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Behavioral reproductive isolation among sympatric strains of *Brachionus plicatilis* Müller 1786: insights into the status of this taxonomic species

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Abstract

We present results on cross-mating experiments using *Brachionus plicatilis* strains collected in three ponds of a coastal marsh (Torreblanca Marsh, Castellón, Spain). These strains were known to differ widely both in morphology and allozyme patterns from a previous study, where they were grouped into three genetically different clonal groups. Although some of the strains co-occurred in the same pond and sexual periods overlapped, no gene flow was found among them. Our first objective was to determine whether behavioral reproductive isolation was responsible for the absence of interbreeding. A second objective was to explore the relationship between sexual isolation and genetic divergence. We performed two experiments. In Experiment 1, we tested five strains from different clonal groups; in Experiment 2, we added a strain from a congeneric species, and strains from different ponds. We recorded male mating behavior in all possible male–female strain pairings. Our data show that males of a strain tend to mate with females of the same strain or genetically similar strains, regardless of the pond they come from. We also found a high positive correlation between isolation distance and genetic distance. These results support the view that mating behavior acts as an important isolating barrier giving cohesion to clonal groups, and structuring populations of this rotifer, and that *Brachionus plicatilis* is a taxon composed of more than one biological species.

Introduction

Brachionus plicatilis O. F. Müller 1786 is a long recognized rotifer species. Apart from extensive morphological variability in size and shape, in recent years evidence is growing about the high levels of physiological, chromosomal, allozymic and total protein variability in this rotifer (e.g. Fu *et al.*, 1991a,b; Rumengan *et al.*, 1991; Carmona *et al.*, 1989). Some researchers have split this species into two groups namely 'S' (small) and 'L' (large), according to morphological, biometrical and allozyme analysis of strains collected all over the world, but a great deal of variation continues to be unexplained (e.g. see Sudzuki, 1987). Fortunately, the relatively high frequency of sexual reproduction in this cyclical parthenogen allowed Snell & Hawkinson (1983) to develop a standardized protocol for assessing the levels of behavioral isolation in rotifers through cross-mating experiments. Using this procedure these authors revealed that strains of *B. plicatilis* collected from different ponds were, to some extent, reproductively isolated, which suggested that a differentiation of their mating recognition signals had taken place. Obviously, the limited tendency of strains to interbreed might maintain genetic differences within this species, which has important evolutionary implications in rotifers (Snell, 1989). Snell (1989) also stressed the need of applying premating tests to as many rotifer species are possible, to disentangle the confused taxonomy of this group at the level of species, which up to now is based primarily on morphological criteria.

In a previous study (Gómez *et al.*, in press), we reported on the genetic structure of *B. plicatilis* populations in Torreblanca Marsh. Three clonal groups, called SS, SM and L, which differed both in their allozyme patterns and morphology, were found in an

annual survey of allozyme variation and population structure. These clonal groups were involved in a seasonal succession in one of the ponds (Poza Sur), and evidence of ecological specialization was also found. Although clonal groups showed partially overlapping sexual periods, allozyme data provided no trace of gene flow between them. Therefore, it was of great interest of ascertain to what extent clonal group genetic cohesion was maintained through a high propensity for momogamic matings (i.e. behavioral or ethological isolation).

Here we report on two experiments focused on behavioral reproductive isolation between strains from Torreblanca Marsh. Although sexual isolation between species or populations has usually been tested using the 'multiple choice method' (e.g. Majerus *et al.*, 1982; Ringo *et al.*, 1986), this experimental design is not suitable in rotifers; thus, we decided to follow the 'no choice' method developed by Snell & Hawkinson (1983) in *B. plicatilis*. In this method, behavioral reproductive isolation between strains is assessed through intra- and inter-strain mating tests, in which males of each strain are tested with females of all strains in successive tests. We applied these procedures to strains collected in Torreblanca Marsh, using strains from different clonal groups and from three different ponds. Two experiments were carried out. In Experiment 1, we aimed to ascertain if sympatric strains from the three clonal groups were more responsive to strains from their own group than to the others. In Experiment 2, we added a strain of *Brachionus quadridentatus* and some other strains to test whether reproductive isolation occurred between strains from the same clonal groups from different ponds, and whether reproductive isolation exists between *B. plicatilis* and *B. quadridentatus*. These experiments allowed us to explore the relationship between sexual isolation and genetic distance.

