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Brachionus calyciflorus is a species complex: Mating behavior and genetic differentiation among four geographically isolated strains

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Abstract

Four geographic strains of *B. calyciflorus* are investigated regarding their genetic similarity and ability to cross-mate. DNA sequence analysis of the mitochondrial *cox*1 gene (694 bp) and the nuclear ribosomal ITS region (735 bp) showed that the Florida and Georgia strains were very similar to each other (0.3% sequence divergence for the 1429 bp) and different from the Texas and Australia strains (~7% and 9% sequence divergence for the 1429 bp, respectively). Consistent with this genetic relatedness, cross-copulation occurred only between the Florida and Georgia strains. Thus, *B. calyciflorus* is a complex of cryptic species. While the Florida, Texas and Australia strains were reproductively isolated from one another, most combinations of cross-strain mating tests showed intense and prolonged male circling behavior following male–female encounters. This suggests that precopulatory male circling and copulation are two separate behaviors that may be controlled by different female chemicals and male coronal receptors. In some cross-strain mating tests, females regularly retracted their corona when circled by a male, indicating that they can recognize 'foreign' males and actively interfere with copulation.

Introduction

Recent studies have shown that zooplankton species previously considered to be cosmopolitan can be complexes of sibling species. This has been clearly demonstrated for the copepod *Eurytemora affinis* (Lee, 2000; Lee & Frost, 2002) and for the rotifer *Brachionus plicatilis* (Ciros-Peréz et al., 2001; Gómez et al., 2002). For the *B. plicatilis* complex within the Iberian Peninsula, three species have been described (Ciros-Peréz et al., 2001) and three more have been identified (Gómez et al., 2002). There is no evidence for any hybridization or introgression among these species, despite frequent sympatry (Gómez et al., 2002).

Brachionus calyciflorus exhibits considerable geographic variation in morphology (Kutikova & Fernando, 1995) and may be a complex of sibling species. The present study begins to address this possibility by examining the affinities of strains from Florida, Georgia, Texas and Australia using both mating tests and DNA sequence analysis. Results linking reproductive isolation and genetic distance can then be compared to those obtained for *B. plicatilis* (Gómez et al., 2002) and *E. affinis* (Lee, 2000).

Materials and methods

Cultures

Four strains of *B. calyciflorus*, from Florida, Georgia, Texas and Australia, were used in this study. The Florida strain was derived from a

pond on the University of Florida campus (Gainesville). Resting eggs were purchased from Florida Aqua Farms Inc. (Dade City). The Georgia strain originated from Piedmont Park Pond (Atlanta). Resting eggs were kindly provided by Terry W. Snell. The Texas strain was collected from a temporary pond at the Hueco Tanks State Historic Site (El Paso Co.) by one of us (E.J.W.) and maintained in culture. The Australian strain was obtained from resting eggs in sediment collected by one of us (J.J.G.) in May 1997 from above the water level of a River Murray billabong-Ryan's 2 (Wodonga, Victoria). Clones of the Florida, Georgia and Australian strains were derived from single resting eggs; a clone of the Texas strain was derived from a single amictic female. The clones tested in this study were: FL 23, 40 and 51 from the Florida strain; GA 3 and 5 from the Georgia strain; TX 1 from the Texas strain, and AUS 5 and 20 from the Australian strain. All clones were cultured on *Cryptomonas erosa* var. *reflexa* in MBL medium at 20 °C in a photoperiod (L:D 16:8), as described elsewhere (Gilbert, 2002, 2004). Mictic females were induced in all strains by crowding (Gilbert, 2002, 2003, 2004).

Live females of the North American strains were extremely similar to each other, but morphologically distinct from those of the Australian strain. Australian females were more bladder-like than those of the North American strains, especially as juveniles (Fig. 1). They were also slightly larger; the mean lorica lengths of live, adult amictic females of one Australian and one Florida clone were 220 μm and 168 μm, respectively (Gilbert, 2003). The Australian strain appeared to be at the low end of the size range recorded for the *B. calyciflorus* from that continent (200–400 μm; Koste & Shiel, 1987).

Mating tests

Young males were obtained by isolating single, ovigerous mictic females in depressions of 96-well tissue culture plates with 200 μl of a suspension of *C. erosa*. Young females were obtained by isolating a group of ovigerous amictic females in 15 ml of a suspension of *C. erosa* and then removing their neonate daughters. All males were less than 22 h (usually 8 h) old and probably had not copulated prior to the tests as they were exposed only to their mothers. All females were less than 8 h (usually 4 h) old and had not been exposed to males prior to the tests. Young individuals of both sexes were used to maximize the potential for mating responses (Gilbert 1963; Snell & Childress, 1987; Snell & Hoff, 1987; Gómez & Serra, 1996).

