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On the taxonomy of three sympatric sibling species of the *Brachionus plicatilis* (Rotifera) complex from Spain, with the description of *B. ibericus* n. sp.

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The accumulation of evidence during the last decade led to the splitting of the Brachionus plicatilis complex (Rotifera) into two morphologically recognizable species: B. plicatilis Müller and B. rotundiformis Tschugunoff, previously referred to as L- and S-type B. plicatilis (s.l.), respectively. However, recent population genetics and molecular studies have revealed that each of these taxa concern cryptic species complexes. In particular, in Torreblanca Marsh, a wetland on the Mediterranean coast of Spain, three genetically distinct groups in this rotifer complex have been shown to co-occur. Differences in genetic markers, ecological preferences, mixis responses, mating behaviour and no evidence of gene flow between them have led to the conclusion that they must be considered as three different biological species. In this study we present a detailed comparative analysis using light and scanning electron microscopy of laboratory strains enabling a morphological characterization of the three species. Fine morphology and morphometry revealed taxonomic characters constant enough to recognize three well-defined morphologies, which always correspond with the respective biological species identified. Since no type material of both B. plicatilis and B. rotundiformis is available, we designed our Spanish clones as neotypes, which would allow further comparative work. Furthermore, B. ibericus n. sp. is described. This study sets the first step for a complete characterization of the biological diversity contained within this economically important species complex.

INTRODUCTION

Brachionus plicatilis (Müller, 1786) is a monogonont rotifer that has long been considered as an ecological generalist with a cosmopolitan distribution in inland and coastal marine habitats (Walker, 1981). Among rotifers, *B. plicatilis* is probably one of the best-studied taxa. It has been widely used as a model for physiological and ecological studies [for examples see (Walker, 1981; Clément and Wurdak, 1991; Nogrady *et al.*, 1993; Kleinow and Wratil, 1996)] and for ecotoxicology [reviewed by (Snell and Janssen, 1995)]. Moreover, it is currently used world-wide in the marine fisheries industry [reviewed by (Lubzens *et al.*, 1987, 2001)]. However, in the last decade, several comparative studies (Fu *et al.*, 1991a, 1991b, 1993; Rumengan *et al.*, 1991; Hagiwara *et al.*, 1995; Rico-Martínez and Snell, 1995) revealed that *B. plicatilis* is not a single species but a complex of at least two morphologically recognizable taxa, the so-called L- (large) and S- (small) type (Oogami, 1976). On the basis of this evidence, Segers (Segers, 1995) re-examined the existing available names and proposed that *B. plicatilis* Müller, 1976 and *B. rotundiformis* Tschugunoff, 1921 were the correct names for the L- and S-types, respectively. Since then, those names have been applied to several strains from all over the world. Although this split enabled a better understanding of the complexity of the species complex, several recent lines of evidence suggest that each of these rotifer taxa are in fact composed of clusters of sibling species (Gómez *et al.*, 1995; Gómez and Snell, 1996; Serra *et al.*, 1998; Ortells *et al.*, 2000; Gómez *et al.*, 2000).

Studies on molecular-markers in *B. plicatilis* at Cabanes-Torreblanca Marsh (Gómez *et al.*, 1995), a coastal brackish area of Eastern Spain, revealed the co-occurrence of

three biological species. These species are involved in a regular pattern of seasonal succession in a single pond of the marsh, with relatively long periods (up to 4 months) of coexistence of two or three species (Ciros-Pérez, unpublished data). Besides being differentiated by several genetic markers, they differ in their general morphology (i.e. body size and shape) (Gómez, *et al.* 1995), sexual reproduction patterns (Carmona, *et al.* 1995; Gómez, *et al.*, 1997), and ecological specialization (Gómez *et al.*, 1995, 1997; Ciros-Pérez *et al.*, 2001). They show assortative mating behaviours (Gómez and Serra, 1995, 1996) and no hybrids have been recorded. The three sibling species were named following Segers' criteria as *B. plicatilis* (*sensu stricto*), *B. rotundiformis* SS and *B. rotundiformis* SM. Despite this body of evidence, greater than for any other rotifer species, the taxonomy of the species had not been established.

Here we present a morphological analysis of three sympatric sibling species belonging to the *B. plicatilis* complex from Spain. Two of the taxa closely match the known species *B. plicatilis* Müller and *B. rotundiformis* Tschugunoff, and these are redescribed in order to facilitate future recognition of the taxa. A third taxon is described as new to science.

METHOD

A total of eight clones from the three sibling species of the *B. plicatilis* complex were investigated. Three clones belong to the *B. plicatilis (*clones L1, L2 and L4), three to *B. rotundiformis* SM (SM2, SM5 and SM11) and two to *B. rotundiformis* SS (SS2 and SHON). The clones are from the rotifer culture collection at the University of Valencia. They consist of parthenogenetically cultured strains founded by isolating single amictic females. They were collected in Torreblanca Marsh, Spain (Gómez *et al.*, 1995), except for the SHON clone that originates from El Hondo de Elche Natural Park (Ortells *et al.*, 2000). Stock cultures were maintained at 23°C, 12 g l^{-1} salinity, fed on *Tetraselmis suecica (*about 10–15 mg C l^{-1}) every 3–4 days, and the medium was renewed weekly, for at least 1 month prior to experimentation. Saline water was made with commercial seasalts (Instant Ocean™, Aquarium Systems).

Since no type material of *B. plicatilis* Müller is available, we tried to obtain animals from the type locality. Müller did not specify type locality (Müller, 1786), referring only to the littoral zone of Denmark. More recently, Thane-Fenchel recorded dense populations of *B. plicatilis* from two localities in this region (Thane-Fenchel, 1968). Accordingly, M. V. Sorensen (Zoological Museum, University of Copenhagen) collected some samples from the localities mentioned by Thane-Fenchel. Several qualitative zooplankton samples (filtered through a 33 μm mesh) fixed with 70% ethanol, collected during spring and summer 2000 from Nivå Bay at Øresund, Denmark (supplied by M. V. Sorensen) were analysed. Additionally, we followed the procedure detailed in Gómez and Carvalho to isolate resting eggs from a sediment sample collected 5 March 2000 from Vellerup Vig (Ise Fjord, Zealand), Denmark (supplied by M. V. Sorensen) (Gómez and Carvalho, 2000).

Morphometry

Culture temperature was 23°C and salinity was 12 g l^{-1}; animals were fed on *T. suecica* (10 mg C l^{-1}) and maintained in constant light conditions (photosynthetically active radiation: ca. 35 μE m^{-2} s^{-1}). Morphometric values were compared using animals of the same age. For each clone, several amictic egg-bearing females were randomly chosen from exponentially growing stock cultures. Twelve groups of 10 rotifers were pipetted into wells (Nunc™ polystyrene 24 well plates) with 1.5 ml of culture medium and were placed in culture conditions. After 3 h of culture, hatched neonates were transferred individually to new wells with 1.5 ml fresh medium (at least 60 for each clone) and placed again in the experimental conditions. After 48 h, rotifers were fixed with formaldehyde (4% final concentration).

For each clone, 20 fixed amictic egg-bearing females (48 ± 3 h old) were randomly chosen and nine characters of the lorica were measured under a Nikon YS2-H microscope. Seven of the characters used in the analysis (*a–g*; Figure 1A) were selected on the basis of Fu *et al.* (Fu *et al.*, 1991a), two others were chosen (*h* and *i*; Figure 1B) because we considered them to be of potential taxonomic importance.

Statistical analyses

All statistical analyses were performed using the SPSS program (release 10. SPSS Inc., Chicago IL). Two stepwise discriminating analyses were performed to discriminate among strains on previously log-transformed (Ln) measurements. Analysis I was performed on the seven characters (*a–g*; Figure 1A) selected on the basis of Fu *et al.* (Fu *et al.*, 1991a), while Analysis II included an alternative set of six characters (*a*, *c*, *e*, *g–i*). Characters used in Analysis II were selected because they appear to be independent of the lorica contraction caused by fixation.

Five characters describing lorica length (*a*), lorica shape (*c*/*a* and *i*/*a*), the relative length dorsal-lateral spines 2 and 3 (*g*/*h*, see Figure 1B) and length of spine 3 in relation to lorica length (*h*/*a*) were analysed with one-way ANOVAs to compare morphological differences among the three sibling species. When differences were found, post-hoc (multiple comparisons among means) Student–Newman–Keuls (Sokal and Rohlf, 1969) tests were carried out.

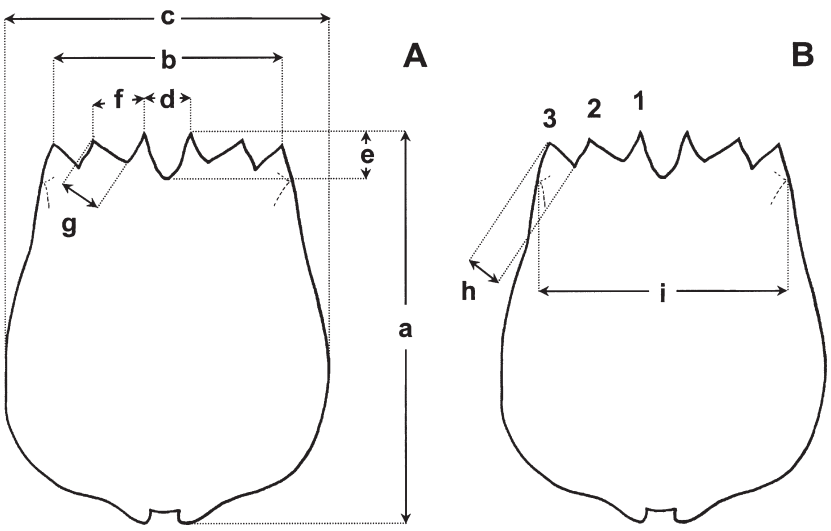

Fig. 1. Characters of *Brachionus* lorica measured in this study. (**A**) Selected characters based on Fu *et al.* (Fu *et al.*, 1991a). (**B**) Additional characters measured in this study. Measurements were made under an Olympus CK-2 microscope. *a–c*, *i* at ×400 magnification, and *d–h* at ×1000 magnification. Anterior dorsal spines are numbered (1–3) as in the text.

