Schröder & Walsh, 2007; Fontaneto *et al.*, 2009; Tang *et al.*, 2012). Within rotifers, the species complex *Brachionus plicatilis* Müller, 1786 (Monogononta, Brachionidae) is a textbook example of the large amount of diversity, which remained hidden or unclear using only morphological taxonomy. With a morphological approach (reviewed by Koste, 1978), a certain amount of variability was known for *B. plicatilis*, mirrored in a number of subspecies, varieties, and species of doubtful taxonomic validity. Oogami (1976), Snell & Carrillo (1984), and Fu, Hirayama & Natsukari (1991) started clarifying such variability, identifying two morphotypes (L, large

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and S, small). Segers (1995) supported the formal re-establishment of the names *Brachionus rotundiformis* Tschugunoff, 1921 and *B. plicatilis sensu stricto* (*s.s.*) for the S and L morphotypes, respectively. Subsequently, more size-forms were detected (Yúfera, 2001), and officially described as independent species (Ciros-Pérez, Gómez & Serra, 2001; Fontaneto *et al.,* 2007): *Brachionus ibericus* Ciros-Pérez, Gómez & Serra, 2001, as a medium-sized morphotype, and *Brachionus manjavacas* Fontaneto, Giordani, Melone & Serra, 2007, as another species in the L morphotype category. The extensive work carried out on this species complex through the application of a wide array of genetic tools and markers improved our knowledge of its diversity. Such works included analyses of microsatellite (Gómez *et al.*, 2002a) and allozyme (Ortells, Gomez & Serra, 2003) loci, sequences of cytochrome *c* oxidase subunit I (COI) and ribosomal internal transcribed spacer 1 (ITS1) genes (Gómez *et al.*, 2002b), restriction fragment length polymorphic DNA (RFLP) analysis of COI (Campillo *et al.*, 2005) and 16SrRNA (Papakostas *et al.*, 2005) genes, denaturing gradient gel electrophoresis (DGGE) of the 16SrRNA gene (Dooms *et al.*, 2007), and genome size variation (Stelzer, Riss & Stadler, 2011). Suatoni *et al.* (2006) and Baer *et al.* (2008), using similarities in COI and ITS1 genes and cross-mating experiments, suggested the existence of at least 15 species within the complex; recent studies increased this number to above 20 (Fontaneto *et al.*, 2009, 2012b; Stelzer *et al.,* 2011). Several of these cryptic taxa may even co-occur in the same water body (Ortells *et al.*, 2003; Campillo *et al.*, 2011).

Resolving complexes of cryptic species is pivotal to inferring ecological and evolutionary mechanisms of diversification in rotifers; different cryptic species may or may not have differing ecological niches (Ortells *et al.*, 2003; Montero-Pau & Serra, 2011; Obertegger, Fontaneto & Flaim, 2012; Leasi *et al.*, 2013) or distinct environmental preferences in aquaculture (Papakostas *et al.*, 2006). Moreover, uncovering the hidden diversity of cryptic species complexes can offset the errors associated with species richness assessments in macroecological studies (Tang *et al.*, 2012). Here, we perform a faunistic survey of the diversity of *Brachionus* in Iranian inland saltwaters comparing quantitative morphometrics with DNA taxonomy.

MATERIAL AND METHODS

Sampling and identification

Several localities were screened for the presence of the *B. plicatilis* species complex in Iran; the species complex was found in six water bodies located in the West Azarbaijan and Hormozgan provinces, described in Malekzadeh-Viayeh (2010) (Table 1, Supporting Information Appendix S1). The sampling was carried out from October 2007 to October 2008. The specimens were collected by filtering the littoral waters through fine-mesh (30–50 μm) plankton net. Rotifer resting eggs were isolated from the surface sediments, collected during the seasonal drought or freezing, by using the sucrose floatation technique (Malekzadeh-Viayeh, 2010). The resting eggs were hatched according to Garcia-Roger, Carmona & Serra (2006). Taxonomic identification was performed by using the standard keys for the genus and the recent species descriptions (Koste, 1978; Ciros-Pérez *et al.*, 2001; Fontaneto *et al.,* 2007). Drawings were obtained for each clonal culture.

Clonal cultures

Clonal cultures were established starting by inoculation of single amictic female rotifers from each

Table 1. List and type of sampling sites and their geographical position

Site name	Code	City	Coordinates	Habitat type	Salinity (‰)
Shatloo	Sht	Makoo	39°36′15″N 44°42′39″E	Temporary pond	19
Zanbil	Zbl	Urmia	37°44′59″N 45°14′44″E	Small pool	15
Eskeleh	Esk	Urmia	37°36′50″N 45°16′03″E	Sinkhole	40
Seyrangul	Segu	Naqadeh	36°50′17″N 45°34′10″E	Permanent lagoon	25
Qoobi	Qo	Mahabad	36°57′23″N 45°53′11″E	Permanent lagoon	20
Bandar Abbas	Ba	Bandar Abbas	27°14′06″N 56°15′32″E	Artificial pond	40

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population into 24-well Plexiglas tissue-culture microplates containing the water taken from their natural habitats. The animals were then acclimated to the laboratory culture conditions with water salinity of 15‰, temperature of 25 °C, pH of 7–7.5 and feeding on *Chlorella vulgaris*. The rotifer cultures were then maintained in 500 mL Erlenmeyer flasks to provide adequate samples for the experiments. Clonal cultures were all kept under the same laboratory conditions in order to minimize the possibility that differences in body shape could be ecologically related, e.g. phenotypic changes resulting from local temperature, salinity, or chemistry.