Mating tests were conducted at room temperature (19–23 °C) using a Wild M 5 stereomicroscope. The tests determined the probability that males would 1) initiate circling behavior with encountered females and 2) copulate with females after circling them. For each replicate, a single female was introduced into a depression of a 96 well plate containing 3–8 males produced there by a single mictic mother. Just before introduction of the newborn female, the mictic mother of the males and about 100 μl of medium were removed, leaving the group of males in about 100 μl of medium. Each newborn female and group of males was used only once. Observation of a group of males ceased either after the first copulation event, or after a set number of encounters (usually 4, but sometimes 10). The number of replicates for a test varied from 3 to 19.

An encounter was said to occur when a male physically contacted the body of a female with the center of his corona. Male precopulatory circling behavior was initiated after an encounter if the male turned around and around the female's body while arching his body and touching her with both his corona and penis. Copulation was said to occur following circling behavior if the male attached his penis to the female (in this study always to her corona) and then released coronal contact with her body while remaining attached to her for some time by his penis before swimming away. In addition, notes usually were made on the approximate duration of circling behavior before copulation occurred or the male swam away, and on the tendency of the female to retract her corona while being circled by the male.

Eight mating-test experiments were conducted at different times. As males of the Florida strain mated as intensively with females of the Georgia strain as with those of their own strain, only the Florida strain was tested with the Texas and Australian strains. In each mating test, males from a clone of one strain were paired with females from a clone of a different strain. More than one clone of the Florida and Australian

