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RESEARCH ARTICLE

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The utility of the 16S gene in investigating cryptic speciation within the *Brachionus plicatilis* species complex

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Abstract Recent reports indicate an extensive amount of molecular evolution separating cryptic taxa as well as significant population structure at a microgeographical scale. Appropriate molecular markers are particularly suitable for distinguishing cryptic biological species. In this study, we examine the phylogenetic utility of 16S rRNA in elucidating the evolutionary relationships within the recently described euryhaline *Brachionus plicatilis* species complex. In addition, we assess the applicability of this marker in the genetic identification and monitoring of rotifer populations. We have sequenced a 378-bp fragment of the mitochondrial 16S rRNA gene in laboratory reference strains, hatchery clones as well as collections from a wild population of the subsaline Lake Koroneia (Northern Greece). Also, restriction fragment length polymorphism (RFLP) analysis was performed with eight restriction endonucleases. Rotifer samples are distinguished into six genetically divergent lineages. Average sequence divergence between lineages is 0.1038. The evolutionary relationships and divergence time-scales revealed with the 16S sequence data are in agreement with previous analyses using different mitochondrial and nuclear markers. The 16S region appears to have several advantages over other regions of the genome regarding use of species-specific primers, ease of amplification from single specimens and undiluted informational content over both recent and more ancient separations. It has also exhibited maximum discriminatory power (100% success) between lineages during RFLP analysis. The 16S assayed region has proven especially informative and consistent in detecting, supporting and establishing the lineage status within the *B. plicatilis* species complex both from a phylogenetic perspective and as an identification tool.

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Introduction

For many years, it has been a rather entrenched notion that the high dispersive potential of aquatic taxa (i.e. passive transport of their diapausing propagules via wind or waterfowl) was likely to result in their entire world populations being nearly panmictic (Mayr 1963). Consequently, zooplanktonic species have long been effectively regarded as geographically invariant due to high rates of gene flow. However, the development and application of molecular tools in the last 2 decades have revealed high levels of population subdivision at a microgeographical scale (De Meester 1996; Hebert 1998). As a result, many aquatic invertebrates are now recognized as cryptic species complexes with a high degree of regional endemism (Colbourne et al. 1997; Serra et al. 1997; Hebert 1998; King and Hanner 1998; Taylor et al. 1998; Witt and Hebert 2000; Abatzopoulos et al. 2002a; Penton et al. 2004; Wellborn and Cothran 2004).

Monogonont rotifers are a striking example of a group of organisms where research from a molecular ecological perspective has overturned long-standing views on patterns of genetic differentiation and adaptive divergence of continental aquatic invertebrates. Recent advances, mainly through application of powerful DNA assays, have considerably refined our knowledge on the evolutionary relationships among these zooplanktonic taxa. Rotifers of the class Monogononta comprise important components of fresh and brackish water ecosystems (Hutchinson 1967). Among monogononts, *Brachionus plicatilis* (Müller) is found at salinities between 2 ppt and 97 ppt (see Lowe et al. 2005) and con-

stitutes a critical food source in marine aquaculture (Lubzens et al. 2001). *B. plicatilis* has been the subject of a plethora of studies focused mainly on the Iberian Peninsula. Morphological and allozyme analyses (Fu et al. 1991a, b) of a large number of *B. plicatilis* strains including other geographical regions suggested the division of *B. plicatilis* into two morphotypes (L for large, S for small), although allowing for an even greater number due to the high within-group genetic variability. On this basis, Segers (1995) proposed that the names *B. plicatilis* and *B. rotundiformis* (Tschugunoff) should be used for the L and S types, respectively. This division helped eliminate some of the uncertainty over the taxonomy and biodiversity of investigated populations. Subsequent field and laboratory studies utilizing among others, morphological characters, allozymic profiles, population growth rates in response to environmental factors, mating preferences and mictic patterns have given convincing evidence that the taxonomical species *B. plicatilis* is in fact a species complex comprised of at least three cryptic species, *B. plicatilis* sensu stricto (s.s.) (type L), *B. rotundiformis* (type SS) and *B. ibericus* Ciros-Pérez et al. 2001 (type SM) (Carmona et al. 1995; Gómez et al. 1995, 1997; Gómez and Snell 1996; Serra et al. 1998; Ortells et al. 2000, 2003). Recently, microsatellite analyses (Gómez and Carvalho 2000; Gómez et al. 2002a) as well as nucleotide sequence variation from both nuclear (ribosomal internal transcribed spacer 1, ITS1) and mitochondrial (cytochrome *c* oxidase subunit I, COI) genes (Gómez et al. 2000, 2002b) have provided detailed information on population structure, phylogeographical patterns and phylogenetic relationships within the *B. plicatilis* species complex. Consequently, an additional six genetically divergent lineages (possibly each deserving a specific status) were identified within the species complex, thus necessitating reassessments of the global biological diversity of this group of zooplankters.