Morphology

Several clones belonging to the three species were studied. All specimens used for the morphological description came from stock cultures or from the experimental cultures used for the morphometric analysis. Examination of rotifer specimens was carried out using a compound microscope (Nikon YS2-H). Drawings were made using a camera lucida. Scanning electron microscopy (SEM) was performed on several complete specimens, resting eggs and trophi from each of the three species using a Hitachi S-4100 microscope. Complete animals and resting eggs were prepared as in Ansellem and Clément (Ansellem and Clément, 1980) with minor modifications. Trophi were prepared for SEM according to De Smet (De Smet, 1998).

RESULTS AND DISCUSSION

Morphometry

Table I shows the results of the discriminant analyses. In both analyses, the first canonical functions (Function 1) accumulated almost all discriminatory power (94.6% and 91.8% of variance, respectively). In Analysis I, Function 1 discriminated the species using major body measurements: lorica length (*a*; correlation coefficients between *a* and Function 1, $r = 0.881$) and lorica width (*c*; $r = 0.817$). Function 2 is mostly correlated with dorsal sinus depth (*e*). In Analysis II, Function 1 is largely correlated with the two major body measurements (*a* and *c*; $r = 0.838$ and 0.77, respectively), but also with head aperture (i.e. *i*, $r = 0.73$). Function 2 is correlated with traits associated to spines, represented by variables *e* (dorsal sinus length; $r = 0.457$) and *h* (spine 3 length; $r = 0.418$). Analysis I showed a clear discrimination of the eight strains into two well-defined groups, one corresponding to the L-type strains and the other to the SS-SM types (Figure 2A); SS strains and SM strains being in a different region of the same cloud. Analysis II was much more efficient in separating the three groups (Figure 2B), each corresponding to one of the three sibling species.

These results indicate that morphometric discrimination of species is highly dependent on the body measurements. In Analysis I, by using the selected characters based on Fu *et al.* (Fu *et al.*, 1991a), the two smaller species were grouped. This seems to be a fixation artefact, as some characters can vary widely as a result of contraction after fixation (Ciros-Pérez, personal observation). This holds especially for the distances between spines (i.e. *b*, *d* and *f*). Accordingly, the most reliable measurements to differentiate between species are those regarding body size and general body shape, and the relative length of the spines.

On the basis of results of the discriminant analyses, we calculated mean values (Table II) of lorica length (*a*) and a set of ratios: relative lorica shape (*c*/*a* and *i*/*a*), relative size of antero-dorsal spines (*h*/*a*), and ratio between antero-dorsal spines 2 and 3 (*g*/*h*). The resulting set of measurements is such that any of them could not be computed from any combination of the rest. The three sibling species clearly separated from each other with respect to lorica length, ratio between antero-dorsal spines, and relative size of antero-dorsal spines. Clones belonging to the

Table I: Stepwise discriminant analyses of Brachionus body measurements. Only values for the first two canonical functions (Function 1 and Function 2) of each discriminant analysis (Analysis I and Analysis II) are shown

	Analysis I				Analysis II			
	Function 1		Function 2		Function 1		Function 2	
	Coeff.	Corr.	Coeff.	Corr.	Coeff.	Corr.	Coeff.	Corr.
Ln(*a*)	0.573	0.881*	0.494	0.317	0.543	0.838*	–0.053	0.111
Ln(*b*)	–0.004	0.509	0.129	0.266	–	–	–	–
Ln(*c*)	0.411	0.817*	–0.333	–0.047	0.231	0.770*	0.569	0.333
Ln(*d*)	0.074	0.169	0.107	0.138	–	–	–	–
Ln(*e*)	0.256	0.197	–1.056	–0.595	0.257	0.177	0.581	0.457
Ln(*f*)	0.313	0.280	–0.051	0.175	–	–	–	–
Ln(*g*)	–0.379	–0.029	0.822	0.194	0.036	–0.027	–0.970	–0.056
Ln(*h*)	–	–	–	–	–0.351	–0.055	0.786	0.418
Ln(*i*)	–	–	–	–	0.416	0.730*	–0.657	–0.256
Eigenvalue	55.057		1.586		61.151		4.186	
% variance	94.6		2.6		91.8		6.3	

Body measurements *a–g* were used for Analysis I, and *a*, *c*, *e*, *g* and *h* were used for Analysis II (see text for details). Coeff. is the standardised coefficient for the canonical discriminant function; Corr. is the pooled within-group correlation coefficient between (1) the body measurement and (2) the canonical discriminant function. No body measurement was excluded by the stepwise analyses.
* indicates largest absolute correlation between each body measurement and any discriminant function.

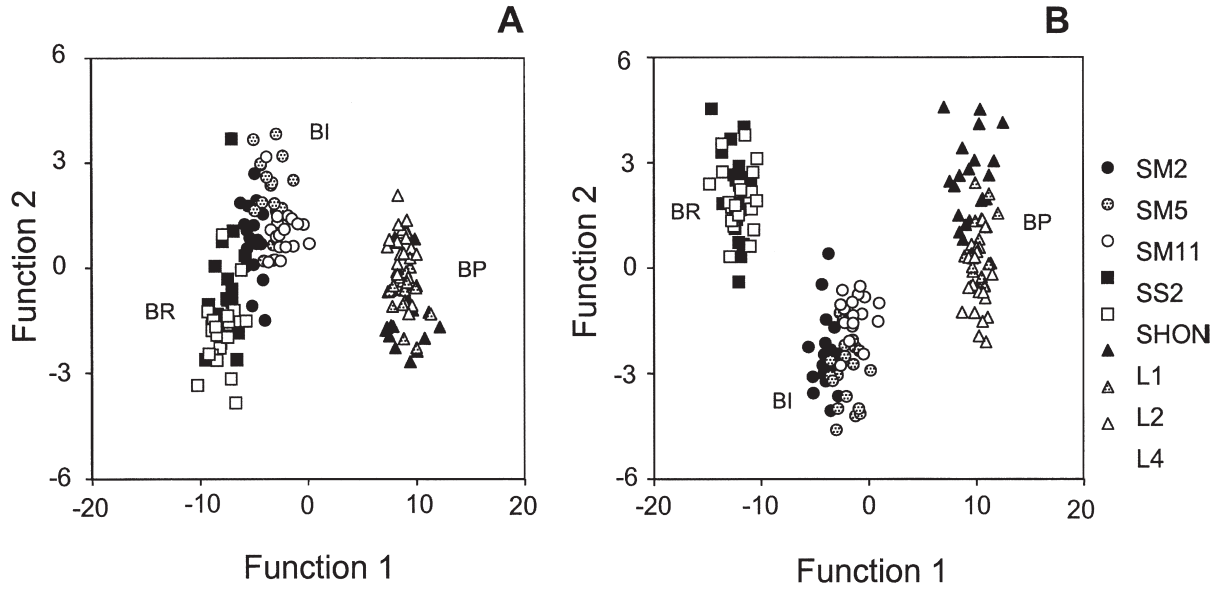

Fig. 2. Scatter plot of the measured individuals in the space defined by two canonical discriminant functions. The functions were obtained by stepwise discriminant analyses performed on several lorica measurements of individuals belonging to eight clones. (**A**) Results from Analysis I performed on seven characters selected according to Fu *et al.* (Fu et al., 1991a) (i.e. *a–g*; see Figure 1A). (**B**) Results from Analysis II performed on an alternative set of characters selected for this work (i.e. *a*, *c*, *e*, *g–i* of Figure 1A,B). Different symbols indicate different species and clones: *B. ibericus* n. sp. (BI and circles), *B. rotundiformis (*BR and squares) and *B. plicatilis (*BP and triangles).

Table II: Mean (SE) of body length (a, in μm) and four body ratios for the three sibling Brachionus species. Sample size (n) is indicated as number of clones times number of individuals per analysed clone. ANOVA results on morphometric variables (log-transformed for a) are also shown

Morphometric variable	*B. rotundiformis* (*n* = 2×20)	*B. ibericus n. sp.* (*n* = 3×20)	*B. plicatilis* (*n* = 3×20)	ANOVA *df*	*F*	*P*
a	148.7(1.3)	193.4(1.4)	299(1.5)	2, 157	2417.7	<0.001
c/a	0.807(0.006)	0.746(0.003)*	0.754(0.004)*	2, 157	53.3	<0.001
i/a	0.481(0.006)*	0.511(0.004)	0.482(0.003)*	2, 157	21.1	<0.001
h/a	0.132(0.002)	0.084(0.001)	0.057(0.001)	2, 157	537.1	<0.001
g/h	0.783(0.01)	1.011(0.01)	0.875(0.013)	2, 157	88.5	<0.001

* indicates non-significant differences between species ($P > 0.05$), as resulting from a post-hoc Student-Newman-Keuls test.

SS-type (SS2 and SHON) were relatively wider and shorter (*c/a*) than the other two species, while the SM-clones (SM2, SM5 and SM11) had the relatively widest head aperture (*i/a*).

According to the results of the present morphometric analysis, as well as to the detailed morphological comparisons provided below, we conclude that the three biological sibling species (Gómez *et al.*, 1995) can be separated as three nominal taxa, each corresponding to a well-differentiated morphology (Figure 3). Detailed descriptions of these three taxa are as follows.