Morphometric analyses

A quantitative morphometric approach describing the shape of the lorica was applied to identify distinct lineages in selected clonal cultures belonging to the *B. plicatilis* species complex. Nine linear dimensions were selected for the measurements according to Ciros-Pérez *et al*. (2001) (Fig. 1). For each of the selected clonal lines, about 20 egg-bearing amictic females were haphazardly taken from the cultures. This procedure was carried out to maximize the within-group variability and to allow us to identify the discriminatory variables that would perform better between groups, even with large within-group variability. The animals were anaesthetized with marcaine (Wallace, Snell & Ricci, 2006), observed under microscope slides, and linear measurements taken from each of the animals under a compound Nikon microscope at 20–40 × magnification.

The linear measurements were analysed through discriminant analyses. In order to identify the most plausible number of potential taxonomic units from the morphometric approach, we used the Calinski criterion from a *K*-means partitioning (Legendre & Legendre, 1998) using a range of values of *K* (different groups) from 2 to the number of analysed clonal lineages. The *K*-means was performed on the axes of the linear discriminant analysis obtained from untransformed measurements, using the clonal lineages as the class for each observation. The discriminant analysis was performed in R 2.14.0 (R Development Core Team, 2011) with the functions *lda* of the package *MASS* 7.3–16 (http://cran.r-project.org/web/packages/MASS/index.html) and the *K*-means partitioning with Calinski criterion with the function *cascade KM* of the R package *vegan* 2.0–2 (Oksanen *et al*., 2011).

DNA taxonomy

Single rotifers were taken from each clonal culture and rinsed in distilled water to remove waste and uneaten algal cells. DNA extraction was performed by using the approach of Gómez *et al*. (2002b): single rotifers were transferred into PCR tubes containing 20 μL of 6% Chelex 100 resin (Bio-Rad, Hemel Hempstead, UK) and 5 μL proteinase K. The mixture were then heated at 55 °C for 2 h, while being vortexed every 10 min, then boiled at 100 °C for 10 min and cooled down at room temperature for 30 min. The samples were centrifuged at 7200 g for 5 min. The extracted DNAs were stored at 4 °C to be used directly in PCR reactions or at −20 °C for long storage periods.

A fragment of the mitochondrial COI gene was amplified by PCR. The PCR reactions were performed

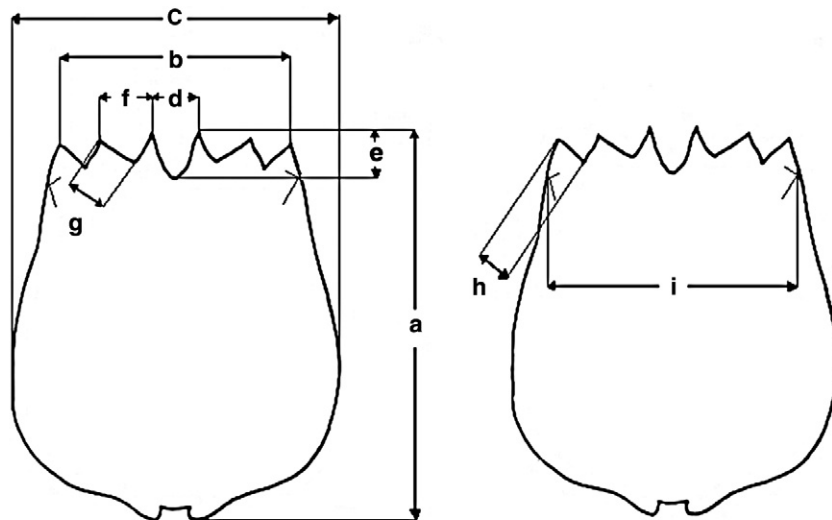

Figure 1. Nine linear measurements of the lorica of *Brachionus* rotifers used in this study, named from (a) to (i) as in Ciros-Pérez *et al*. (2001).