It is currently accepted that the number of evolutionary independent lineages within the complex remains largely undetermined. There is also the possibility of additional species complexes within the genus. Elucidation of patterns of diversification and population structure is based upon reliable identification and description of sibling species. Firm establishment of specific status for apparently divergent strains requires phylogenetic assessments, which will help clarify the course of evolution and provide the basis for a refined taxonomy. This is expected also to contribute to applied research in these abundant, widespread and economically important organisms. To this end, molecular tools are particularly germane offering the advantages of bypassing the subtlety of morphological differences between these rotifer taxa and distinguishing phenotypic plasticity and intraspecific variation from cases of cryptic speciation. Mitochondrial ribosomal RNA genes have been highly informative in providing phylogenetic signals across a wide spectrum, from divergence of ancient lineages to relationships between closely related species (Hillis et al. 1996 and references therein; Palumbi 1996; Daniels et al. 2002; Haye et al. 2002; Chu et al. 2003; Darst and Cannatella 2004; Gajardo et al. 2004; Lavery et al. 2004; Baxevanis et al. 2005; Triantafyllidis et al. 2005). To date, 16S sequence divergence studies have been mainly directed towards the elucidation of the evolutionary relationships between the three classes of Rotifera and other major groups (Garey et al. 1998; Derry et al. 2003). In view of the cryptic nature of biodiversity within the *B. plicatilis* group, additional interspecific surveys are needed, which will consequently shed more light to higher-level phylogenetic affinities.

Under this perspective, we have used sequencing of a fragment of the mitochondrial 16S rRNA gene on a clade representative sampling design in order to (a) assess the phylogenetic utility of 16S and re-examine the relationships between the recently described lineages within the *B. plicatilis* species complex and (b) evaluate the degree of congruence between our results and those of other studies (Gómez et al. 2002b) using different genes. In addition, we have used the observed sequence variation in the 16S fragment to develop species-specific restriction fragment length polymorphism (RFLP) markers as a rapid, reliable and cost-effective method for genetic identification and monitoring of rotifer populations.

Materials and methods

Samples analyzed

Representative populations for six of nine genetically divergent lineages within the *B. plicatilis* species complex were investigated. They included laboratory reference strains, hatchery clones as well as collections from a wild population of the subsaline Lake Koroneia (Northern Greece). The latter, is located near the city of Thessaloniki (23°04′–23°14′E, 40°07′–40°43′N) at an altitude of 75 m above sea level (Michaloudi and Kostecka 2004). It is a highly eutrophic lake with a surface area of 30 km^2 and a maximum depth of <1 m. Lake Koroneia is protected by the RAMSAR convention and it has been proposed for inclusion in the protected areas of NATURA 2000. The laboratory strains were obtained from the University of Valencia (Spain) and the Norwegian University of Science and Technology, Trondheim (Norway). The hatchery clones were obtained from the Salt Research Institute (SRI), Tianjin (P.R. China) and a Greek hatchery (Plagton, Central Greece). A 16S *B. plicatilis* haplotype (GenBank accession number: AF108106) from Garey et al. (1998) was also included for comparative purposes. Sample sizes, codes and origins are shown in Table 1.

DNA extraction, amplification and sequencing

During DNA extraction, single rotifers were transferred into PCR tubes containing 50 µl of 6% Chelex 100 resin (BioRad, Hemel Hempstead, UK). The tubes were placed

Table 1 Codes, morphological type, presumptive species status, number of individuals scored (*n*) per strain and origins of the investigated *Brachionus* strains

Strain code	Morphological type	Presumptive species/biotype	Number of individuals scored (*n*)	Origin
Bp1	L	*B. plicatilis* s.s.	5	University of Valencia[a]
Bp3	L	*B. plicatilis* s.s.	5	University of Valencia[a]
Bp2	L	*B. plicatilis* s.s.	5	Plagton[b]
Aus	L	Austria	17	Lake Koroneia[c]
Mnj	L	Manjavacas	14	Plagton[b]
BrG	L	*B. plicatilis*	–	Garey et al. (1998)*
Nev	L	Nevada	15	Norwegian University of Science and Technology[a]
Cay	SM	Cayman	16	Salt Research Institute[b]
Ibe	SM	*B. ibericus*	15	Lake Koroneia[c]
Cal	NA	*B. calyciflorus*	13	Lake Koroneia[c]

[a]laboratory, [b]hatchery, [c]wild population, *unknown, NA = - not applicable

in a thermocycler where a program consisting of 5 min at 56°C, 10 min at 99°C and 30 min at 4°C was executed. The samples were then spun for 1 min at 8,000 rpm and stored at −35°C until required for PCR amplification.

Individual rotifers were assigned to species or strain using three criteria. Initially, morphological inspection categorized samples into two morphotypes, namely L and SM (Gómez and Snell 1996). In a next step, microsatellite amplification (7 loci) was used to discriminate *B. plicatilis* s.s. strains according to the protocols described in Gómez et al. (1998). Finally, we sequenced a PCR-amplified 713-bp region of the cytochrome *c* oxidase I (COI) mitochondrial gene, using the primers LCO1490 and HCO2198 (Folmer et al. 1994) and the protocols described in Gómez et al. (2002b). The obtained sequences were compared with representative COI haplotypes of the *B. plicatilis* species complex deposited in GenBank and subjected to BLAST searches for verification of species status.

The set of primers Br16SL (5′ TGAAAAGATAATCCAACATCGAG 3′) and Br16SH (5′ CAAAGTATCTTCTGCCCACTGA 3′) was designed on the basis of the published 16S partial sequence by Garey et al. (1998) and was used for amplification of a 378-bp fragment of the 16S mitochondrial gene on an Eppendorf Mastercycler. For each amplification, the total reaction volume of 50 µl consisted of 10 µl template DNA, 5 µl×10 reaction buffer (670 mM Tris–HCl, pH 8.8, 166 mM $(NH_4)_2SO_4$, 0.1% Tween-20), 2 µl $MgCl_2$ (50 mM), 0.5 µl dNTPs (100 mM), 0.5 µl of each primer (50 pmol), 3.75 U of *Taq* DNA polymerase and 30.75 µl of sterile double-distilled H_2O. Cycling conditions for amplification were the following: 2 min at 94°C, 35 cycles of 20 s at 94°C, 30 s at 60°C, 40 s at 72°C and a final extension of 3 min at 72°C. PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide and visualized under UV light. DNA fragments were excised from the gel and purified using the NucleoSpin Extract kit (Macherey–Nagel). Cycle sequencing was performed on a Li-COR 4,200 DNA analyzer with the Br16SL primer using the Excell II DNA sequencing kit (EPICENTRE). All sequences were deposited in GenBank (accession numbers: AY647198 - AY647206).