Materials and methods

Rotifer clones were maintained in duplicate in 15 ml glass tubes at 25 °C (±1 °C) and constant light conditions (PAR: approx. 35 $\mu EM^{-2}\ s^{-1}$). All rotifer cultures were fed by changing half of the culture with fresh growth medium every other day. Growth medium consisted of a culture of *Tetraselmis suecica* in 10 $g\ l^{-1}$ salinity water made with Instant Ocean salts and fertilized with f/2 solution (Guillard & Ryther, 1962). The strains were acclimated to experimental temperatures and salinities for at least a week before the trials.

Males were produced spontaneously by all strains in these experimental conditions, and can be readily collected. Only active-swimming males were used in the experiments. Neither male nor female's age was controlled. Individuals were washed in fresh saline medium just prior to the test. In each test, 25 randomly chosen females (including ovigerous females) of the appropriate strain were placed in a small well (96 well clusters with round base, COSTA, USA) containing 50 μl of Instant Ocean medium (10 $g\ l^{-1}$). One male was then introduced in the well and his behavior observed continuously during 5 min at 12× under a microscope.

Three behavioral categories were used in the mating tests: encounter, circling and copulation (Fig. 1; for description of mating behavior see Gilbert, 1963; Snell & Hawkinson, 1983). Male–female encounters happen at random as male rotifers are not attracted to females at a distance (Gilbert, 1963; Snell & Garman, 1986). If males detect a specific mate recognition pheromone on the female's body through coronal contact chemoreception (reviewed in Snell & Morris, 1993), circling behavior is displayed by the male, consisting of quick, conspicuous swimming in tight circles around the female's body, maintaining continuous coronal contact. Then the male locates the female's corona or foot opening during circling and copulation takes place. The sequence of mating behavior may be broken in any place, the male usually swimming away.

We recorded an encounter as a head-on contact between male's corona and female's body; the criterion for circling was established as male turning around the female's body in one or more complete revolutions; finally, a copulation was recorded if male attached his penis into the female's body and male coronal contact was lost. In each test, six replicate males were tested successively on the same set of females. All possible intra- and interstrain pairings were made using a counterbalanced design. All behaviors were recorded by the same observer.

Composite genotypes for the clones used in the experiments and ponds where they had been collected are shown in Table 1. Methods for obtaining zymogram patterns, and other features of these strains can be found in Gómez *et al.* (in press). These clones are representative of composite genotypes found in high frequency during their occurrence in each pond (Gómez *et al.*, in press). In Experiment 1 we used five

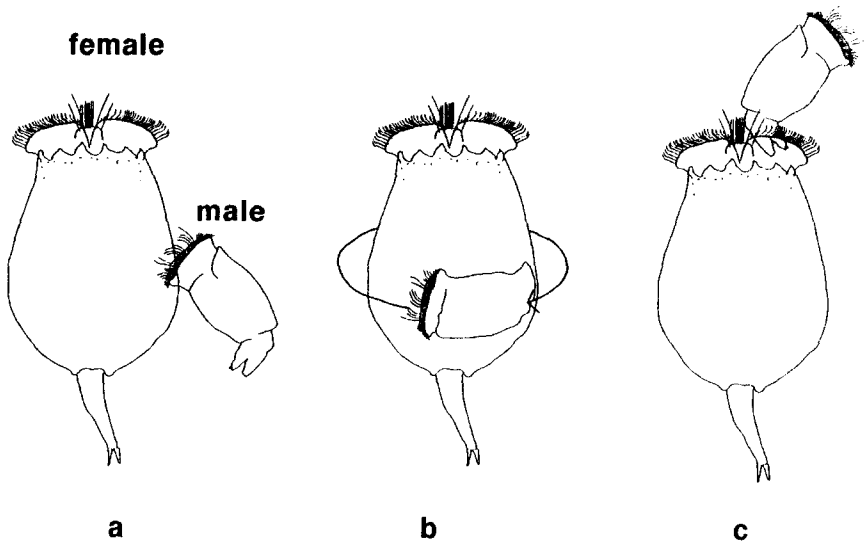

Fig. 1. Schematic drawing showing male behavioral categories recorded during the mating tests. a: encounter; b: circling; c: copulation.

clones obtained from Poza Sur in Summer and Autumn 1992, when maximum overlapping of sexual periods took place. These clones were two SS clones (SS1 and SS2), a SM clone (SM1), and two L clones (L1 and L2). In Experiment 2, seven strains, including some from different ponds, were tested. We selected SM2, SM3, and SM4 strains, all of them SM strains, from Poza Sur, Poza Norte, and Canal Central respectively, and members of the other groups from Poza Sur (L1, SS1 and SS2), together with a clone (BQ) of a congeneric species, *B. quadridentatus*, as an 'outgroup', also isolated from Poza Norte.