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Table 2. Clonal populations of the *Brachionus plicatilis* species complex from Iran, locality of origin, GenBank accessions and putative taxon according to the morphotype and the generalized mixed Yule coalescent model (see Fig. 6). Asterisks after the name of the clonal lines identify the ones used also for the morphometric analyses

Clonal line	GenBank	Morphotype	Species
Ba	JX293040	Small	B03
Esk*	JX293039	Small	B03
Qo_S2	JX293038	Small	B03
Segu_L1	JX293045	Large	*Brachionus* 'Austria'
Segu_L2*	JX293044	Large	*B.* 'Austria'
Segu_S1	JX293035	Small	*Brachionus* 'Tiscar'
Segu_S2*	JX293036	Small	*B.* 'Tiscar'
Sht_L1*	JX293047	Large	*B. plicatilis s.s.*
Sht_L2*	JX293046	Large	*B. plicatilis s.s.*
Sht_S1*	JX293041	Small	B03
Zbl1_L*	JX293050	Large	*B. plicatilis s.s.*
Zbl1_S	JX293042	Small	B03
Zbl3_1*	JX293049	Large	*B. plicatilis s.s.*
Zbl3_2*	JX293048	Large	*B. plicatilis s.s.*
Zbl4_S	JX293037	Small	B03

L, large and S, small morphotypes.

in 10 μL final volume containing 2 μL template DNA, 1.5 mM $MgCl_2$, 200 mM of each nucleotide, 2.5 pmol of each primer, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 °C), 0.01% Tween-20 buffer, and 0.125 U of *Taq* DNA polymerase (CinnaGen, Iran). The primers for the amplification were LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAA AAATCA-3′) (Folmer *et al.,* 1994). PCR cycling conditions were as follows: denaturation at 95 °C for 3 min, 35 cycles of 95 °C for 1 min, annealing at 46–48 °C for 1 min, extension at 72 °C for 1.5 min, followed by a post-amplification incubation at 72 °C for 7 min. The reactions were performed in a Bioer XP (China) thermal cycler. The PCR products were separated by electrophoresis on a 1% agarose gel against a 1 kb DNA ladder to ensure the production of the expected fragments. A volume of 25 μL of each amplified fragment was sequenced by BioNeer (South Korea). Chromatograms were controlled by eye and sequences checked and edited with MacClade 4.08 (http://macclade.org/), looking for potential reading errors such as frame shifts, stop codons, and gaps.

In order to identify the species in the *B. plicatilis* species complex found in Iran, we used a DNA taxonomy approach, in the framework of previously published sequences and information. The DNA sequences that we obtained (Table 2) were aligned with all the available COI sequences for the *B. plicatilis* species complex in GenBank (Table 3). The complete data sets were then reduced to haplotypes only, in order to avoid redundancy. The alignment for COI in rotifers is trivial, as no insertions or deletions are present; thus, a text editor was used to obtain the alignments.

Phylogenetic trees were reconstructed to look for evidence of independently evolving entities with a maximum likelihood approach (Pons *et al.*, 2006). The selected model of evolution for the phylogenetic reconstructions was Hasegawa-Kishino-Yano+Invariant+ Gamma (HKY + I + G), chosen by Akaike information criterion and Bayesian information criterion (BIC) in MODEL GENERATOR v. 0.85 (Keane *et al.*, 2006). This model was implemented in BEAST v. 1.6.1 (Drummond & Rambaut, 2007), including an uncorrelated lognormal relaxed clock with estimated rate and coalescent prior as suggested by Ceccarelli, Sharkey & Zaldívar-Riverón (2012) for DNA taxonomy with COI data sets. The analysis was run for 50 000 000 generations, keeping all other default settings, and the first 20% of trees were discarded when obtaining the consensus tree. The rotifer *Epiphanes senta* (GenBank accession DQ089728) was used as outgroup. The generalized mixed Yule coalescent (GMYC) model (Pons *et al.*, 2006; Fujisawa & Barraclough, 2013) was then applied to test for the presence of cryptic species. The method uses a maximum likelihood approach to optimize the threshold identifying the shift in the branching patterns of the gene tree from interspecific branches (Yule model) to intraspecific branches (coalescent). It identifies clusters of sequences corresponding to independently evolving entities. The maximum likelihood approach also provides a 95% confidence interval around the

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Table 3. GenBank accession numbers for the sequence of the *Brachionus plicatilis* species complex used for the phylogenetic reconstruction. The data set for *B. plicatilis* includes the already-described *B. ibericus*, *B. manjavacas*, *B. plicatilis s.s.*, and *B. rotundiformis*. The original papers and the countries of origin for the sequences are listed

GenBank accessions	Source	Country of origin
AF266853–AF266950, AF387246–AF387261, AF387270–AF387280, AF387287–AF387293, AF387244–AF387245, AF387262–AF387269, AF387281–AF387286, AF499054–AF499069, AM180752, AY785174–AY785219, AY785220–AY785235, DQ089826–DQ089998, DQ140386, DQ314556–DQ314566, DQ346198–DQ346204, DQ664507, DQ865457, DQ865458, DQ865459, EF017608–EF017655, EF524543–EF524555, EU289219, HQ444171–HQ444172.	Gómez & Carvalho (2000), Gómez *et al*. (2002b, 2007), Campillo *et al*. (2005), Papakostas *et al*. (2006), Suatoni *et al*. (2006), Lowe *et al*. (2007), Mills, Lunt & Gómez (2007), Baer *et al*. (2008), Curini-Galletti *et al*. (2012).	Australia, Austria, Indonesia, Canada, China, Israel, Italy, Japan, Kazakhstan, Malaysia, Russia, Spain, Tunisia, Turkey, UK, USA

maximum likelihood solution. We used the conservative approach of identifying the minimum number of entities within the 95% confidence interval in order not to over-split the species complex. The GMYC model was performed with the R package *splits* 1.0–11 (https://r-forge.r-project.org/projects/splits/).