Description of *Brachionus ibericus* n. sp. (Figures 3C,D, 4 and 5)

Type locality

Poza Sur is a man-made shallow pond (maximum size about 30 m long, 7 m wide and maximum depth in winter about 1.5 m), located in the Prat de Cabanes-Torreblanca Marsh (Castellón, Spain; 40°10′04″N, 0°10′57″E), a brackish water area next to the coast.

Material examined

Holotype: A parthenogenetic female, taken from a clonal population (strain SM2) maintained in the rotifer culture collection at the Institut Cavanilles de Biodiversitat i Biologia Evolutiva, University of Valencia (ICBIBE-UV), Spain, originally founded from a single amictic female collected in Cabanes-Torreblanca Marsh, May 6, 1993 (Gómez *et al.* 1995), was fixed (95%) and preserved (70%, with a drop of glycerine) with ethanol; vial deposited in the Natural History Museum (NHM; London, UK); catalogue number: NHM-2000.2929.

Paratypes: Thirty parthenogenetic females belonging to each of the strains SM2 (collection data as holotype), SM5 (collected May 27, 1993) and SM11 (collected September 17, 1992), originated from the type locality. Specimens were fixed and preserved as the holotype. Vials deposited in the NHM; catalogue numbers: NHM-2000.2930–2959, NHM-2000.2960–2989 and NHM-2000.2990–3019. One parthenogenetic female on a permanent glycerine glass slide sealed with Permount™ mounting medium, and 30 females (strain SM2) preserved with 70% ethanol into a vial, both deposited in the Academy of Natural Sciences (ANS; Philadelphia, USA); catalogue numbers: ANSP RO-1046 and RO-1049. One parthenogenetic female on a permanent glycerine glass slide sealed with Permount™ mounting medium, and 30 females (strain SM2) preserved with 70% ethanol into a vial, both deposited in the National Museum of Natural History (NMNH; Washington, USA); catalogue numbers: USNM 189272–189273.

Further material examined: Many more specimens, amictic and mictic females (entire, trophi and resting eggs), and males obtained from the experimental and stock cultures belonging to strains SM2, SM5 and SM11. Six trophi as SEM preparations are deposited at ICBIBE-UV. All clones are currently maintained in the rotifer culture collection at ICBIBE-UV.

Etymology

The species is named after the Iberian Peninsula. Its name derives from its ancient inhabitants whom the Greeks called Iberians, probably after the Ebro (*Iberus*) river that flows into the Mediterranean Sea close to the type locality.

Differential diagnosis

Brachionus ibericus n. sp. can be distinguished from the two other species belonging to the *B. plicatilis* complex in three ways. First, the antero-dorsal spine pattern (Figure 4C,D): the three pair of spines are similar in length; the median spine (i.e. spine 2 in Figure 1B) is shaped like an equilateral triangle (Figure 4E,F), in contrast to both *B. plicatilis* and

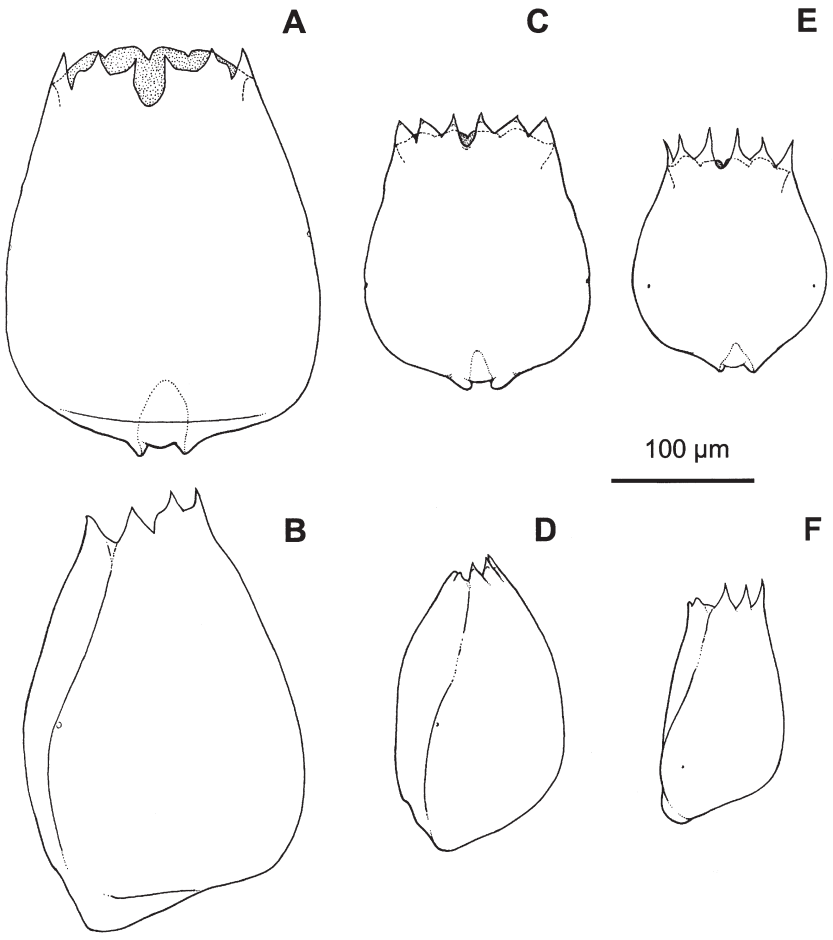

Fig. 3. Camera lucida drawing of the three sibling species. (**A**,**B**) *B. plicatilis* Müller. (**A**) dorsal view, (**B**) ventral view. (**C**,**D**) *B. ibericus* n.sp. (**C**) dorsal view, (**D**) ventral view. (**E**,**F**) *B. rotundiformis* Tschugunoff. (**E**) dorsal view, (**F**) dorsal view.

B. rotundiformis (Figures 6E,F and 8E,F). Second, by the shape and surface topography of resting egg (Figure 5E,F) which is ovoid in shape, with a characteristic rough surface pattern of anastomosing wavy ridges uniform in size, and with pores densely distributed on the entire egg surface. The egg shape differs from that described by Munuswamy *et al.* [(Munuswamy *et al.*, 1996); see their Figures 5 and 6] for *B. rotundiformis*. Third, the mode of carrying the resting eggs is different. The resting eggs remain inside the lorica (one single resting egg produced per female).

Description

Parthenogenetic female: Lorica relatively soft and flexible, ovoid shaped (Figure 4A) head aperture relatively wide. Lorica surface smooth. Anterior dorsal margin with six pointed, triangular spines, all similar in size, three on each side of a V-shaped sinus. Spine arrangement is constant in all analysed clones; the inner spines (i.e., spine 1 in Figure 1B) the most prominent, the external ones (i.e. spine 3 in Figure 1B) least developed (see Figure 4D). Median spines shaped like an equilateral triangle (Figure 4E,F). Anterior ventral margin with two pairs of lobules flanking a narrow sinus (Figure 4B); the external lobules slightly wider than the inner ones. Lateral antennae located slightly posterior to the lorica midpoint. Foot aperture sub-terminal, on ventral plate.

Trophi: Malleate and symmetrical (Figure 5A). Fulcrum short. Rami similar to a rectangular tetrahedron, with a ventral, flat surface (Figure 5B); anterior processi soft, lamellate. Unci plate-like, with five solid ridges having four teeth-like structures proximally (Figure 5C). Subuncus brush-like, consisting of several rows of small teeth or spines located at the inner side of the proximal ends of the unci (Figure 5D). Manubria flattened plate-like structures (Figure 5A), highly twisted and bent distally; with three opened proximal cavities.

Resting egg: A single resting egg carried within the lorica. Mictic egg ovoid (Figure 5E), slightly flattened on