Two indexes of reproductive isolation were computed. First, percent mating initiation, i.e., percent of male–female encounters ending in circling, was calculated for each mating test. Statistical significance of this index was assessed using G-tests of independence (Sokal & Rohlf, 1981) in pair-wise comparisons between male responses to females of his own strain and to females of other strains. Second, a modified Y index, originally developed by Yule (discussed in Spieth & Ringo, 1983) was estimated as follows:

$$Y = \frac{[(p_{ii} \cdot p_{jj})^{1/2} - (p_{ij} \cdot p_{ji})^{1/2}]}{[(p_{ii} \cdot p_{jj})^{1/2} + (p_{ij} \cdot p_{ji})^{1/2}]};$$

where p_{ij} equals the proportion of male–female encounters resulting in circling in a test where a male of the strain i is tested with females of the strain j. To estimate Yule's index we used percents of mating initiation in the four mating tests (two homogamic, p_{ii} and p_{jj} and two heterogamic p_{ij} and p_{ji}) carried out with two given strains. Yule's index ranges from -1 to $+1$. The index is $+1$ for complete sexual isolation between the strains, 0 for random mating and -1 if mating is completely heterogamic (that is, when the other strain is preferred). Yule's Y index measures the degree of reproductive isolation between two strains eliminating artifacts owed to differences in mating activity among them (Spieth & Ringo, 1983).

To evaluate the relationship between sexual isolation and genetic distance we used allozyme data from Gómez *et al.* (in press) to compute Prevosti genetic distance (Wright, 1978) between strains. We also did a correlation analysis between Prevosti genetic distance and Yule's index estimates from Experiments 1 and 2; when we had data from both experiments, we used mean Yule's indexes. Adjusted G-tests were computed by using a Turbo-PASCAL program developed by M. Kramer, and Prevosti distance was calculated using BIOSYS-1 release 1.7 (Swofford & Selander, 1989). Significance level was set at 0.05 in all tests.

Table 1. Genetic characterization of the tested strains at 9 allozyme loci. Letter/s in each clone's name indicate the clonal group to which they belong (either SS, SM or L). BQ is a *Brachionus quadridentatus* clone. Alleles are disignated alphabetically according to decreasing anodal mobility. J in BQ indicates different alleles than in the rest of the strains. Pond and season where the strain was isolated is also given (PS: Poza Sur; PN: Poza Norte; CC: Canal Central; su: summer; sp: spring; f: fall).

			Locus/Genotype								
Clone	Pond	Season	*Pgi*	*Pgm*	*Mdh-1*	*Mdh-2*	*Ldh*	*Apk-1*	*Apk-2*	*Apk-3*	*6-Pgd*
SM1	PS	su	AA	AA	AA	BB	AA	BB	BB	BB	CC
SM2	PS	sp	AA	AA	AA	BB	AA	BB	BB	BB	CC
SM3	PN	f	AA	AA	AA	BB	AA	BB	BB	BB	CC
SM4	CC	su	AA	AA	AA	BB	AA	BB	BB	BB	CC
SS1	PS	su	EE	DD	CC	OO	CC	BB	CC	CC	AA
SS1	PS	su	EE	DD	CC	OO	–	BB	CC	CB	AA
L1	PS	f	CC	CC	CC	AA	BB	AA	AO	AO	BB
L2	PS	f	CD	CC	CC	AA	BB	AA	AA	AA	BB
BQ	PN	f	JJ	BB	JJ	BB	JJ	JJ	JJ	JJ	–