In order to compare the results of DNA taxonomy and the morphometric data, we assessed the number of individuals correctly predicted as belonging to the different DNA taxonomy units (GMYC entities) on the basis of the axes of the discriminant analysis on the morphometric variables. This was carried out with the function *predict.lda* of the R package *MASS*, using the identity of the DNA taxonomy units as the grouping variable.

RESULTS

In total, 15 clonal lineages of the *B. plicatilis* species complex were sampled from six sampling sites. Only nine clonal lineages grew well enough to provide adequate numbers of egg-bearing animals for the morphometric studies. The examined rotifers showed remarkable morphological variability (Fig. 2): seven cultures belonged to the L morphotype (adult lorica length > 200 μm) and eight to the S morphotype (adult lorica length < 200 μm). Such bimodality in lorica length is clearly present in the measurements from the nine analysed clonal lineages in Figure 3.

MORPHOMETRICS

Twenty individuals from each of the nine clonal lineages of the *B. plicatilis* species complex were used for the morphometric analyses. Overall, 187 animals were measured (Supporting Information Appendix S2). The discriminant analysis provided eight axes of variation, the first two cumulatively explaining 88.7% of the total variance (75.3 and 13.4%, respectively), and visually separated groups of clonal lines (Fig. 4). The Calinski criterion on the *K*-means partitioning gave two groups as the best solution (Fig. 5). The two groups corresponded almost perfectly to the L and S morphotypes: all S individuals (*N* = 61) clustered in one group, and 122 out of 126 L individuals (96.8%) clustered in the other group; only four L individuals of the clonal lineage Segu-L2 grouped with the S individuals. Repeating the *K*-means partitioning separately for the L and the S morphotypes, two groups were found within the L morphotype, and three groups within the S morphotype (Supporting Information Appendix S3).

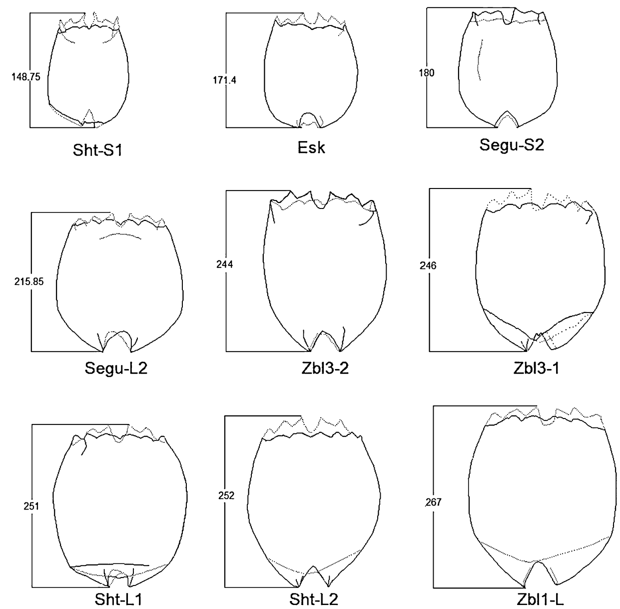

Figure 2. Morphological diversity of the Iranian populations of *Brachionus plicatilis*. Lorica lengths are given in micrometres. Refer to Table 1 for abbreviations. L, large morphotype; S, small morphotype.

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DNA TAXONOMY

A 661-bp region of the COI gene was amplified. The 488 sequences downloaded from GenBank for the *B. plicatilis* species complex resulted in 105 haplotypes; the 15 clonal lineages from Iran added eight further haplotypes to the data set, giving a total of 113 haplotypes. The phylogenetic reconstruction (Fig. 6) provided evidence of at least 22 independent entities, potential cryptic species, in the species complex [GMYC model: likelihood of the null model = 606.09 (one entity), maximum likelihood of GMYC model = 624.54, number of entities = 39, 95% confidence interval = 22–44 entities]. Eight of the GMYC entities are identified by single haplotypes, whereas the most diverse entity, *B. plicatilis s.s.*, has more than 40 haplotypes (Fig. 6, Table 4). Four of the 22 GMYC entities have already been named and described formally as *B. ibericus*, *B. manjavacas*, *B. plicatilis s.s.*, and *B. rotundiformis*; another nine entities as yet have only unofficial names, *B.* 'Almenara', *B.* 'Austria', *B.* 'Cayman' (comprising two GMYC entities), *B.* 'Coyrecupiensis', *B.* 'Harvey', *B.* 'Lost', *B.* 'Nevada' (comprising three GMYC entities), *B.* 'Tiscar', and *B.* 'Towerinniensis'. Six additional potential cryptic species have been identified by the GMYC model but have no name yet.