Phylogenetic analysis

Multiple alignments were performed using Clustal X. Default gap opening and extension parameters were implemented for the treatment of single base indels in two haplotypes (see Results). Phylogenetic analysis was conducted with the PHYLIP software package (Version 3.6b, Felsenstein 2004) and topologies were inferred using distance (neighbor-joining, NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods.

For the NJ algorithm, the Kimura two-parameter model of nucleotide substitution was employed. For MP, examined program options were ordinary parsimony, all steps versus transversional changes only, equal site weighing and thorough searches for extended rearrangements on all parts of tied trees. Gaps were treated as a fifth nucleotide state. For ML, all sites were taken into consideration including those that were conserved. Transition/transversion ratios of 2.000 and 1.000 were tested based on the input (empirical) base frequencies. Both constant and user-defined rates of evolution among sites were implemented. For the latter, different coefficients of variation of the rates of substitution among sites were checked, each determined by setting the values of 200, 50, 2 and 0.5 to the shape parameter α of the Gamma distribution. These values were also examined on a fraction of 50% as well as 75% of invariant sites.

Reliability of the inferred phylogenetic trees was assessed by bootstrap analysis using 1,000 pseudoreplicates. Consensus trees were constructed based on the 50% majority rule criterion. In all cases, *B. calyciflorus* (Pallas) was used as an outgroup taxon.

RFLP typing

Restriction maps for more than 20 restriction endonucleases, of all taxa identified by sequencing, were constructed. The objective here was to deduce species status by the most parsimonious combination of enzymes as well as to assess species composition and possible intraspecific polymorphism in a representative number of individuals (105 in total) from all sampling

sites. The BioEdit Sequence Alignment Editor software (Hall 1999) was used for generating restriction maps. Individual digestions were carried out in 20 µl total reaction volumes following the protocol described in Baxevanis and Abatzopoulos (2004). DNA fragments were routinely electrophoresed in 2.5% agarose gels and visualized under UV light after ethidium bromide staining.

Results

16S sequence variability and phylogeny

Mean alignment length was 341.3 bp. Two single base insertions were identified in the Cayman lineage at positions 120 (T added) and 304 (C added). For *B. calyciflorus*, there were three insertions, two of them at the same positions as Cayman (A and C, respectively) and a third at position 202 (T) as well as two single base deletions.

Out of 339 sites considered (excluding sites with alignment gaps), 265 were conserved and 74 were polymorphic. Within the latter, 36 were singleton polymorphic and 38 were phylogenetically informative under parsimony criteria (Fig. 1). Mean base frequencies were 0.34 (A), 0.14 (G), 0.18 (C) and 0.34 (T). There was no evidence of heterogeneity in nucleotide composition among lineages (R × C test of independence, $G = 4.50$, df = 27, $P > 0.99$).

Average nucleotide sequence divergence between lineages was 0.1038 (±0.014). Within the *B. plicatilis* s.s. group of haplotypes, mean nucleotide divergence was 0.0039 (±0.002), a value somewhat lower than the estimate (0.0053) obtained by Derry et al. (2003). The pairwise 16S sequence divergence matrix is shown in Table 2. Two main groups were identified by the NJ analysis. The first included *B. plicatilis* s.s. (Bp1, Bp3, Bp2), Nev (Nevada), Aus (Austria) and Mnj, BrG (Manjavacas) lineages while the second included Cay (Cayman) and Ibe (*B. ibericus*) (Fig. 2a). Between-group divergence was 0.1298 (±0.020). The

Fig. 1 Variable sites within a 344-bp aligned segment of the 16S rRNA gene across *Brachionus* lineages (strain codes as in Table 1). Parsimony informative sites are indicated with an *asterisk* and singleton polymorphic sites with *bold*

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                                        **  ***  **    ******    *** ***
          **  * *** *    ***   1111 1111111111 1111111111 1111111222
    11134444 4445555666 6777780001 1111111122 3666666668 8999999000
   6734671245 6792456456 7567847890 1234567923 4012345683 5145689014
Bp1 CTTTTGCGTA ATTTAAATTT GATTATACCC TTGAAAAGGA TACGCTTGCC AGCACTTATT
Bp3 .......... ....G..... .......... .......... .......... ..........
Bp2 .......... ....G..... ..G....... .......... .......... ..........
Aus .......... ..A...T... .GG.GG..A. ...GC..... .GAA..A... T.A.......
Mnj .......... ..A...T... A.......T. ..A.G..... .GGA...... TA....GC..
Nev .......A.. ..A...T... .G......A. .......... .GTT..A... .A..T..G..
Cay .C.....TA. .GAA..T... A.G......A AA.G.TTT.. .GGA.AC... TA...CA...
Ibe ..C..ATTA. TCA....... A.GA....TA CAA.T..T.. CGA.TAA... T....AA..C
Cal T..CC..TAG T..ATTTAAC A.....GGAG .AA...TCAG .GAAT..ATT ..AGTA..G.