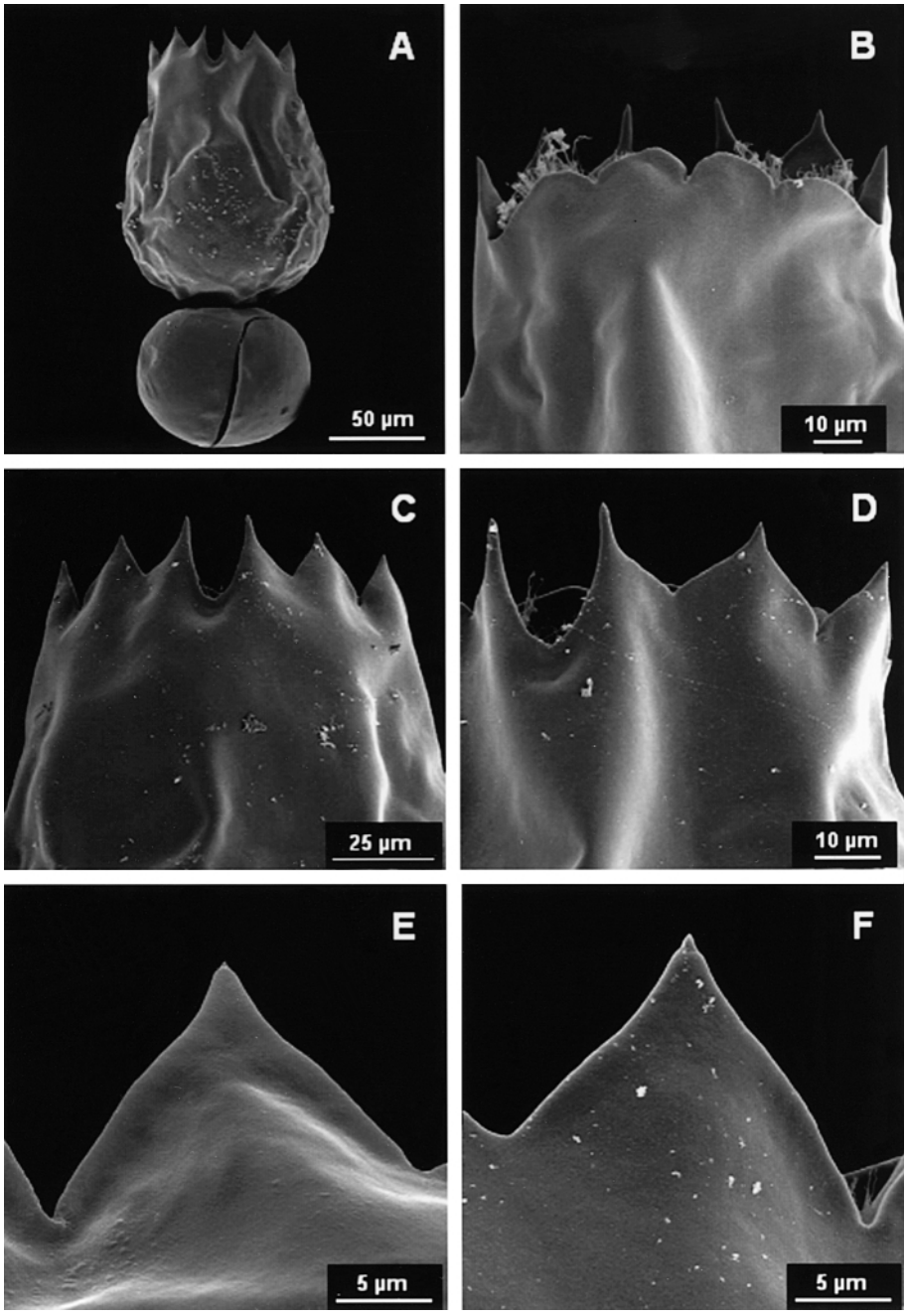

Fig. 4. *Brachionus ibericus* n. sp. (**A**) Amictic female, dorsal view; (**B**) head aperture, ventral view; (**C**) head aperture, dorsal view; (**D**) detail of a set of anterior dorsal spines; (**E**,**F**) Median anterior dorsal spine (spine 2, see Figure 1B).

both sides. Operculum located in a slight depression, defining a skullcap-like structure on one end of the egg. Surface topography showing an anastomosing pattern of granulated, wavy ridges, uniform in size, pores densely distributed on both the ridges and the depressions (Figure 5F).

Measurements (range and, in parenthesis, mean ± SE; in µm) of adult (48 ± 3 h old) animals cultured at 23ºC, 12 g l^{-1} salinity: Female lorica length, 175.5–220.0 (193.5 ± 2.5); width, 126.0–163.0 (144.5 ± 2.5); head aperture, 84.0–113.5 (99.0 ± 2.5); depth of dorsal sinus, 18–25 (21 ± 1); length of dorsal anterior spine 2 (median),

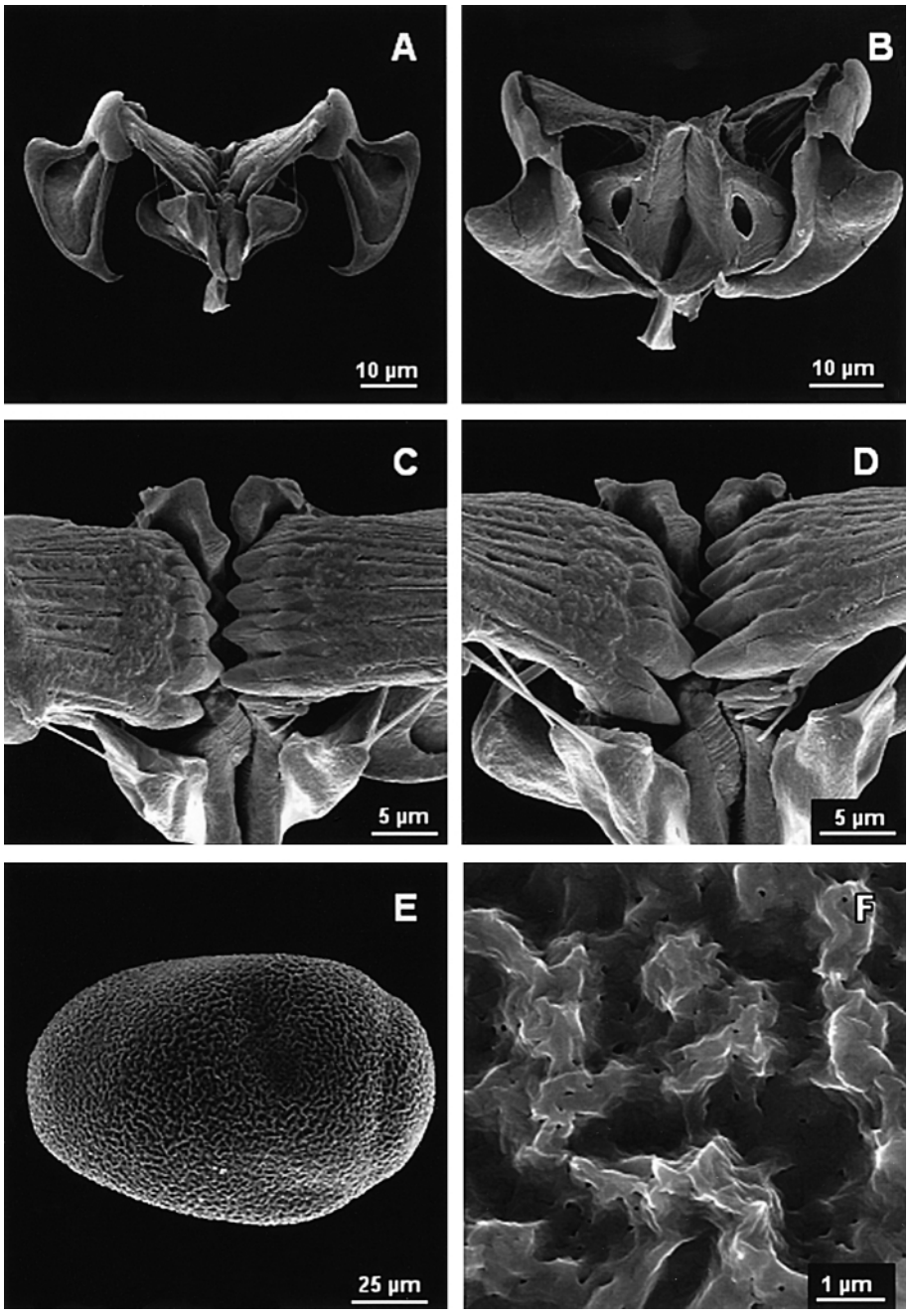

Fig. 5. *Brachionus ibericus* n. sp. (**A**) Trophi, dorsal view; (**B**) trophi, ventral view; (**C**) trophi seen from above, detail of the unci; (**D**) close up dorsal view of the middle part of the trophi; (**E**) resting egg; (**F**) detail of the resting egg surface.

12–22 (16 ± 1); length of dorsal-anterior spine 3 (external), 12–21 (16 ± 1). Trophi length, 30.6–31.3 (30.9 ± 0.2); fulcrum length, 6.4–7.9 (7.2 ± 0.4); manubrium length, 33.9–35.0 (34.8 ± 0.4); rami width, 31.8–34.0 (32.9 ± 0.6); uncus length, 19.0–20.9 (20.4 ± 0.4). Resting egg length, 128.3–137.2 (132.3 ± 2.6); width, 90.3–93.5 (91.6 ± 1.0). Male length, 96.5–116.0 (109.0 ± 2.5); width 59.5–66.5 (62.0 ± 2.5).

Comments

Brachionus ibericus n. sp. has been previously referred as *B. rotundiformis* SM (Gómez *et al.*, 1995). The differentiation

between this and the other two sibling species belonging to the *B. plicatilis* complex is based on genetic markers, assortative mating behaviour, etc. [reviewed in (Serra *et al.*, 1998)]. For a morphological comparison, see the redescription of both *B. plicatilis* Müller and *B. rotundiformis* Tschugunoff below.

According to the Principle of Priority of the International Code of Zoological Nomenclature, and following Segers' criteria used with *B. plicatilis* (Segers, 1995), the oldest available name applying to a taxon should be used for a stable nomenclature. Accordingly, one of the junior synonyms of *B. plicatilis* [reviewed in (Segers, 1995)] could have been used for the SM-type animals. Unfortunately, as Segers pointed out, no type material is available for any of those names and the original descriptions do not permit recognition of the taxa. However, when comparing with some of the published drawings a resemblance between *B. ibericus* n. sp. and *B. plicatilis* f. *longicornis* Fadeev, 1925 can be observed [see (Koste, 1978), T. 9, Figure 1b; (Koste, 1980), p.152, Figure 4]. Nevertheless, this superficial similarity with *B. plicatilis* f. *longicornis* is insufficient to establish that this taxa and *B. ibericus* n. sp. are the same species, given that we are splitting species on very narrow morphological grounds that would not necessarily be reproduced in others' drawings. So, we prefer to use a new specific-name for our SM-type animals. Another reason to disregard using this nominal taxa (i.e. *B. longicornis*) is because there is evidence (Ortells *et al.*, 2000; Gómez unpublished data; Ciros-Pérez, unpublished data) that more genetically and morphologically distinct species (different to the clones analysed here) exist with a morphology close to the SM-type '*B. plicatilis*'. So, the identity of *B. plicatilis* f. *longicornis* may be different from our Spanish animals, since very similar species can occur in a relatively reduced geographic region.