Genetic distances (uncorrected pairwise distances) within each entity ranged from 0.1 to 9.0%, whereas distances between them ranged from 7.9 to 23.9% (Table 4). Two unofficially named taxa, *B.* 'Cayman' and *B.* 'Nevada', have been split into more entities by the GMYC model: genetic distances between the two GMYC entities within *B.* 'Cayman' ranged from 10.1 to 12.7%, whereas amongst the three GMYC entities within *B.* 'Nevada' ranged from 9.4 to 13.1%.

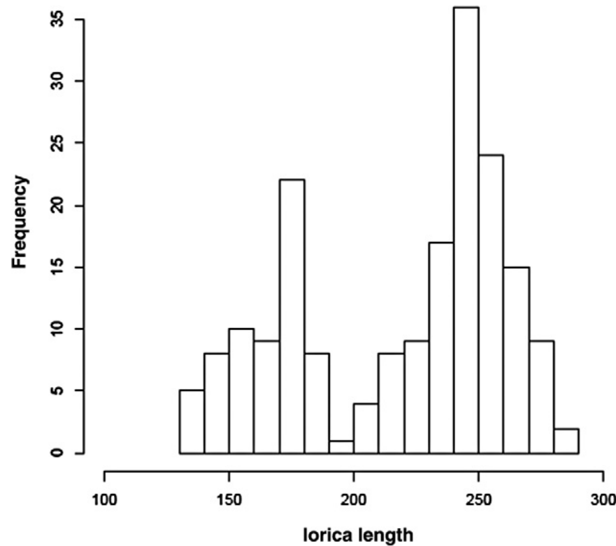

Figure 3. Frequency distribution of the rotifers based on their lorica length in micrometres.

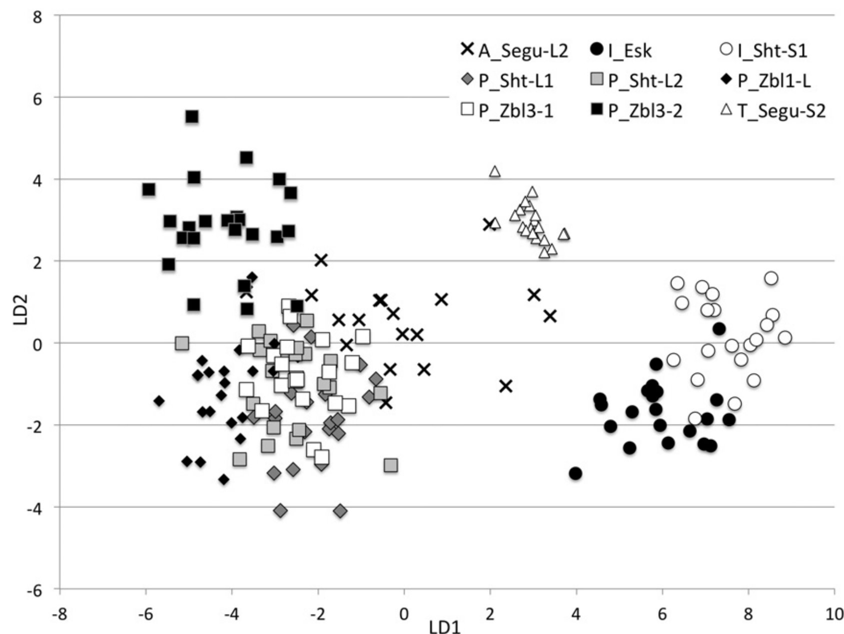

Figure 4. Scatter plot of the measured individuals in the space defined by the first two discriminant axes. Names of the clonal lines refer to those of Table 1, but start with an additional capital letter, identifying the species according to DNA taxonomy (A, *Brachionus* 'Austria'; I, B03; P, *Brachionus plicatilis s.s.*; T, *Brachionus* 'Tiscar'). LD1, 2, Linear Discriminant axes.

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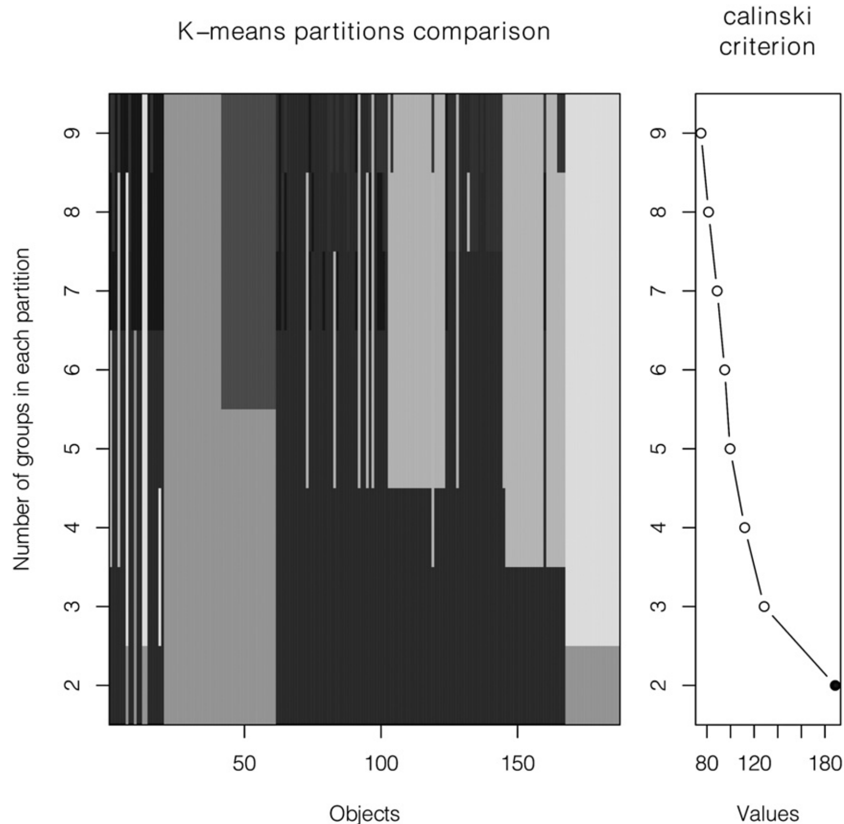