    **   ** ** ** *
    2222222223 3333
    0011122780 0011
    5637937395 8901
Bp1 GGTTCTTAGA CGAT
Bp3 .......... ....
Bp2 .......... ....
Aus A........T T...
Mnj A....C.... TTG.
Nev A.......A. ....
Cay .A...CA.A. TTTA
Ibe .AGA.CAGAT T..G
Cal .A..TAA.A. TT.A
```

Table 2 Pairwise 16S sequence divergence estimates for all lineages based on Kimura's two-parameter model (Gamma rates, $\alpha = 0.5$)

Lineage	Bp1	Bp3	Bp2	Aus	Mnj	BrG	Nev	Cay	Ibe	Cal
Bp1	–									
Bp3	0.0030	–								
Bp2	0.0060	0.0030	–							
Aus	0.0595	0.0633	0.0596	–						
Mnj	0.0563	0.0602	0.0638	0.0596	–					
BrG	0.0673	0.0712	0.0750	0.0708	0.0184	–				
Nev	0.0456	0.0493	0.0528	0.0524	0.0561	0.0671	–			
Cay	0.1162	0.1207	0.1162	0.1162	0.0949	0.1164	0.1162	–		
Ibe	0.1396	0.1446	0.1399	0.1407	0.1399	0.1647	0.1545	0.1125	–	
Cal	0.1763	0.1763	0.1814	0.1978	0.1814	0.2094	0.1814	0.1669	0.2054	–

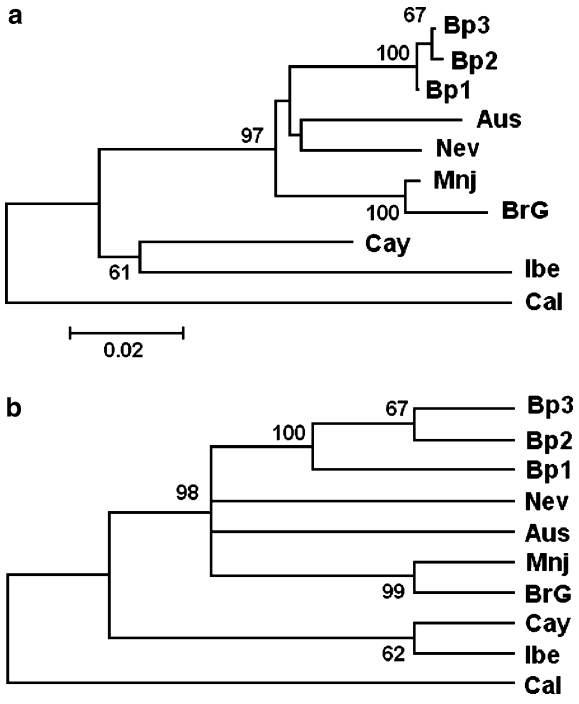

Fig. 2 **a** Neighbor-joining tree of 16S sequences based on pairwise divergence estimates obtained after implementation of the Kimura two-parameter model (Gamma rates, $\alpha = 0.5$). **b** Fifty percent majority rule consensus tree. *Numbers* indicate percent bootstrap support (only values $> 50\%$ are shown) out of 1,000 pseudoreplicates. Both trees are rooted with *B. calyciflorus* (Cal)

same estimates within-groups were 0.0512 (± 0.009) and 0.1117 (± 0.023) for the first and second group, respectively. These two main branches bear an absolute correspondence to those identified by Gómez et al. (2002b) based on ITS1 and COI molecular phylogeny (with the exception of *B. rotundiformis*, which is not included in the current study) as well as to the accepted morphological taxonomy in the *B. plicatilis* species complex (Ciros-Pérez et al. 2001). Our morphological characterization was also concordant with the composition of taxa within each group (data not shown). In this respect, the NJ tree (Fig. 2a) performed well in recovering the phylogenetic relationships between lineages. However, the Nevada–Austria relationship appeared to be not fully resolved as judged by the low value of bootstrap support, thus forcing a polytomy in the 50% majority rule tree (see Fig. 2b). Some sensitivity in the resolution of the Cayman–*B. ibericus* clade was also evident.

Certain facets of the analysis, regarding the support of particular groupings were crystallized when MP was used. Five equally parsimonious trees were obtained with a length of 143 steps and a consistency index CI = 0.78. These trees differed in the branching order of Austria–Nevada and Cayman–*B. ibericus* clades, an ambiguity already marked by the NJ analysis, whereas, the Manjavacas clade remained unaffected. One of these trees is shown in Fig. 3. The 50% majority rule consensus tree (not shown), produced after bootstrapping 1,000 pseudoreplicate data sets accommodated polytomies for the clades mentioned above as a result of low nodal support. These topological particularities were also largely retained in the ML analysis. For the latter, it is noteworthy that rate variation effects were evident between some lineages (i.e., Ibe vs Bp3, Bp2, Aus, Mnj, BrG) over a range of α parameter values from 0.5 to 200 and classes of 50% and 75% of invariant sites. When a disparity index (ID) test (Kumar and Gadagkar 2001) for homogeneity in nucleotide substitution was applied to the previous comparisons, the null hypothesis could not be rejected at the 1% significance level.

A salient feature of the analysis is the inclusion of the *B. plicatilis* haplotype (BrG) by Garey et al. (1998) in the

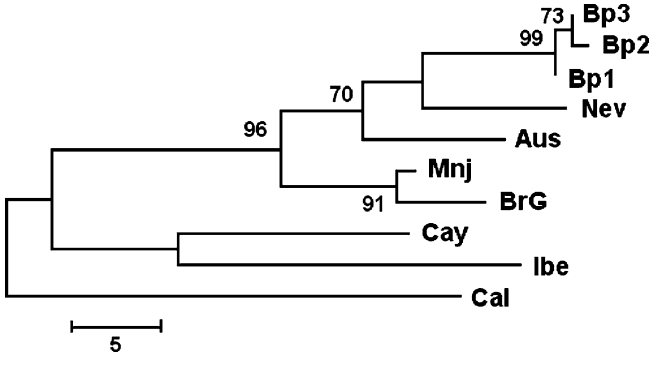

Fig. 3 One of five equally parsimonious trees produced with the min-mini heuristic search algorithm. Above 50% bootstrap support values (1,000 pseudoreplicates) are indicated for each node. *Bar* and branch lengths are scaled to five substitutions. The tree is rooted with *B. calyciflorus* (Cal)

Manjavacas clade. The within-clade divergence estimate was 0.0184 (see Table 2). This grouping was impressively robust (bootstrap support of >99% using NJ) as well as insensitive to the other methods (MP, ML, despite a decline in bootstrap support) of phylogeny reconstruction. It is therefore obvious that the supposed *B. plicatilis* strain used by Garey et al. (1998) was in fact an undetected Manjavacas biotype. The average sequence divergence between the *B. plicatilis* s.s. and Manjavacas clades was 0.0652 (±0.014).

RFLP analysis