Results

With few exceptions, copulations were observed only within strains and between genetically related strains, where percent mating initiation was usually over 50% (Tables 2 and 3). Thus, 90% of copulations observed during the two experiments ($n = 87$) took place between members of the same clonal group. From 44 copulations where we recorded the site of penis attachment, 16% were in the female's foot opening, and 84% were in the coronal region. These figures are remarkably similar to data reported by Snell & Hoff (1987) for *B. plicatilis* (strain RUS). We observed that males seemed not to discriminate between mictic or amictic females, or between old and young females, since copulations were seen with any kind of female. Although females are noth thought to play an active role on mating (Gilbert, 1963; Snell & Hawkinson, 1983), we often observed that females seemed to interfere with male mating behavior by swimming quikly, shaking suddenly the foot or body, or by withdrawing their foot or corona into the lorica. All these behaviors may cause a courting male to lose contact with the female, and terminate mating behavior. Another previously undescribed pattern we observed was the apparent formation of a thin thread between courting males and females, which may help the male maintain contact with the female.

With respect to Experiment 1, males from most strains displayed a higher percent of mating initiations to females from their own clonal group (that is, within SS, SM or L; Table 2). Thus, 15 out of 16 mating tests between clonal groups showed significantly lower percent mating initiations than the corresponding intra-strain mating test. In contrast, when strains belonging to the same clonal group are considered, only one out of four comparisons between intra-strain and inter-strain was statistically significant. Intra-strain mating per-

Table 2. Comparison of mating among *B. plicatilis* clones. Percent mating initiation (percent of encounters resulting in circling) is given for each pairing; standard error within parenthesis. The associated probability of pairwise comparison (adjusted G-test statistic) between intrastrain and inter-strain responses ('encounters not ending in circling' and 'encounters ending in circling') is also given. $* = P < 0.05$, $** = P < 0.005$.

	Female clones				
Male clones	L1	L2	SM1	SS1	SS2
L1	70.4c	72.2c	40.8*	31.3**	28.7**
	(9.6)	(8.2)	(8.9)	(6.8)	(10.5)
L2	41.5c**	78.0c	34.8**	25.6**	14.4c**
	(6.5)	(4.1)	(10.6)	(5.9)	(5.8)
SM1	8.3**	16.6**	75.8c	81.8c	32.5*
	(5.8)	(5.8)	(11.9)	(5.3)	(7.6)
SS1	23.3**	9.9**	45.8*	88.9c	85.0c
	(9.6)	(4.6)	(10.5)	(8.2)	(8.1)
SS2	14.4**	11.7**	50.6*	66.2	85.3c
	(7.8)	(3.1)	(7.8)	(6.5)	(3.6)

c: observation of at least one copulation during the test.

Table 3. Comparison of mating among *B. plicatilis* clones and *B. quadridentatus*. Numbers given are percent mating initiations (SE in parenthesis). The probability associated to pairwise comparisons (adjusted G-test statistic) between intrastrain and interstrain responses ('encounters not ending in circling' and 'encounters ending in circling') is also given. Significance of G-test statistic is only given when males showed lower preference for females of the other strain than for their own strain females. * = $P < 0.05$, ** = $P < 0.005$.

	Female clones						
Male clones	SM2	SM3	SM4	SS1	SS2	L1	BQ
SM2	52.2c	71.1c	76.5c	79.3	89.2	28.7**	25.4
	(10.6)	(6.0)	(7.8)	(6.0)	(4.9)	(8.5)	(8.7)
SM3	59.9c**	84.7c	63.7c*	67.7c*	75.7	5.9**	44.4**
	(7.1)	(5.4)	(8.8)	(1.2)	(10.0)	(2.5)	(4.5)
SM4	85.1c	79.7c	80.9c	79.6	62.8	23.1**	33.5**
	(6.1)	(5.1)	(4.6)	(3.3)	(14.3)	(4.2)	(2.4)
SS1	33.3**	43.2*	37.4**	65.6	56.8	28.1**	8.6**
	(9.8)	(9.6)	(4.4)	(12.5)	(10.4)	(6.2)	(4.4)
SS2	15.7**	23.9**	30.8**	50.2	71.1c	12.2**	19.1**
	(7.6)	(8.5)	(12.0)	(11.4)	(12.2)	(4.7)	(1.8)
L1	17.3**	44.8*	20.8**	30.3**	17.3**	72.0	22.4**
	(5.1)	(6.8)	(4.0)	(2.2)	(3.7)	(9.2)	(9.4)
BQ	11.8*	12.2*	21.3	7.5*	11.1	3.8**	34.6
	(5.4)	(3.0)	(7.9)	(4.8)	(5.1)	(2.8)	(8.1)

c: observation of at least one copulation during the test.