Figure 5. Output of the *K*-means partitioning and choice of the best fit according to the Calinski criterion. The greyscale codes for the *K*-means partitioning indicate the identity of each individual (objects on the *x*-axis) in the groups from 2 to 9 on the *y*-axis. Individuals are numbered from 1 to 187 as in Appendix S2 (1 to 20 is *Brachionus* 'Austria', 21 to 61 is B03, 62 to 167 is *Brachionus plicatilis s.s.*, and 168 to 187 is *Brachionus* 'Tiscar').The most likely value of the criterion is marked as a filled circle.

Four GMYC entities have been found in Iran within the *B. plicatilis* species complex, namely *B.* 'Austria', *B. plicatilis s.s.*, *B.* 'Tiscar', and a new lineage, common and widespread in Iran (B03 in Fig. 6). The newly identified lineage is closely related to other two clades, *B.* 'Tiscar' and an as-yet unnamed one (B02 in Fig. 6). The genetic distances between the newly identified lineage B03 and the closely related taxa are 8.5% with B02 and above 10% with *B.* 'Tiscar'.

DNA taxonomy vs. morphometrics

We used the eight axes obtained by the discriminant analysis on the nine clonal cultures to assess the predictive power of morphology on the entities identified by the GMYC model. In the shape space, *B.* 'Austria' was positioned in between *B. plicatilis s.s.* and *B.* 'Tiscar' (Fig. 4). The discriminant analysis correctly attributed 100% of the individuals of B03 and of *B.* 'Tiscar' to their entities. Three of the 106 individuals of *B. plicatilis s.s.* were mistakenly attributed to *B.* 'Austria' (97.1% correct discrimination), whereas two of the 20 individuals of *B.* 'Austria' were mistakenly attributed to *B. plicatilis s.s.* and another two to *B.* 'Tiscar' (only 80% correctly discriminated).

Comparing DNA taxonomy with the results of the *K*-means partitioning, within the L group, the distinction of the two species (*B.* 'Austria' and *B. plicatilis s.s.*) was clear. Within the S group, the best solution of the *K*-means partitioning revealed three groups, which corresponded to the three analysed clonal lineages and not to the two species from DNA taxonomy (*B.* 'Tiscar' and B03).

DISCUSSION

Our faunistic survey of the *B. plicatilis* species complex in Iran provided evidence of four different taxa from DNA taxonomy, whereas only two morphotypes, corresponding to the S and L types, could be unambiguously identified from discriminant analysis on the morphometric data. Once DNA taxonomy identified the four potential entities, morphometrics supported the existence of such entities with relatively