An extensive survey of sequence variability and cross-checking of cleavage sites across taxa reduced the number of restriction endonucleases from more than 20, for which restriction maps had been initially constructed, down to eight (Table 3). The latter, were chosen on the basis of low cost, stability, satisfactory resolution of specific bands and/or digestion profiles and reproducibility of patterns.

An average of 15 individuals from each lineage (six *B. plicatilis* species complex and *B. calyciflorus*) were screened with these eight endonucleases. The RFLP survey revealed neither intraspecific polymorphism (with the exception of *Acc*I) nor any signs of heteroplasmy. In all cases, the obtained digestion profiles were unambiguous and exhibited exceptional band resolution as low as 20 bp (Fig. 4). This was mainly due to the high quality of PCR product and the proper standardization of conditions (digestion times, dilution of restriction mix) for the avoidance of partial digestion of amplicons.

The battery of eight restriction endonucleases exhibited a total of 22 cleavage sites, corresponding to 30 fragments, an average of 2.75 fragments per enzyme. A desirable property in RFLP analysis is the presence of an adequate number of cleavage sites for the effective screening of sequence variability. This permitted the fast and accurate discrimination (100% success) of the different taxa. In fact, due to the inequality of probabilities for gain versus loss of a restriction site it was decided that single-cutters (e.g. *Acc*I, *Afl*II) should be underrepresented. Thus, out of eight endonucleases used, six had at least two restriction sites. Five enzymes, namely *Swa*I, *Apo*I, *Taq*[a]I, *Afl*II and *Dpn*II, are specific for Nev, Cay, Aus, Mnj and Ibe, respectively. *B. calyciflorus* (Cal) can be identified by more than one endonuclease (*Apo*I, *Dra*I and *Dpn*II). The same is true for *B. plicatilis* s.s. (strains Bp2, Bp1 and Bp3, see Table 3) using *Dra*I and *Ssp*I. Furthermore, certain enzyme combinations can be used in cases where additional taxon confirmation is required. For instance, digestions with *Apo*I and *Acc*I can decisively discriminate Cayman from all others.

The RFLP analysis (i.e., choice of enzymes, interpretation of restriction profiles, etc.) was designed under the criterion of maximizing sequence screening. Overall, a proportion of 48.6% of sequence variability among taxa (36 of 74 polymorphic sites) was screened using restriction endonucleases.

Table 3 Digestion profiles for eight restriction endonucleases (recognition sequences alongside, m = A or C, k = G or T, r = A or G, y = C or T) across taxa

Endonuclease	Site	Taxon/strain								
		Bp1	Bp3	Bp2	Aus	Mnj	Nev	Cay	Ibe	Cal
*Acc*I$_{GT\downarrow mkAC}$	78	−	−	+	−	−	−	+	−	−
*Afl*II$_{C\downarrow TTAAG}$	159	−	−	−	−	+[a]	−	−	−	−
*Apo*I$_{r\downarrow AATTy}$	100	−	−	−	−	−	−	−	−	+[a]
	147	+	+	+	+	+	+	+	+	+
	209	−	−	−	−	−	−	−	−	+[a]
	218	+	+	+	+	+	+	+	+	+
	237	−	−	−	−	+	+	+	−	−
	353	−	−	−	−	−	−	+[a]	−	−
*Dpn*II$_{\downarrow GATC}$	172	+	+	+	+	+	+	+	+	−[a]
	361	−	−	−	−	−	−	−	+[a]	−
*Dra*I$_{TTT\downarrow AAA}$	56	−	−	−	−	−	−	−	−	+[a]
	146	+	+	+	+	+	+	+	+	+
	153	+	+	+	+	+	+	+	+	−[a]
	206	+	+	+	−	−	−	−	−	−
	237	−	−	−	−	+	+	+	−	−
*Ssp*I$_{AAT\downarrow ATT}$	60	+	+	+	+	+	+	+	−	−
	96	+	+	+	−	−	−	−	−	−
*Swa*I$_{ATTT\downarrow AAAT}$	146	+	+	+	+	+	+	+	+	+
	237	−	−	−	−	−	+[a]	−	−	−
Taq[a]I$_{T\downarrow CGA}$	20	+	+	+	+	+	+	+	+	+
	41	−	−	−	+[a]	−	−	−	−	−
	131	−	−	−	+[a]	−	−	−	−	−

Cleavage sites (+ = presence, − = absence) of the 378-bp 16S amplified fragment are indicated. [a]denote taxon-specific presence or absence of sites

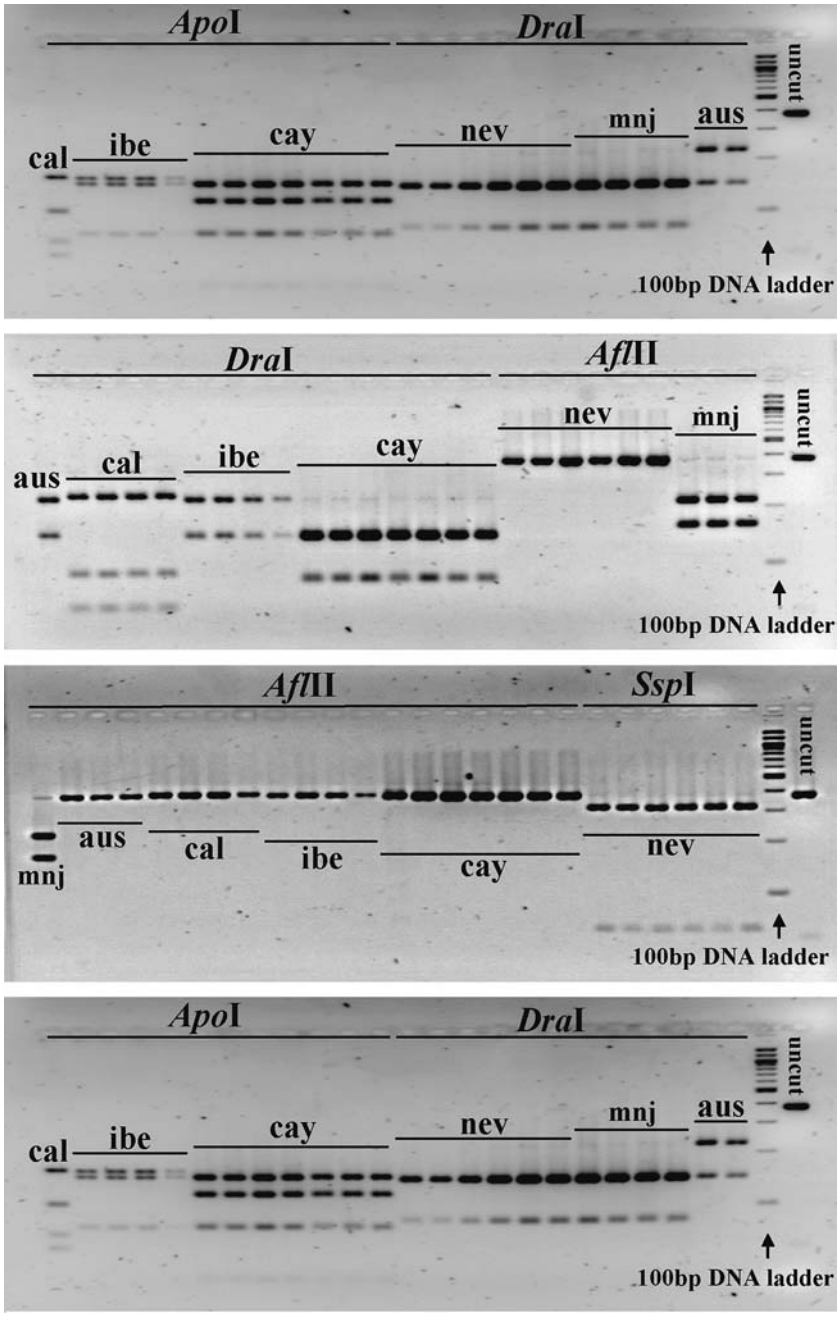

Fig. 4 Examples of digestion profiles in 2.5% agarose gels between taxa for selected endonucleases

Discussion

Phylogenetic assessment

To our knowledge, this is the first study where the phylogenetic relationships within the recently described *B. plicatilis* species complex are explicitly addressed using mitochondrial 16S rRNA sequence data. Previous sequencing approaches (Garey et al. 1998; Derry et al. 2003) had mainly focused on higher-level systematics of Rotifera as a whole. Recent research (Taylor et al. 1998; Witt and Hebert 2000; Penton et al. 2004) has shown that the particularities and dynamics of cryptic speciation are more appropriately assessed by a molecular toolbox capable of unveiling the encrypted information and the evolutionary signatures within closely related lineages. The present study adds to this direction by providing useful markers for a more complete biogeographical description of *B. plicatilis* species complex and by ventilating the utility of molecular methods in both basic and applied work.