cents were similar among the five strains and ranged from 70.4 in L1 strain to 88.9 in SS1 strain (adjusted G statistic = 8.84, 4 d.f., n.s.). Homogeneity of intra-strain responses simplifies the analysis of mating results, as effects due to differences in male responsiveness are minimized. SS strain males showed the highest percent mating initiations to SS strain females and copulated only with them; percent mating initiations were significantly lower to SM strains and L strain females. L strain males were also typical in their responses. Both SS and SM females elicited significantly less percent mating initiations, while L females were readily courted and copulations were observed. However, L2 males seemed to discriminate against L1 females, but to a lower extent than against SM and SS strains. SM strain males showed an unexpected behavior. Although females from own strain received a high response, they devoted maximum percent mating initiation and copulations to SS1 females; but SS2 and L females received significantly lower percent mating initiation.

In Experiment 2, intra-strain percent mating initiation ranged from 34.6 in BQ strain to 84.7 in SM3. We found that SM males from the three strains behaved similarly: they copulated with SM females from any pond and percent mating initiations were also high (Table 3). Only SM3 strain seemed to discriminate its own strain from the others with respect to percent mating initiations but not to copulations. However, as in Experiment 1, SM males displayed high percent mating initiations in response to SS females, although only 2 copulations were recorded between SM males and SS females, suggesting that some mechanism preventing copulation might exist. On the other hand, L females received significantly fewer percent mating initiations from SM males and no copulations were observed. Surprisingly, BQ females (a *B. quadridentatus* strain) elicited higher percent mating initiations from SM males than L female did. The SS and L males behaved very similarly as in Experiment 1, with maximum percent mating initiation to related strains. Low percent mating initiations were devoted to BQ females. Finally, although BQ males were not very active, they displayed greater preference for females from their own strain. BQ males devoted intermediate responses to SS and SM females and the lowest to L

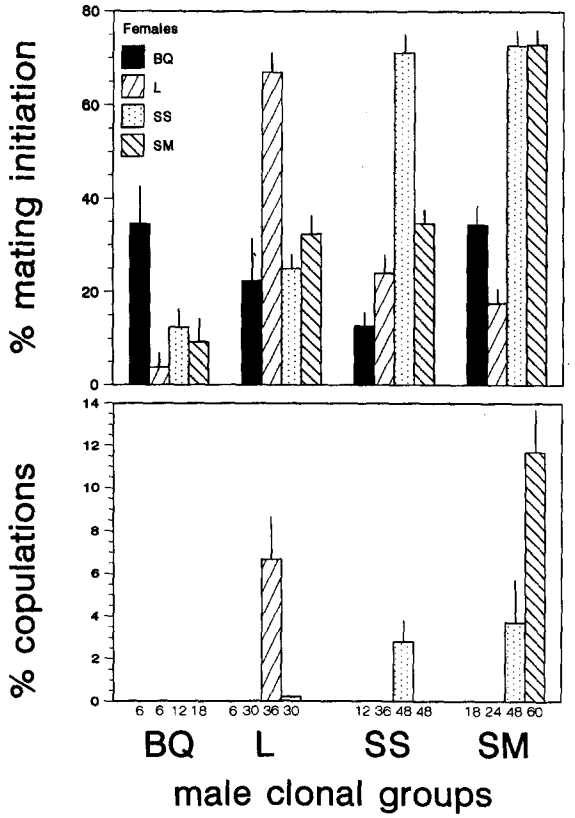

Fig. 2. Mating behavior of clonal groups of *Brachionus plicatilis* and *B. quadridentatus* in Experiments 1 and 2. Upper panel shows the average male percent mating initiations (circling to total encounters) from each clonal group and BQ towards each kind of female. Bottom panel shows average copulations per encounter in each kind of cross. Vertical lines indicate SE. Sample size (males) for each estimate is shown on X-axis.

females. In some homogamic tests we did not record any copulation (BQ, L1, and SS1, see Table 3) what is not surprising if we take into account that copulation rates were low in these strains (Fig. 2, bottom panel).