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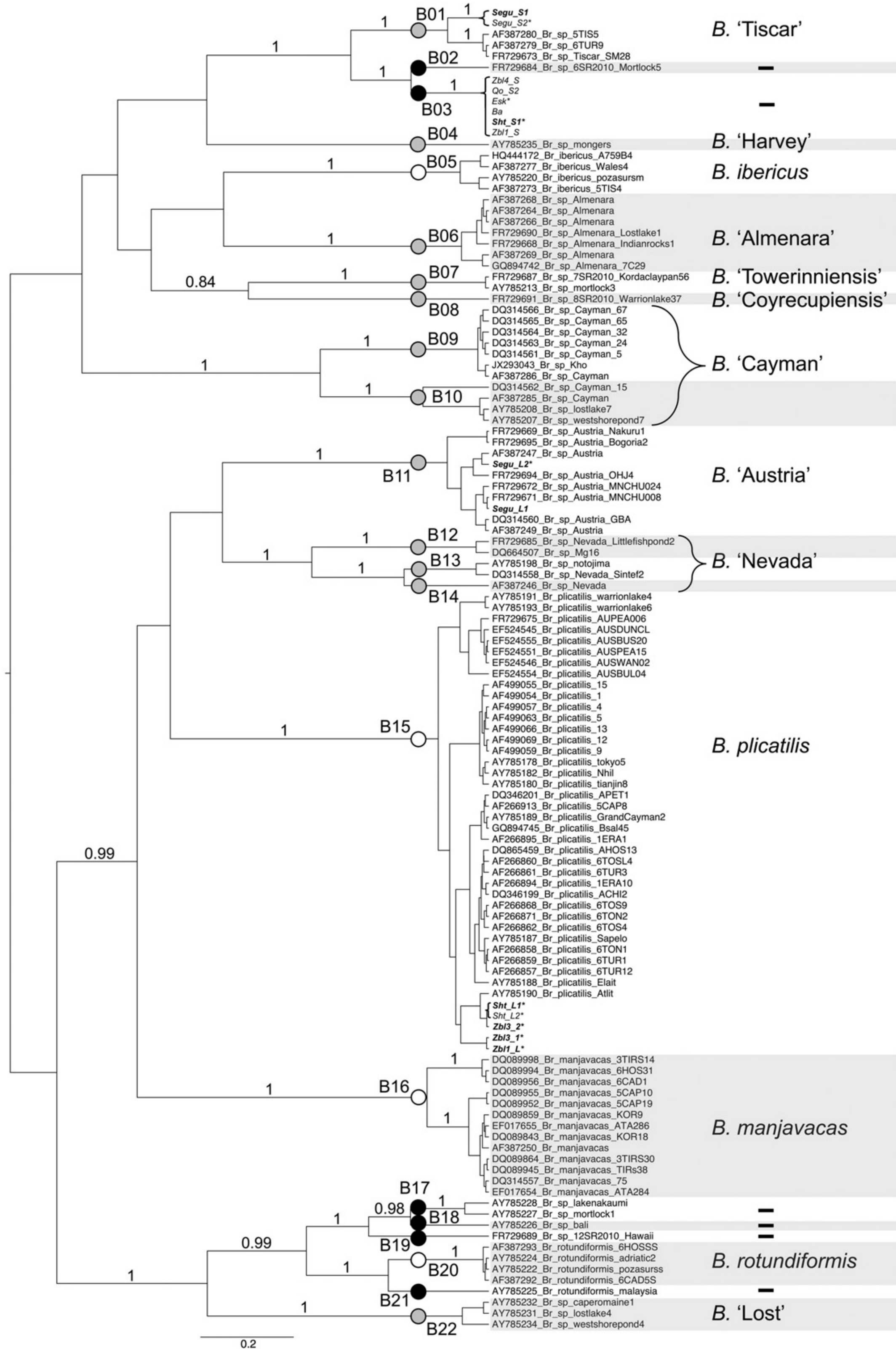

Figure 6. See legend on next page.

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Table 4. Uncorrected pairwise genetic distances within and between the clades identified by the generalized mixed Yule coalescent (GMYC) model as units of diversity in the *Brachionus plicatilis* species complex

GMYC entity	Name	No. of haplotypes	Mean	Median	Min.	Max.
B01	*Brachionus* 'Tiscar'	4	0.028	0.028	0.002	0.053
B02	Unnamed	1	–	–	–	–
B03	Unnamed	1	–	–	–	–
B04	*Brachionus* 'Harvey'	1	–	–	–	–
B05	*Brachionus ibericus*	4	0.030	0.037	0.012	0.040
B06	*Brachionus* 'Almenara'	7	0.020	0.015	0.003	0.036
B07	*Brachionus* 'Towerinniensis'	2	0.003	0.003	0.003	0.003
B08	*Brachionus* 'Coyrecupiensis'	1	–	–	–	–
B09	*Brachionus* 'Cayman'	7	0.010	0.009	0.003	0.020
B10	*B.* 'Cayman'	4	0.035	0.036	0.006	0.063
B11	*Brachionus* 'Austria'	10	0.035	0.038	0.001	0.056
B12	*Brachionus* 'Nevada'	2	0.017	0.017	0.017	0.017
B13	*B.* 'Nevada'	2	0.018	0.018	0.018	0.018
B14	*B.* 'Nevada'	1	–	–	–	–
B15	*B. plicatilis s.s.*	41	0.045	0.049	0.001	0.090
B16	*Brachionus manjavacas*	13	0.036	0.023	0.001	0.080
B17	Unnamed	2	0.033	0.033	0.033	0.033
B18	Unnamed	1	–	–	–	–
B19	Unnamed	1	–	–	–	–
B20	*Brachionus rotundiformis*	4	0.004	0.004	0.002	0.007
B21	Unnamed	1	–	–	–	–
B22	*Brachionus* 'Lost'	3	0.025	0.032	0.006	0.036
	Amongst all	–	0.187	0.189	0.079	0.239
	Between the two *B.* 'Cayman'	–	0.112	0.113	0.101	0.126
	Amongst the three *B.* 'Nevada'	–	0.120	0.126	0.095	0.131

good, albeit not complete, predictive power. Thus, morphometrics alone cannot be used as a reliable taxonomic tool for the studied rotifer group, even though it can statistically support the morphological distinctiveness of taxonomic entities identified with DNA taxonomy. Only an integrative approach, merging the results from morphological and molecular approaches in taxonomy, is able to provide support to describe the actual diversity of saltwater *Brachionus* in faunistic surveys such as the one we performed in Iran. However, it still needs to be tested if such an integrative approach would be reliable for all the more than 20 cryptic taxa in the *B. plicatilis* species complex. Although cryptic species have also been reported in other zooplankton groups (e.g. *Daphnia*: Nilssen *et al.*, 2007) and in most microscopic animals of the meiofauna (Tang *et al.*, 2012), the amount of hidden diversity in rotifers is high (Gómez *et al.*, 2002b; Schröder & Walsh, 2007; Fontaneto *et al.*, 2009). Moreover, for rotifers, it has been demonstrated that using morphological species or DNA-taxonomy species as the units of diversity for ecological analyses can provide different results (Obertegger *et al.*, 2012).