Distribution and ecology

Brachionus ibericus n. sp. is known from several coastal ponds, lagoons and marshes located in Eastern Spain (Ortells *et al.*, 2000), that include three ponds in Prat de Cabanes-Torreblanca Marsh [Poza Sur, Poza Norte and Canal Central, see (Gómez *et al.*, 1995)], Estany d'en Turies (Parc Natural dels Aigüamolls de l'Ampurdà), Laguna de San Lorenzo, El Basset de l'Altet, and two ponds (Charca Sur and Charca Poniente) in El Hondo de Elche Natural Park. These habitats vary from oligohaline to euryhaline, some of them are temporary while others are permanent [see (Ortells *et al.*, 2000) for a detailed description and location of each site].

The spatial and temporal distribution of *B. ibericus* n. sp. in Cabanes-Torreblanca Marsh (type locality) has been well characterized (Gómez *et al.*, 1995, Ciros-Pérez, unpublished data). This species occurs at medium to high salinities (from 8 to 50 g l^{-1}) and at high temperatures (>15°C) during spring and summer. It has been observed co-occurring for relatively long periods (ca. 4 months) with one, or with both other sibling species of the *B. plicatilis* complex (i.e. *B. plicatilis* s.s. and *B. rotundiformis*), as well as with other congeners (*B. urceolaris*). It has been observed in other waterbodies (i.e. Laguna de Almenara, and two ponds at El Hondo de Elche Natural Park) co-occurring with *B. angularis*, *B. calyciflorus*, *B. quadridentatus*, *B. bidentatus* and *B. leydigi* (Ortells, personal communication). The zooplankton assemblage accompanying the new species in Cabanes-Torreblanca Marsh also included some other monogonont rotifers (e.g. *Notholca salina*, *N. marina*, *Colurella salina*, *C. dicentra*, *Lecane grandis*, *Synchaeta cecilia valentina*, *S.* cf. *oblonga*, *Encentrum marinum*, *Testudinella* cf. *parva* and *T. obscura*) and a copepod (*Diacyclops bicuspidatus odessanus*).

Description of *Brachionus plicatilis* O. F. Müller, 1786 (Figures 3A,B, 6 and 7) (*Brachionus plicatilis* O. F. Müller, 1786, p. 344, Fig. 50: 1–8)

Designation of neotype

Since it has been established that no type material of *B. plicatilis* is available, and because we could not obtain topotypical material or isolate this taxon from the type locality (Denmark) to compare it genetically and morphologically with our Spanish clones, we decided to assign our *B. plicatilis* strains as neotype material. This decision was also taken considering that this morphospecies [*sensu* (Segers, 1995)] seems to be a group of at least two different species (Gómez *et al.*, 1998; Ortells *et al.*, 2000), which reveals that this taxon is in fact a species complex. This redescription allows us to appropriately compare among our three sibling species. Deposit of reference material in public collections would permit further comparative research (i.e., morphology, genetics) that should clarify the actual status of this taxon.

Material examined

Neotype: A parthenogenetic female, from a clonal population (strain L1) maintained in the rotifer culture collection at the ICBIBE-UV, originally founded from a single amictic female collected in Prat de Cabanes-Torreblanca Marsh, October 8, 1992 (Gómez *et al.* 1995). Ethanol fixed (95%), and preserved (ethanol 70%) with a drop of glycerine, vial deposited in the NHM (London, UK); catalogue number: NHM-2000.3020.

Further material examined: Many more specimens, amictic and mictic females, and males obtained from the experimental and stock cultures belonging to strains L1, L2 and L4. Thirty parthenogenetic females belonging to each of the strains L1 (collection data as neotype), L2

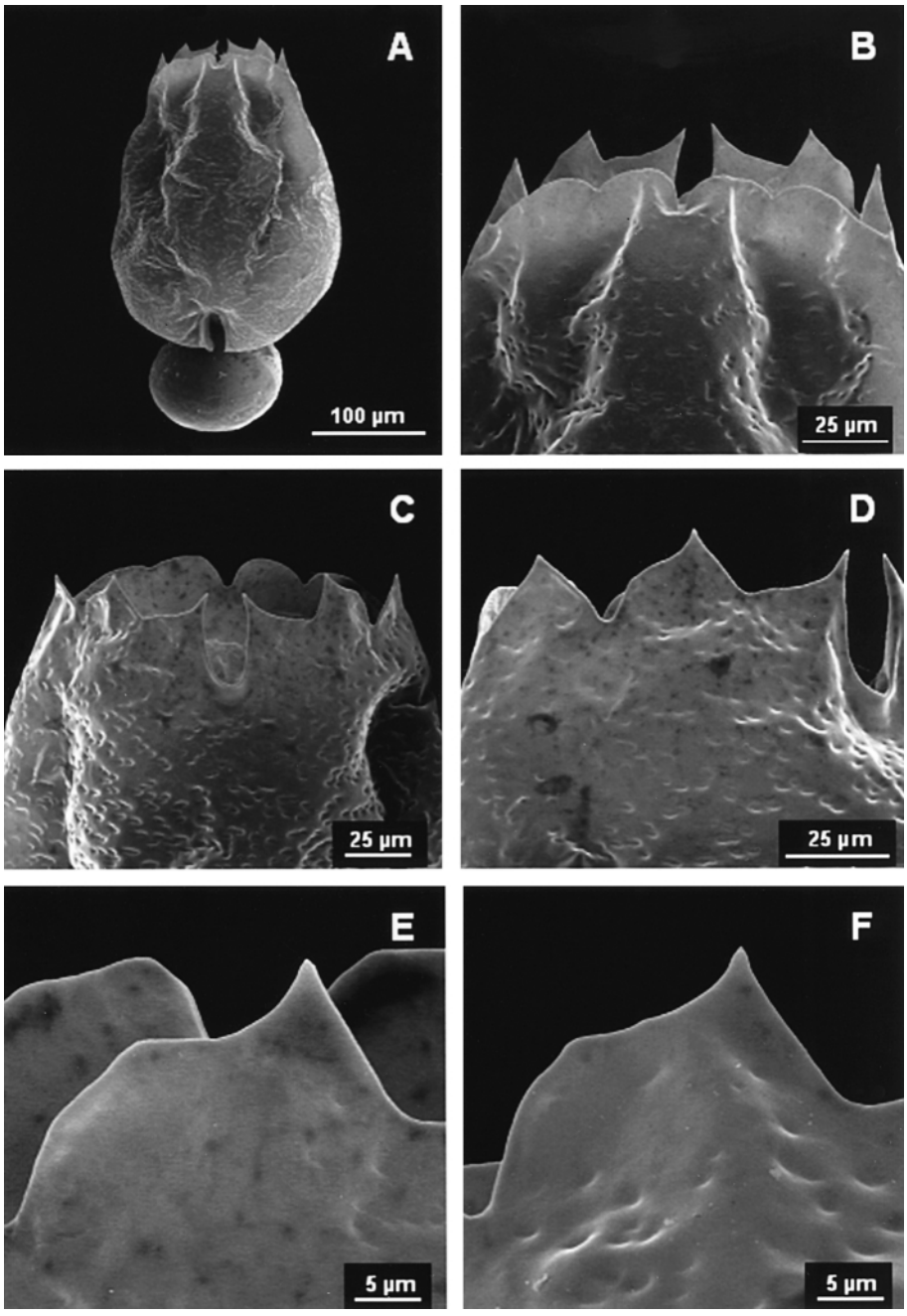

Fig. 6. *B. plicatilis* Müller. (**A**) Amictic female, ventral view; (**B**) head aperture, ventral view; (**C**) head aperture, dorsal view; (**D**) detail of a set of anterior dorsal spines; (**E**,**F**) Median anterior dorsal spine (spine 2, see Figure 1B).

(collected November 2, 1992) and L4 (collected November 22, 1992), from the neotype locality. Specimens fixed and preserved as the neotype. Vials deposited in the NHM; catalogue numbers: NHM-2000.3021–3050, NHM-2000.3051–3080 and NHM-2000.3081–3110. One parthenogenetic female on a permanent glycerine glass slide sealed with Permount™ mounting medium, and 30 females (strain L1) preserved with 70% ethanol into a vial, both deposited in the ANS (Philadelphia, USA); catalogue numbers: ANSP RO-1044 and RO-1047. One parthenogenetic female on a permanent glycerine glass slide sealed with Permount™ mounting