As Experiment 1 was carried out in March 1993 and Experiment 2 in December 1993, the experimental conditions could be somewhat different. Thus, to assess the consistency of our results, nine cross-mating tests involving three strains (SS1, SS2, and L1) were replicated, as they were common to Experiments 1 and 2. Independence G-tests revealed no statistically significant differences between male responses in 8 out of 9 repeated crosses.

Results of Experiment 1 and 2 are summarized on Fig. 2. Mean percent mating initiations of males, and

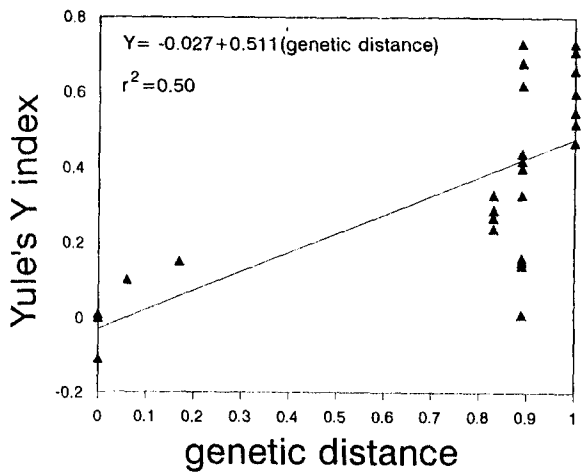

Fig. 3. Plot showing the relationship between Yule's Y index (mating isolation) and Prevosti genetic distance.

percent copulations per encounter of each clonal group (L, SS and SM) and BQ directed towards each kind of female are shown. The general pattern of a higher response to females of their own clonal group (both percent mating initiations and copulations) is broken only by the high percent mating initiation of SM males to SS females. Nevertheless, SM males showed higher copulation rates toward their own females than to SS females. Hence, the main explanatory variable in the pattern of mating and copulation shown by these strains seems to be the clonal group (i.e. genetic composition).

The relationship between Yule's Y isolation index and Prevosti genetic distance for the strains used in both experiments is shown in Fig. 3. Yule's Y estimates emphasize the behavioral reproductive isolation between clonal groups. Correlation between Prevosti genetic distance and Yule's Y isolation index was quite high (Pearson's correlation coefficient = 0.70, $n = 28$; $P < 0.001$).

Discussion

Not surprisingly, cross-mating test results were almost completely consistent to those yielded by the previous allozyme study and support the view that behavioral isolation is an important mechanism for rotifer speciation and population structuring (Snell, 1989). The genotypes studied here belong to the previous-

ly described 'S' and 'L' morphotypes. Thus, SS and SM clonal groups can be recognized as 'S' forms, and L clonal group is a 'L' form (*sensu* Fu *et al.*, 1991a). Recently, in a study made with seven 'S' and 'L' strains, no resting eggs were found when cross-breeding 'S' and 'L' strains, while most within–S or within–L crosses were successful (Fu *et al.*, 1993). These authors suggest further that 'S' and 'L' forms should be classified into two different species. Moreover, there is evidence indicating differentiation of the mate recognition pheromone between 'S' and 'L' morphotypes (Rico–Martínez & Snell, 1995). However, in both studies the authors reported some anomalies in the expected reproductive behaviors. Our study, stresses the differences between 'S' and 'L' morphotypes through the inclusion of the congeneric *B. quadridentatus* strain in Experiment 2. Both SS and SM strains were more or at least equally distant from BQ than from L strains. As *B. quadridentatus* is undoubtedly a different species from what has been called *B. plicatilis*, our data indicate that L strains from Torreblanca Marsh are as isolated from SS and SM strains as from a congeneric species.

On the other hand, our results, along with those of Gómez *et al.* (in press) show that biologically relevant diversity within *B. plicatilis* is still ignored in the 'S' and 'L' classification, which was also suggested by Carmona *et al.* (1989). Gómez and coworkers found two genetically different clonal groups (SS and SM) having 'S' morphology, that according to the present results, show behavioral reproductive isolation, although to a lower extent than to L group. These differences among 'S' strains could be useful in explaining the anomalies found in cross-mating experiments (Fu *et al.*, 1993; Rico–Martínez & Snell, 1995). Hence, we propose that three good species are currently included into the taxon *B. plicatilis*. Additional tests, involving allopatric strains from different clonal groups, and experiments on postzygotic reproductive isolation will help to confirm the species status of each of the clonal groups found in Torreblanca Marsh.