Figure 6. Phylogenetic relationships in the *Brachionus plicatilis* species complex. The consensus of 40 000 sampled trees from Bayesian analysis of the cytochrome *c* oxidase subunit I data set is shown, displaying all compatible groupings and with average branch lengths proportional to numbers of substitutions per site under a HKY + I + G substitution model. Posterior probabilities higher than 0.80 are shown above each branch. Support values for within-species relationships are not shown for very short branches. The outgroup is not shown. Circles on branches indicate generalized mixed Yule coalescent entities akin to cryptic species; white circles correspond to species that have already been described; grey circles correspond to species that have only an unofficial name; black circles correspond to putative new taxa, still not named. Fifteen Iranian rotifer lineages are shown in italics. Bold italics are the eight new haplotypes used in the phylogenetic reconstruction. The additional seven taxa in italics represent populations with the same haplotypes as those already present. They were added manually to the tree for graphical representation only. Asterisks after the names of the Iranian rotifers identify the ones used for the morphometric analyses.

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The case of the *B. plicatilis* species complex may well be an extreme instance of hidden diversity; almost every study employing a molecular approach identifies new clades that could be putative new species (Gómez *et al.*, 2002b; Suatoni *et al.*, 2006; Baer *et al.*, 2008; Stelzer *et al.*, 2011). Along the same lines, we here identify the new strain B03, closely related to *B.* 'Tiscar'. The GMYC model supports the identity of the new lineage from Iran as an independent entity; the genetic distances to its most closely related species are above 8%, even higher than what is known between species of rotifers and of other animals, usually above 3% (Hajibabaei *et al.*, 2006; Fontaneto *et al.*, 2009; Zou *et al.*, 2011; Tang *et al.*, 2012). Our study also suggests that two other strains, *B.* 'Cayman' and *B.* 'Nevada', should be split. It has to be noted, however, that the GMYC model had a fairly large confidence interval for its solutions. Between 22 and 44 entities could be supported, and it is already known that this model can provide unclear results in specific cases (Lohse, 2009; Esselstyn *et al.*, 2012). We chose the most conservative estimate of 22 taxa. This choice includes entities that are comparable to previous studies and that are supported by cross-mating experiments (Suatoni *et al.*, 2006).

Our study confirms that the *B. plicatilis* complex is species-rich, and that further work should still be performed on its ecology, biogeography, and taxonomy at all levels to unravel the actual diversity within the complex. The relatively limited number of analysed populations did not allow us to statistically analyse and support any ecological or biogeographical pattern. Nevertheless, our data will be useful for future meta-analyses gathering such information for a larger data set.

There is an urgent need to stabilize the large number of unofficial names that have been given to the genetic lineages, to prevent taxonomic confusion, given that they are not valid and cannot be used unambiguously. The large amount of unexplored and unresolved diversity is rather surprising, given that this taxon has attracted lots of attention and funding because of its use as food for fish farming (Theilacker & McMaster, 1971; Yoshimura *et al.*, 1996), and as a model system for various biological studies including cyclical reproduction (Snell, 2011; Stelzer, 2011) and dormancy resistance (Denekamp *et al.*, 2009; Clark *et al.*, 2012). The existence of large amounts of hidden diversity in the taxon should be carefully considered when ecological and/or evolutionary inferences are made based on the differences amongst studies; there is always the possibility that distinct species within the species complex have been considered in different studies. Thus, a precise assessment of the identity of the cryptic species of *B. plicatilis* through a DNA taxonomy approach, now available as COI and/or ITS1 gene polymorphism, should always be provided, given that the morphological features and the morphometric parameters of the lorica (as suggested in our study) and even the shape of the trophi (e.g. Fontaneto *et al.*, 2007) are insufficient for correct identification of the taxon.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Map of Iran and position of sampling localities. Sampling sites of two rotifer strains (Ti and Ba) are shown on the southern part of the country. Refer to Table 1 for abbreviations.

Appendix S2. Original measurements (a–i) in micrometres for the 187 animals of the nine clonal lineages of *Brachionus plicatilis* used in the study.

Appendix S3. Output of the *K*-means partitioning and choice of the best fit according to the Calinski criterion. Analyses are limited to (A) the large and (B) the small morphotypes within the *Brachionus plicatilis* species complex. The colour codes for the *K*-means partitioning identify the identity of each individual (objects on the *x*-axis) in the groups from 2 to 9 on the *y*-axis. Individuals are numbered from 1 to 187 as in Appendix S2. The most likely value of the criterion is marked in red.