The divergence estimates presented here reaffirm the currently accepted taxonomic status of *B. plicatilis* species complex. Our results show a high degree of con-

gruence to those of Gómez et al. (2002b) based on ITS1 and COI sequencing, with similar values of topological support for the main clades. Additionally, in most between-lineage comparisons (see Table 2) sequence divergence values exceed 4.5% (range 4.56–16.47%). Therefore, the phylogenetic signals depicted by three DNA markers (ITS1, COI and 16S in this study) are concordant and consistent with distinctive evolutionary histories of these lineages. In this respect, molecular sequences are conclusive and contribute greatly to previous inferences made by allozymic, morphological and mating behavior data (Gómez et al. 1995; Gómez and Serra 1995; Gómez and Snell 1996; Ortells et al. 2000; Ciros-Pérez et al. 2001) regarding lineage relationships within the species complex.

The 16S sequence data have proved informative in delineating the major groups within the *B. plicatilis* species complex. Compared to ITS1 and COI sequences used by Gómez et al. (2002b), the 16S mitochondrial region performed in an intermediate, yet superior way by (a) retrieving satisfactorily both shallow and deep branches, (b) minimizing conflicting relationships and resultant multifurcating patterns like those seen in the former study, (c) displaying a consistent phylogenetic signal among different tree-building algorithms and distance measures and (d) exhibiting considerable power in inferring the expected tree with a restricted amount of data. These qualities stem from the characteristics of sequence change (i.e. absence of transition saturation effects, homogeneous distribution among taxa of hypervariable domains, see Fig. 1) and an "appropriate" evolutionary pace of 16S, thus validating its suitability for phylogenetic inference in the *B. plicatilis* species complex. In addition, the 16S region assayed here was amplified by species-specific primers.

A number of reasons have conspired to limit the robustness of mainly the Austria–Nevada clade and to a lesser extent that of Cayman–*B. ibericus*. Sequence divergences for *B. plicatilis* s.s. versus Nevada and Austria were 0.0524 (±0.013) and 0.0602 (±0.014), respectively, exhibiting a considerable overlap. The same estimate between *B. plicatilis* s.s. and Nevada–Austria (taken as a single group) was 0.0563 (±0.012), while the net estimate amounts to about 47.2% (0.0266) of the latter. Thus, the Nevada lineage either is grouped together with Austria or comes out as a sister taxon closer to *B. plicatilis* s.s. (see Figs. 2a and 3). It is noteworthy, that Nevada is separated from *B. plicatilis* s.s. by, impressively enough, the same amount of distance (0.0524) as it is from Austria (see Table 2). Neither phenetic (NJ) or cladistic (MP, ML) approach nor any model parameterization during the analysis was able to decisively resolve this ambiguity. The instability of this particular node was evident even in the combined analysis of ITS1 and COI by Gómez et al. (2002b). In the present case, it seems that different phylogenetic algorithms can do no more than outlining a varying propinquity of relationship of Nevada to *B. plicatilis* s.s. and Austria (contrast Figs. 2a and 3). In a similar vein, the reduced bootstrap support for the Cayman–*B. ibericus* clade is presumably a result of the sampling practice used. Based on the findings of Gómez et al. (2002b), this clade is actually comprised of two pairs of sister groups, namely Cayman–Tiscar and Almenara–*B. ibericus*. It is therefore quite conceivable that synapomorphic characters were not fully sampled here, which resulted in a paraphyletic picture for the former clade. Therefore, inclusion of more sequences is expected to better resolve this problematic node as well as to completely eliminate long-branch-attraction effects (detected with ML) of Cayman and *B. ibericus* towards *B. calyciflorus* (Cal).