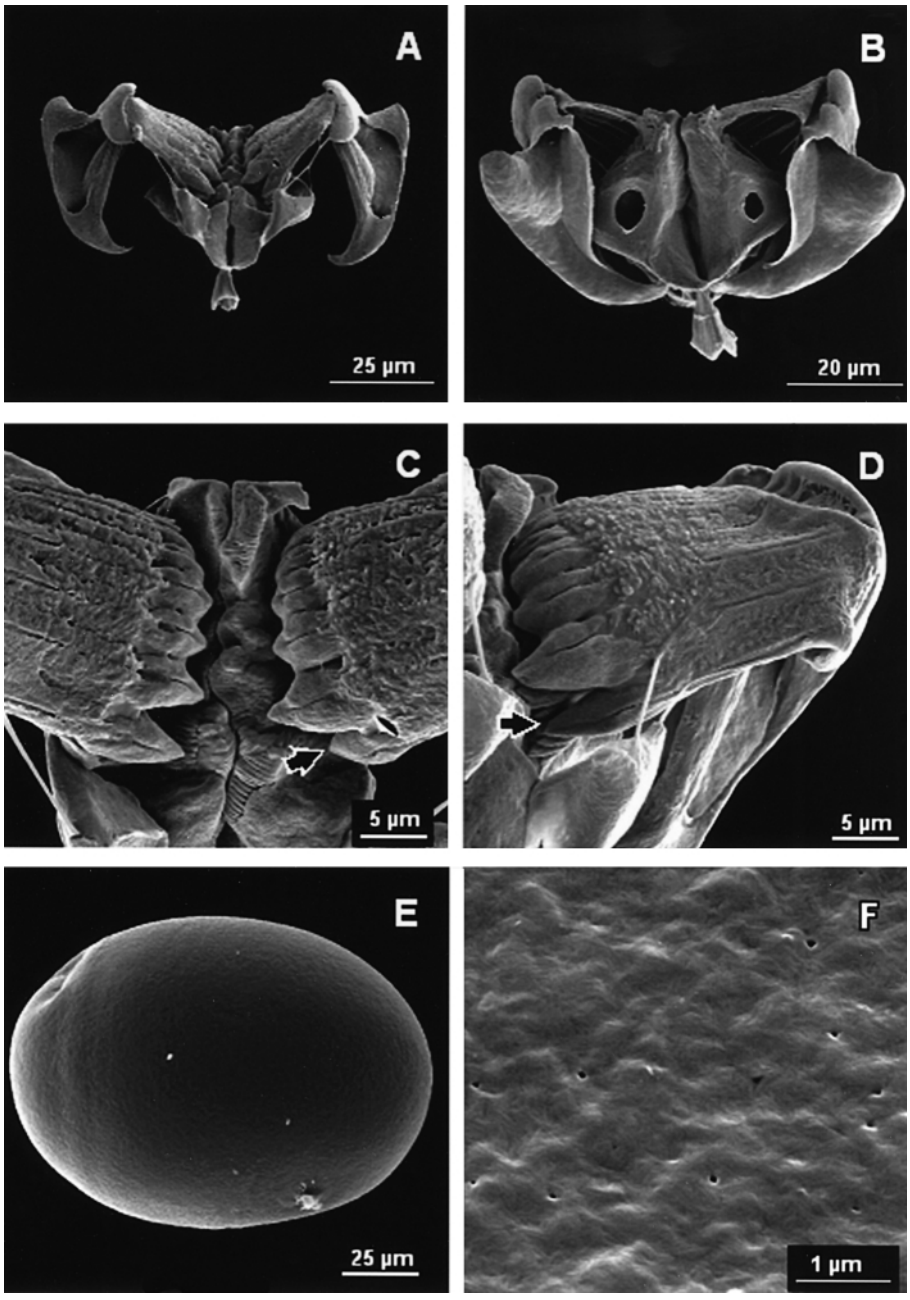

Fig. 7. *B. plicatilis* Müller. (**A**) Trophi, dorsal view; (**B**) trophi, ventral view; (**C**) trophi seen from above, detail of the unci; (**D**) close up dorsal view of right uncus, stressing the position of the most dorsal tooth (see arrow); (**E**) resting egg; (**F**) detail of the resting egg surface.

medium, and 30 females (strain L1) preserved with 70% ethanol into a vial, both deposited in the NMNH (Washington, USA); catalogue numbers: USNM 189274–189275. Six trophi as SEM preparations, deposited at ICBIBE-UV. All clones are currently maintained in the rotifer culture collection at ICBIBE-UV.

Differential diagnosis

Brachionus plicatilis Müller differs from the other two sibling species in the following ways. First, its antero-dorsal spine pattern (Figure 6C–F): three pairs of spines, all of them similar in length; the inner and outer spines (i.e. spine 1 and 3 in Figure 1B) roughly triangular with a wide base; median spine (i.e. spine 2 in Figure 1B) triangular in shape,

but with a sigmoid outer margin. Second, shape and surface topography of resting eggs (Figure 6E,F). Resting egg ovoid, with a fairly smooth surface with relatively few pores distributed on the entire egg surface. Surface arrangement differs from the description for *B. plicatilis* given in Munuswamy *et al.* (Munuswamy *et al.*, 1996). Third, body size is larger than *B. ibericus* n. sp. and *B. rotundiformis*.

Description

Parthenogenetic female: Lorica soft, pear-like shape (Figure 6A). Dorsal and ventral plates fused laterally and posteriorly. Lorica surface coarsely smooth or dotted. Anterior dorsal margin with three pairs of spines flanking the U-shaped sinus (Figure 6C); all spines roughly triangular similar in length, with wide base and relatively sharp apices (Figure 6D); outer margin of median spines sigmoid with sharp apexes (Figure 6E,F). Anterior ventral margin of the lorica with two pairs of rounded lobules flanking a slender sinus (Fig. 6B); outer lobules having a wider (about 1.5 times) base than the inner ones. Lateral antennae medially located. Foot aperture sub-terminal, on ventral plate.

Trophi: Malleate and symmetrical (Figure 7A). Morphology generally according to the description by Kleinow *et al.* (Kleinow *et al.*, 1990). Fulcrum short and hollow, shaped like a truncated cone. Rami roughly rectangular-tetrahedron in shape, with a ventral flat surface (Figure 7B); anterior processes soft and lamellate. Unci plate-like, with six or seven solid ridges; ridges having five teeth-like structures [four in the description by (Kleinow *et al.* 1990)] decreasing in size toward the anterior end (Figure 7C), these structures followed by a flattened molar-like structure (the last ridge tip, or as a result of the fusion of the last two). All unci teeth arranged in the same plane except the most dorsal one of the right uncus (see arrows on Figure 7C,D); right uncus slightly directed to the inner side. Subuncus brush-like. Manubria flattened, highly twisted and bent distally, plate-like, nearly triangular shaped (Figure 7A); three proximal cavities opened.

Resting egg: Attached to the posterior part of the lorica when carried. Resting egg oval (Figure 7E), slightly flattened on both sides. Operculum located in a scar-like depression at one end of the egg. Surface fairly rough with relatively few pores distributed on the entire egg surface (Figure 7F).

Measurements (range and, in parenthesis, mean ± SE; in μm) of adult (48 ± 3 h) animals cultured at 23ºC, 12 g l^{-1} salinity: Female lorica length, 274.0–341.0 (299.0 ± 2.5); width, 200.0–269.0 (225.5 ± 2.5); head aperture, 121.0–155.5 (144.0 ± 2.5); depth of dorsal sinus, 22–36 (28 ± 1); length of dorsal anterior spine 2 (median), 11–20 (15 ± 1); length of dorsal-anterior spine 3 (external), 12–26 (17 ± 1). Trophi length, 42.3–45.7 (43.7 ± 0.7); fulcrum length, 10.6–13.1 (11.9 ± 0.4): manubrium length, 38.2–45.2 (42.9 ± 0.7); rami width, 39.1–42.2 (40.8 ± 0.5); uncus length, 22.0–26.0 (24.0 ± 0.5). Resting egg length, 134.2–139.9 (137.0 ± 2.8); width, 99.5–1020 (100.8 ± 1.2). Male length, 121.0–141.0 (130.5 ± 2.5); width, 70.0–84.0 (82.0 ± 2.5).

Comments

Recently, Segers (Segers, 1995) suggested that *B. plicatilis* Müller is the correct name for the so-called *B. plicatilis* L-type, which can be morphologically distinguished from the S-type or *B. rotundiformis* Tschugunoff. Based on morphological comparisons, several synonyms have been recorded for this taxon [for a review see (Segers, 1995)], that includes *B. muelleri* Ehrenberg, *B. hepatotomus* Gosse, *B. plicatilis asplanchnoides* Charin, *B. plicatilis longicornis* Faveed and *B. orientalis* Rodewald. However, this *B. plicatilis* [*sensu* (Segers, 1995)] is more diverse than previously thought (Gómez *et al.*, 2000, 2001; Ortells *et al.*, 2000; Rong *et al.*, 1998), and deep genetic divergence has been reported within this taxon. As a result, we cannot assess, for the time being, the identity of all the nominal taxa presently listed as synonyms of *B. plicatilis* Müller without a careful and detailed analysis. The original description (i.e. published drawing) of *B. plicatilis* by O. F. Müller (Müller, 1786; Koste and Hollowday, 1993), that correspond to the so-called *B. plicatilis plicatilis* by Koste [(Koste, 1978); Plate 9, Figure 1c,f), is in good agreement with the morphology of our animals. But since no type material is available for comparison, we decided to redescribe this taxon from our Spanish material, in order to establish a base for enabling easier further comparative research that should clarify the identity of those synonyms and *species inquirendae* listed by Segers (Segers, 1995).

Distribution and ecology

B. plicatilis Müller has been found inhabiting several brackish and saline ponds, lagoons, lakes and marshes in the central, southern and eastern regions of the Iberian Peninsula, which are distributed in five endorheic basins and in the coastal plain [see the so-called 'cluster A' in (Ortells *et al.*, 2000; Gómez, *et al.* 2000)]. These habitats vary from oligohaline to euryhaline (3–55 g l^{-1}), some of them are temporary while others are permanent [for further details of each site see (Ortells *et al.*, 2000)]. Waterbodies where this species has been recorded are the following.

(1) Coastal lagoons of Spain: Cabanes-Torreblanca Marsh [Poza Sur, Poza Norte and Canal Central, see (Gómez et al., 1995)], Parc Natural dels Aigüamolls de l'Ampurdà (Estany d'en Turies), Laguna de Almenara, Marjal de Pego-Oliva (Charca Barranquet), Laguna

de San Lorenzo, El Basset de l'Altet, El Clot de Galvany, El Hondo de Elche Natural Park (Charca Norte, Charca Sur and Charca Poniente) and Cádiz (Charca Temporal Universidad de Cádiz).

(2) Ebro Basin: Laguna Salada de Chipriana, Balsa de Santed and Laguna de Gallocanta.

(3) Duero Basin: Laguna de las Eras.