From our results and other data presented at the VII International Rotifer Symposium, *Brachionus plicatilis* was split into two species (Segers, 1995). L morphotypes will keep the older name *Brachionus plicatilis* Müller, 1786, and S morphotypes should be called *B. rotundiformis* Tschugunoff, 1921.

The evidence presented up to now should be used to revise the taxonomic status of *B. plicatilis* according to recent views on species concepts. Thus, Van Valen (1976) and, with a more inclusive view, Templeton (1989) have stressed the importance of ecological similarity (what Templeton calls demographic exchangeability) in maintaining cohesion among populations from the same species, which would be outstanding in populations with low levels of bisexual reproduction. This cohesion mechanism seems to be relevant in maintaining sharp limits among genetic groups within *B. plicatilis*, as evidence for ecological specialization has been found between clonal groups in Torreblanca Marsh (Gómez *et al.*, in press). On the other hand, and related to Mayr's Biological Species Concept (1970), Paterson (1985) claims that a species must be defined in terms of the cohesion promoted by common fertilization devices, not in terms of an always relational concept of 'isolation from other reproductive groups'. Characterization of the mate recognition pheromone in rotifers (reviewed in Snell & Morris, 1993) makes this idea particularly workable in rotifers, and may be useful to recognize species within the taxon *B. plicatilis*. Moreover, the features of clonal groups belonging to *B. plicatilis* may give support to Templeton's ideas. Thus, in cyclical parthenogens, genetic drift and selection working during the parthenogenetic growth phase would play a role as a cohesive force maintaining genetic similarity within groups of populations having demographic exchangeability, while reproductive isolation would be an important cohesive mechanism during the bisexual phase.

Some methodological considerations are also relevant. Snell & Hawkinson (1983) did not report on the occurrence of copulations in their cross-mating experiments. In our experiments, copulations, which give undoubtedly a more conclusive index of genetic cohesion and percent mating initiation followed similar patterns of occurrence within groups. This, of course, is not a rule, as mating tests results show that low percent mating initiation can be found in strains where copulations are frequent (in some crosses SM–SM), and, on the contrary, a high percent mating initiations is not associated necessarily to greater copulation probabilities (e.g., in SM–SS crosses). In summary, by the ease of its performance, mating tests, as developed by Snell & Hawkinson (1983) are a good procedure to get a first impression about the structure of rotifer populations, but, by its importance, actual copulation should be reported, if observed, in any study of behavioral reproductive isolation.

The temporal and spatial distribution of clonal groups is relevant to understanding what selective pressures could have shaped the mating behavior of clonal groups. Thus, SS and L clonal groups, on one hand,

and SM and L groups on the other, for which overlapping in bisexual periods was observed, showed strong behavioral isolation. In fact, male tendency to mate with females of the same clonal group may be a sufficient condition to explain why hybrids between these clonal groups were not found in Poza Sur. In contrast, spatial and temporal distribution, and sexual periods of SS and SM clonal groups seems to be more segregated, as laboratory and field observations suggest that both groups differ in their ecological requirements. Consequently, habitat or seasonal reproductive isolation already present might account for the lower levels of behavioral isolation between SS and SM. These features are predicted by the hypothesis of the evolution of behavioral isolation as a way to avoid hybridization in sympatric populations (Butlin, 1989; Endler, 1989), and not merely as a by-product of genetic divergence in allopatry. This hypothesis also predicts relatively low positive correlation between genetic distance and prezygotic isolation, as other causal factors – taking place in historical periods of sympatry – such as reinforcement or reproductive character displacement would be working on prezygotic isolation (Butlin, 1989). According to the correlation found (0.70), Prevosti genetic distance predicts nearly 50% of the variation in Yule's index, thus, at least part of the remaining variance might be explained through processes as those mentioned taking place in sympatry. As a tentative conclusion, in accordance to the results reported by Snell & Hawkinson (1983), we propose that the level of behavioral isolation found between clonal groups is not only a consequence of the genetic distance between groups, but also that overlapping of sexual periods strenghten behavioral reproductive isolation as a way to reduce hybridization.

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