The obtained distance estimates can be used to gain an estimation of the divergence times for the species complex. Although molecular clock estimates should be exercised with great caution (see Hebert et al. 2002), they are useful in providing approximations for an evolutionary time framework of significant phenomena like the antiquity of cryptic species complexes (see Vaïnöla et al. 1994; King and Hanner 1998; Witt and Hebert 2000). Employment of Schubart et al. (1998) 16S molecular clock (0.4–0.9% pairwise sequence divergence per million years) gives an estimated split for the two main ingroup assemblages (see Fig. 2a) in a range of 14.4–32.4 million years ago. The separation of the two groups as a whole from *B. calyciflorus* (the outgroup taxon) is estimated at 16.6–37.5 million years ago. Compared with the dates obtained by Gómez et al. (2002b) on COI distances, our calibrations are very similar (especially if we employ the lower estimate of 0.4%) and yield further evidence for the ancient origin of this species complex.

Species-level taxonomy and higher-level phylogeny are not entirely decoupled (Knowlton 1993). The fact that the *B. plicatilis* strain studied by Garey et al. (1998) was actually a Manjavacas biotype is an illustration of the complexities of cryptic speciation. In our analysis, the Manjavacas clade (see Figs. 2, 3) was particularly immune to any tree-building method or distance measure used. At the level of sequence divergence obtained using 16S (average 0.1038, *B. plicatilis* s.s. vs Manjavacas 0.0652), the consequences of undetected biodiversity may be nontrivial. This is expected to have a larger effect at generic level investigations, when certain taxa are taken as proxies for the clades they belong to (see Hillis 1998; Rosenberg and Kumar 2003).

An intriguing point regarding *B. calyciflorus* (from Lake Koroneia) deserves special reference. The sequence used throughout the analysis is represented by two haplotypes, which were collapsed to one (Cal) due to 100% identity. However, these two sequenced *B. calyciflorus* individuals, collected at the same site of Lake Koroneia in the same collection trip, were characterized by remarkable morphological divergence (most notably in the shape and orientation of the two median lorica spines) as well as such prominent size differences so as to be nicknamed as "large" and "small" individuals. Everything else being equal (identical physicochemical conditions, developmental stage), this pronounced

intraspecific morphological diversity requires a more thorough examination and explanations based on environmentally induced variation should be carefully sought.

Ill-defined taxa are the symptoms of cryptic biodiversity. The major source of erroneous inferences of this sort is the subtle morphological differences, common in many zooplanktonic organisms (Knowlton 1993; Hebert 1998). The confounding effects of intraspecific morphological variation and interspecific morphological similarity have made the study of cryptic biological species a contentious enterprise. This patterning is exemplified in this work, not only by the *B. plicatilis*–Manjavacas errancy, but also by the observation of extensive morphological diversity between, otherwise identical in sequence, *B. calyciflorus* individuals.

Several studies have reported intraspecific morphological variation in rotifers. Gilbert (1966, 1967) identified cyclomorphosis in the posterolateral spine development of *B. calyciflorus* induced by chemicals released by *Asplanchna*. Serra and Miracle (1987) studied the increase in lorica length in *B. plicatilis* in response to temperature decrease. Kutikova and Fernando (1995) investigated the extensive morphological variation among widely distributed *B. calyciflorus* isolates and suggested a subspecies status of multiple forms. In general, such phenotypic variation either can be environmentally induced (phenotypic plasticity), caused by intraspecific genetic factors or related to unrecognized sibling species engaged in seasonal succession (Serra et al. 1997). The latter possibility should be explored in more depth since in three collection trips to Lake Koroneia, there were differences in the abundance of the sampled taxa (Austria, *B. ibericus*, *B. calyciflorus*). This observation agrees with previous data (Michaloudi and Kostecka 2004), on patterns of seasonal dynamics of the zooplankton community in Lake Koroneia and seems to echo the patterns of seasonal succession observed in Torreblanca Marsh (Gómez et al. 1995). The *B. plicatilis* species complex is in a sense unique since two constitutive aspects of its biogeography, spatial and ecological, are so interweaved that have probably come to dominate its entire evolution. Certainly, more analytical inquiries and input from several fields are needed for proper descriptions of the taxa involved and their ranges.