(4) Guadiana Basin: Laguna de Manjavacas, Laguna de Peña Hueca, Laguna de Tirez and Laguna Camino de Villafranca.

(5) Júcar-Segura Basin: Laguna de Salobrejo, Laguna del Saladar, Laguna de Pétrola, Laguna de Mojón Blanco, Laguna de Hoya Rasa, Laguna de Casa Nueva II and Laguna de la Atalaya de los Ojicos.

(6) Guadalquivir Basin: Laguna de Capacete and Laguna de Fuente de Piedra.

The spatial and temporal distribution of *B. plicatilis* Müller in Cabanes-Torreblanca Marsh (site from which neotype comes from) has been well characterized (Gómez, *et al.*, 1995; Ciros-Pérez, unpublished data). This species occurs from low to high salinities (from 3 to 45 g l^{-1}) and at temperatures below 25ºC, during autumn, winter and spring. It co-occurs with one or with both two sibling species of the *B. plicatilis* group (i.e. *B. ibericus* n. sp. and *B. rotundiformis*), as well as with other congeners (i.e. *B. urceolaris*, *B. quadridentatus*, *B. angularis* and *B. calyciflorus*) (Ortells, personal communication).

Brachionus rotundiformis Tschugunoff, 1921 (Figures 3E,F, 8, 9) (*Brachionus muelleri* var. *rotundiformis* Tschugunoff, 1921, p. 160, Figure 12)

Designation of neotype

Since no original type material of *B. rotundiformis* is available, and for the same reasons stressed above for the *B. plicatilis* Müller redescription, neotype material has been deposited and described from our Spanish strains. Deposit of reference material in public collections would allow further comparative research (morphology or genetics) that should clarify the actual status of this taxon.

Material examined

Neotype: A parthenogenetic female, from a clonal population (strain SS2) maintained in the rotifer culture collection at the ICBIBE-UV, originally founded from a single amictic female collected in Cabanes-Torreblanca Marsh, September 17, 1993 (Gómez *et al.*, 1995). Fixed (95%) and preserved (70%, with some drops o glycerine) with ethanol, vial deposited in the NHM (London, UK); catalogue number: NHM-2000.3111.

Further material examined: Many more specimens, amictic and mictic females, and males obtained from the experimental and stock cultures belonging to strains SS2 (collection data as the neotype) and SHON (collected August 26, 1998). Thirty parthenogenetic females belonging to the SS2 strain from Cabanes-Torreblanca Marsh, and 30 parthenogenetic females of the SHON strain, from El Hondo de Elche National Park (Charca Norte). Specimens fixed and preserved as the neotype. Vials deposited in the NHM; catalogue numbers: NHM-2000.3112–3141 and NHM-2000.3142–3171. One parthenogenetic female on a permanent glycerine glass slide sealed with Permount™ mounting medium, and 30 females (strain SS2) preserved with 70% ethanol into a vial, both deposited in the ANS (Philadelphia, USA); catalogue numbers: ANSP RO-1045 and RO-1048. One parthenogenetic female on a permanent glycerine glass slide sealed with Permount™ mounting medium, and 30 females (strain SS2) preserved with 70% ethanol into a vial, both deposited in the NMNH (Washington, USA); catalogue numbers: USNM 189276–189277. Six trophi as SEM preparations, deposited in the ICBIBE-UV. Both of the clones are currently maintained in the rotifer culture collection at the ICBIBE-UV.

Differential diagnosis

Brachionus rotundiformis differs from the other two sibling species in four ways. First, the antero-dorsal spine pattern (Figure 8C–F). Three pairs of antero-dorsal triangular spines, all sharply pointed; the median spine (i.e. spine 2 in Figure 1B) shorter than the others. Second, lorica semicircular shaped and dorsal-ventrally compressed; lateral antenna dorsally and slightly posteriorly located. Third, the body size is smaller than *B. ibericus* n. sp. and *B. plicatilis*. Fourth, shape and surface topography of resting eggs (Figure 9E,F). Resting egg is kidney shaped, with a fairly rough surface, abundant pores irregularly distributed on the entire egg surface. Surface topography and egg shape differ from those described by Munuswamy *et al.* [(Munuswamy *et al.*, 1996); see their Figures 5 and 6] for *B. rotundiformis*.

Description

Parthenogenetic female: Lorica soft, almost circular in profile (Figure 8A) and dorso-ventrally compressed (Figure 3F). Dorsal and ventral plates fused laterally and posteriorly. Lorica surface smooth. Anterior dorsal margin with three pairs of spines, all triangular and sharply pointed flanking a U-shaped sinus (Figure 8C). Median spines the shortest (Figure 8D), having a characteristic acuminate shape (Figure 8E,F). Anterior ventral margins with two pairs of lobules flanking a slender sinus; inner lobes roughly quadrangular shaped, and external ones slightly rounded; the latter followed by a straight margin

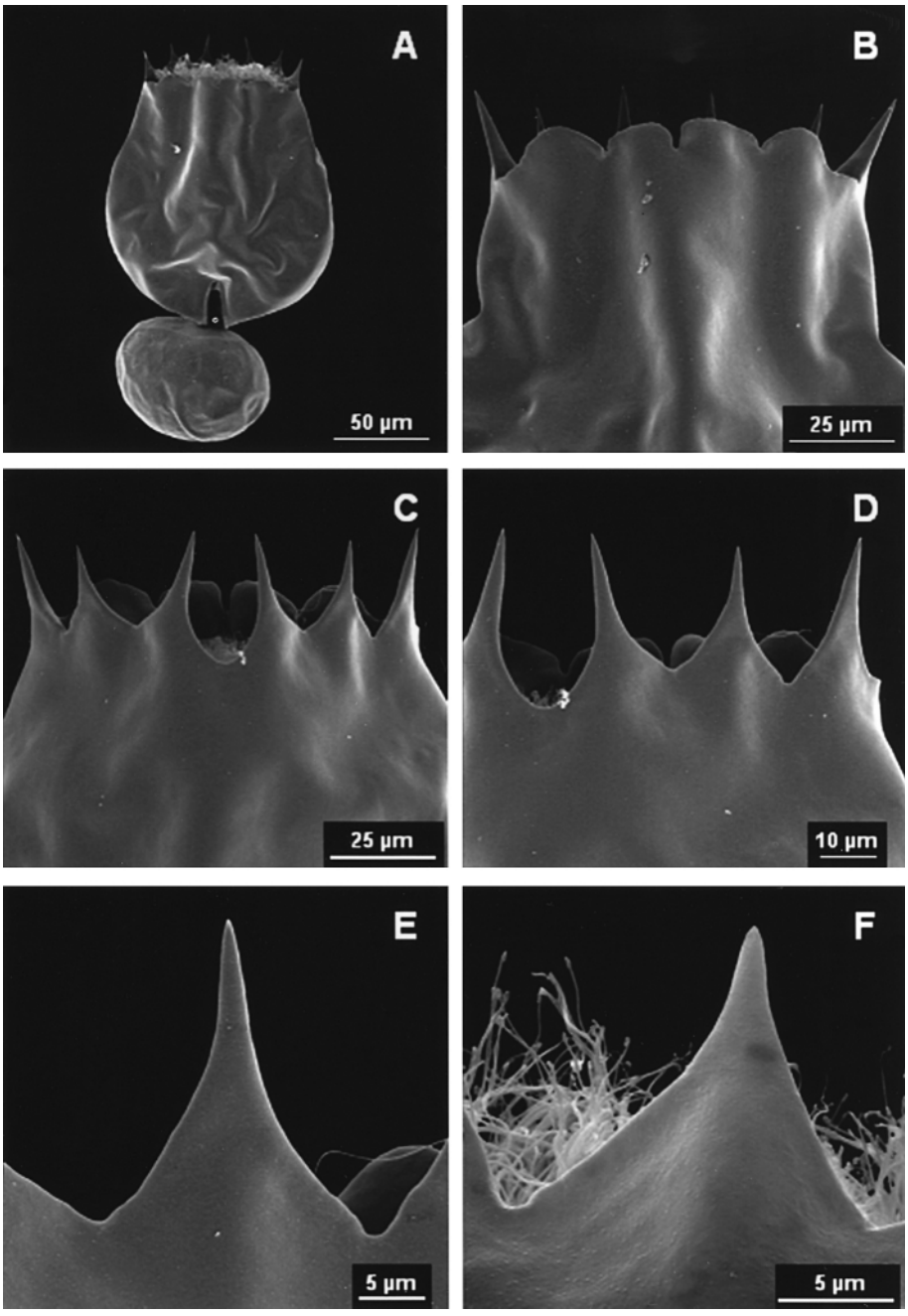

Fig. 8. *Brachionus rotundiformis* Tschugunoff. (**A**) Amictic female, ventral view; (**B**) head aperture, ventral view; (**C**) head aperture, dorsal view; (**D**) detail of a set of anterior dorsal spines; (**E**,**F**) Median anterior dorsal spine (spine 2, see Figure 1B).

that reaches the lateral margins of the lorica (Figure 8B). Lateral antenna located on the dorsal lorica, at about the posterior third. Foot aperture sub-terminal, on the ventral plate.

Trophi: General morphology (Figure 9A) similar to that of *B. ibericus* n. sp. (Figure 5A). Fulcrum short. Rami roughly rectangular tetrahedron shaped (Figure 9B); anterior processi soft and lamellate. Unci with four teeth-like structures proximally (Figure 9D). Subuncus brush-like. Manubria flattened plate-like structures, highly twisted and bent distally (Figure 9A); three proximal cavities opened.