The approaches used for the investigation of *B. plicatilis* species complex should probably expand to also include other species of the genus. According to Ciros-Pérez et al. (2001), more strain-inclusive analyses are expected to facilitate differentiation of biological from taxonomical species. Extending further this notion, the 16S results have pointed out that the error involved in phylogeny reconstruction using shorter sequences is minor. Instead, increased confidence for offending nodes within the *B. plicatilis* species complex can be achieved by a practice incorporating a large number of taxa per clade. Such sampling designs are particularly relevant to phylogenetic investigations involving cryptic species.

RFLP markers

Research in aquatic planktonic fauna has recently made significant inroads in issues regarding local adaptation, population differentiation and coexistence. It is probably true that questions still exceed answers on these important evolutionary patterns and mechanisms. However, with the advent of powerful molecular tools, discrimination of distinct sympatric lineages from conspecific clonal groups has become more mundane, at least for aquaculturalists. The RFLP analysis has proved highly efficient in the current study for the reliable diagnosis of *B. plicatilis* species complex individual lineages or groups (see Table 3 and Fig. 4). Complementary information from the previous sequencing assay has enabled the selection of the most appropriate restriction enzymes for the diagnostic purpose.

The chosen endonucleases had predominantly AT-rich recognition sequences that matched the AT content (on average $>60\%$) of the investigated sequences. Beyond $\backsim 2\%$ sequence divergence, it becomes too difficult to identify gains or losses of cleavage sites (Dowling et al. 1996) making 5- or 6-bp recognizing restriction enzymes more suitable for the analysis. At this point and due to the low frequency of indels in our sequences, a reasonable balance was achieved between small and large generated fragments. Two enzymes (*Acc*I, *Apo*I) had multiple or variable cleavage sites to safeguard against noncleavage or uniformity of fragment patterns, exploiting in this way the most of interspecific variability. This was particularly important for *Acc*I, enabling the delineation of the different *B. plicatilis* s.s. strains.

The RFLP markers developed in this study constitute a ready-to-use tool of significant applicability. Preliminary indications have shown that Cayman seems to be very frequent in most investigated hatcheries and that the 16S RFLP markers can still discriminate cryptic species within the *B. plicatilis* species complex as well as other taxa (*B. quadridentatus*, *B. angularis*, *B. urceolaris*, *B. dimidiatus*) not belonging to the *B. plicatilis* group (unpublished data). Therefore, despite the absence of Tiscar, Almenara and *B. rotundiformis* from our survey, the application of the proposed RFLP assay may still be at least instructive, if not entirely conclusive. Restriction analysis and other DNA-based approaches (Hebert et al. 2003) can contribute greatly to both basic and applied work, integrate these fields, initiate further research (especially in cases where sequencing is unavailable or RFLP marker studies are scarce) and form the basis for a broader identification system of controversial or cryptic taxa.

We have assessed the utility of 16S rRNA in *B. plicatilis* species complex both from a phylogenetic perspective and as an identification tool. The information harbored within the investigated region is a balanced aggregation of invariant and polymorphic sites not saturated by change. This renders 16S a powerful marker for unraveling the evolutionary relationships between

sibling *Brachionus* taxa on a broad divergence scale. The strong biogeographical context of cryptic speciation requires more analytical, multidisciplinary descriptions (see Triantaphyllidis et al. 1997; Abatzopoulos et al. 2002b; Kappas et al. 2004). As additional cases of cryptic biological species are coming into light, issues like the dynamics of coexistence, morphological stasis and the role of phenotypic plasticity should receive greater scrutiny in order to achieve a more coherent evolutionary theory of such phenomena.

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