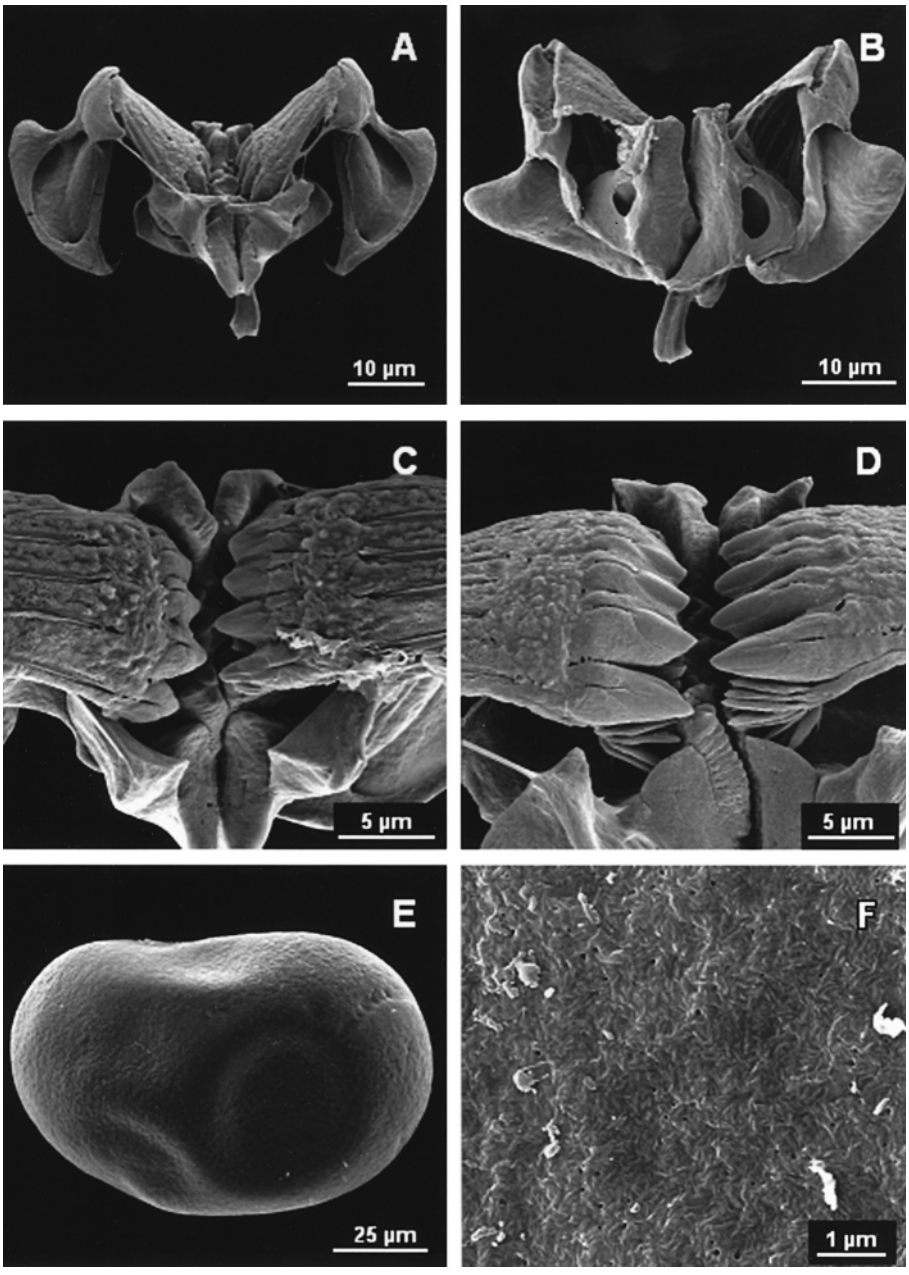

Fig. 9. *Brachionus rotundiformis* Tschugunoff. (**A**) Trophi, dorsal view; (**B**) trophi, ventral view; (**C**) trophi seen from above, detail of the unci; (**D**) close up dorsal view of the middle part of the trophi; (**E**) resting egg; (**F**) detail of the resting egg surface.

Resting egg: Attached to the posterior part of the lorica when carried. Resting egg kidney shaped (Figure 9E). Operculum with a fine scar-like structure, located at one end of the egg. Surface fairly rough with abundant pores irregularly distributed on the whole egg surface (Figure 9F).

Measurements (range and, in parenthesis, mean ± SE; in µm) of adult (48 ± 3 h) animals cultured at 23°C, 12 g l^{-1} salinity: Female lorica length, 131.0–165.5 (148.5 ± 2.5); width, 106.0–128.5 (120.0 ± 2.5); head aperture, 62.0–79.0 (71.0 ± 2.5); depth of dorsal sinus, 14–26 (22 ± 1); length of dorsal anterior spine 2 (median), 11–20 (15 ± 1); length of dorsal-anterior spine 3 (outer), 15–22 (20 ± 1). Trophi length, 25.0–28.4 (26.6 ± 0.7); fulcrum length, 7.1–8.6 (7.8 ± 0.4); manubrium length, 25.6–29.2 (27.1 ± 0.5); rami width, 23.3–26.5 (24.4 ± 0.8);

uncus length, 113.2–113.8 13.3–16.6 (15.1 ± 0.4). Resting egg length, (113.5 ± 0.5); width 74.0–75.9 (75.0 ± 1.0). Male length, 89.0–111.0 (98.0 ± 2.5); width, 49.0–59.5 (55.0 ± 2.5).

Comments

As previously explained, from a re-examination of the available published names for the *B. plicatilis* morphospecies, Segers (Segers, 1995) established that the correct name for the so-called S-type was *B. rotundiformis* Tschugunoff, originally described from the Caspian Sea (Tschugunoff, 1921). Although this reassignment was an important step, we now know that this morphospecies [*sensu* (Segers, 1995)] is not a single biological species, but is a complex of several cryptic taxa, each probably having a more restricted geographical distribution than the whole complex. It is worth pointing out that more than two cryptic species (i.e. *B. ibericus* and *B. rotundiformis*) have probably been included in *B. rotundiformis sensu* Segers. This would explain disagreements in morphological descriptions available in the literature [see for instance (Sudzuki, 1987; Munuswamy *et al.*, 1996), and descriptions in this paper]. Besides these, no type material is available for comparison, and the probability of find the same animal species described by Tschugunoff (Tschugunoff, 1921) is low because several species belonging to this species complex might coexist in the type locality. Consequently, we decided to re-describe this taxon from our Spanish material. This would allow further comparative works. Our clones correspond well to the original description by Tschugunoff (Tschugunoff, 1921).

Distribution and ecology

Brachionus rotundiformis Tschugunoff has been found inhabiting several ponds in two brackish marshes in the Eastern coast of Spain, [see the so-called 'cluster 0' in (Ortells *et al.*, 2000)], that include three ponds in the Cabanes-Torreblanca Marsh [Poza Sur, Poza Norte and Canal Central, see (Gómez *et al.*, 1995)], and a single pond in El Hondo de Elche National Park (Charca Norte). Additionally, it has been observed (Gómez, personal observations) in Albufera de Pollensa, a pond in Cádiz (Charca Temporal, Universidad de Cádiz) and a 'sebkhet' in Korba (Tunisia). These habitats vary from mesohaline to euryhaline, being temporary or semipermanent (Ortells *et al.*, 2000), with a highly variable regime.

The spatial and temporal distribution of *B. rotundiformis* has been well characterized in Cabanes-Torreblanca Marsh (Gómez, *et al.*, 1995; Ciros-Pérez, unpublished data). This species occurs at medium to high salinities (from 10 to 57 g l^{-1}) and high temperatures (from 10 to 30°C) during spring, summer and autumn.

REMARKS

Since Segers (Segers, 1995) proposed the use of *B. plicatilis* Müller and *B. rotundiformis* Tschugunoff as the correct names for the designated S- and L-morphotypes, several researchers have been using these names to denominate their own clones (isolated from all over the world), assuming with this that both taxa are cosmopolitan. Nevertheless, until now, nobody knew what those animals described by Müller or Tschugunoff really were (Müller, 1786; Tschugunoff, 1921). The main problem arises because several published data suggest that these rotifer taxa are clusters of various sibling species (Gómez and Snell, 1996; Serra *et al.*, 1998; Ortells *et al.*, 2000), with probably more restricted distribution than previously thought. Since no type material for either of these two specific names was available, many superficially similar strains were classified as one or other. For these reasons, this study contributes to establish a reference base for further comparative (taxonomy, behavioural, or genetic) works, to try to elucidate the actual species identity of those related *B. plicatilis*-like animals.

Several studies of the *B. plicatilis* species complex [for a critical review see (Serra *et al.*, 1998)] have typically neglected the differences within the so-called S- and L-morphotypes, assuming *a priori* that only two species exist. The S- vs. L-type classification is probably an important phylogenetic division, but it does not mean that only two species belong to this species complex. In this sense, the analysis based on populations (several strains belonging to each population) rather than on single strains (each belonging to a different population) might provide more information to differentiate biological and taxonomic species.

By distinguishing and characterizing three nominal species of the *B. plicatilis* complex our analysis bridged the gap between rotifer classical taxonomy and modern approaches. Our results show that morphometry can be a powerful tool to differentiate similar species when biological species are recognized based on molecular, ecological and physiological data. However, care must be taken since the discriminating power of a morphometric approach critically depends on the selection of the characters measured; in our case, various aspects of the lorica. Although our data were obtained from clones cultivated in laboratory conditions, the result should pertain to field populations as well, since the characters described as differentially diagnostic are quite constant, at least in our Spanish collections (Ciros-Pérez, unpublished data). Nevertheless, as there are undoubtedly other species within the *B. plicatilis* complex awaiting description, our data should not be used to consequently define animals from other locations, without performing a detailed comparison.

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