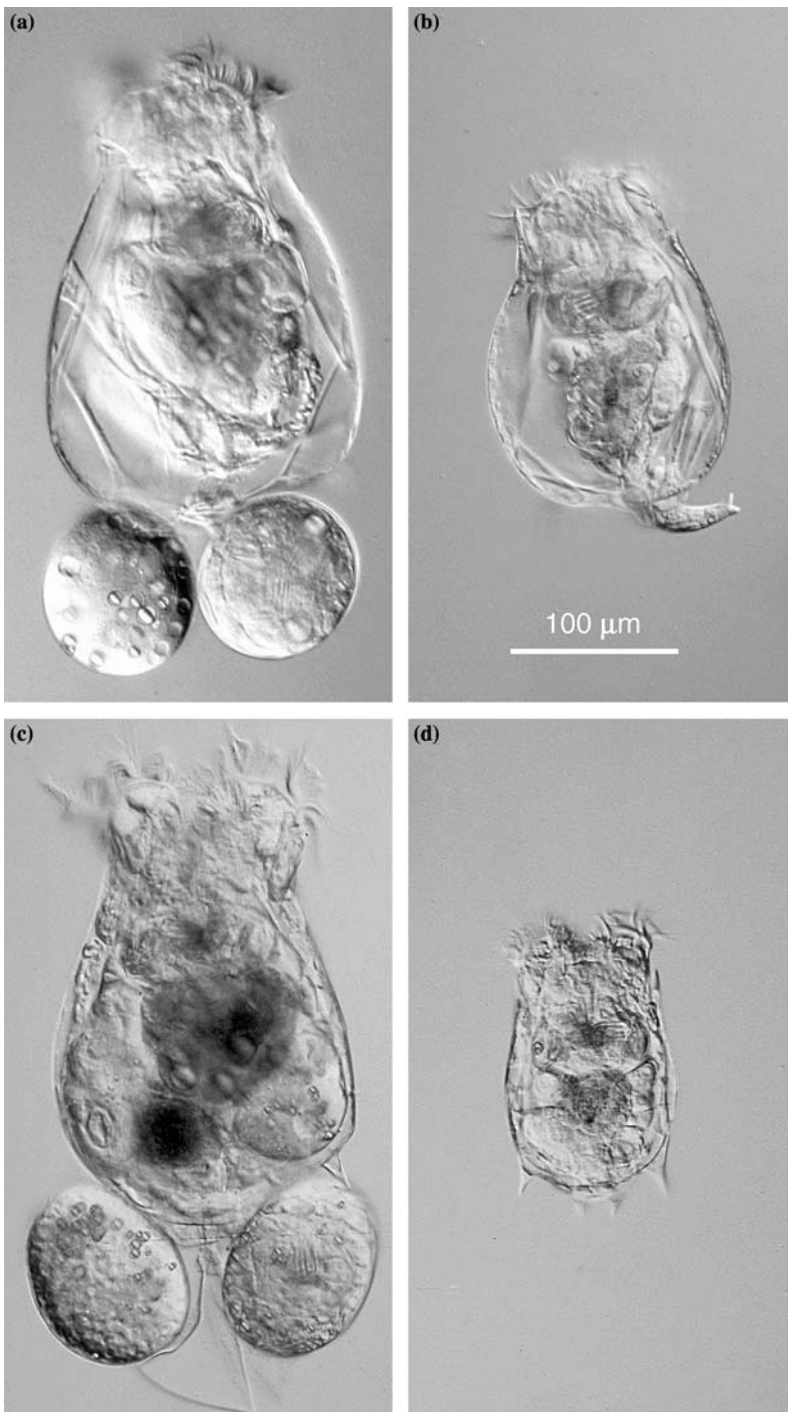

Figure 1. Photographs of live *Brachionus calyciflorus* (Nomarski interference contrast optics and flash illumination) showing difference in shape between females from Australia clone 20 (a, adult; b, juvenile) and Florida clone 40 (c, adult; d, juvenile).

strains were used because cultures of some clones were discontinued. No tests paired different clones within strains. In most experiments, mating behaviors of males with females of a different strain were compared to those with females of the same strain. For these pair-wise comparisons,

probabilities of male circling behavior and copulation were statistically evaluated using Mann–Whitney tests corrected for ties (Zar, 1999).

DNA sequence analysis

DNA was isolated and amplified from axenized fresh or ethanol preserved animals by homogenizing 100 to 150 individuals in 100–150 μl 5% Chelex-100 (BioRad) followed by boiling for 7 min. Primers used to amplify the mitochondrial *cox*1 gene (LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′, HCO2198: 5′-TAAACT TCAGGGTGACCAAAAAATCA-3′; Folmer et al., 1994) and the nuclear ribosomal ITS region (ITS4: 5′-TCCTCCGCTTATTGATATGC-3′, ITS5; 5′-GGAAGTAAAAGTCGTAACAAGG-3′; White et al., 1990) each amplify approximately 800 bp. The ITS primers amplify ITS1, 5.8S, and ITS2 regions of the nuclear ribosomal gene complex. All amplifications included a negative control to detect amplification of contaminating DNA. Amplification products were examined by electrophoresis to verify their size and cleaned with GeneClean kits (Bio101) before sequencing. Amplified products were sequenced directly using SequiTherm kits (EpiCentre Tech) and run on a LI-COR 4200 Series automated sequencer. All gene regions or genes were sequenced at least twice in both directions. Sequences were aligned using Clustal W (Thompson et al., 1994) and then adjusted by eye. Uncorrected Genetic distance (''p'') calculations were done using PAUP*4.0b10 (Swofford, 2002) using UPGMA.

Results

Results of the eight mating experiments are shown in Table 1 and summarized in Table 2. In crosses between the Florida and Georgia strains, male circling behavior was initiated after each encounter, and the males always copulated with the females (Table 1), usually after no more than several seconds. In most between-strain crosses with the Florida, Texas and Australian strains, male circling behavior was observed but never led to copulation (Tables 1 and 2). Despite this complete absence of copulation, male circling behavior in some of the crosses was very strong and often persisted for more than a minute before the male released contact with the female. Such strong circling behavior typically was observed when Florida males encountered Texas or Australian females, and when Texas males encountered Australian females. In all within-strain crosses using the Florida, Texas and Australian strains, the probability of copulation was very high (0.65–1.0; Table 1), and copulation usually occurred shortly after initiation of male circling behavior.

Relatively low probabilities of male circling behavior occurred only when Australian males encountered Florida or Texas females (Table 1). The probability of AUS 5 males circling FL 23 females was 0.49 (vs. 1.0 for AUS 5 females), and the probability of AUS 20 males circling FL 40 females was 0 (vs. 1.0 for AUS females). Similarly, the probability of AUS 20 males circling TX 1 females was only 0.25 (vs. 0.86 for AUS 20 females). In all of these pair-wise comparisons, the difference in probabilities was statistically significant.

Females from the Texas and Australian strains showed a pronounced tendency to retract their corona when circled by Florida males. The same was true when Australian females were circled by Texas males. For example, in Experiment 7 with Texas females and Florida males (Table 1), the Texas females retracted their corona throughout prolonged circling behavior in 14 of the 16 cases, and intermittently during this behavior in the other 2 cases. In contrast, Florida females rarely retracted their corona when circled by Texas or Australian males. Similarly, female coronal retraction was rare when Texas or Australian females were circled by males of their own strain.

A total of 1429 bp were sequenced, aligned and analyzed from each of the strains (Table 3). The *cox*1 region showed slightly higher levels of sequence divergence (mean 11.5%) as compared to the ITS region (mean 5.2%). The Georgia and Florida strains were identical in their *cox*1 sequences and showed only 5 differences in the ITS region. Compared to these two strains, the Texas strain showed 4% and 10% sequence divergence in the ITS and *cox*1 regions, respectively. The Australian strain showed the greatest sequence divergence, ranging from approximately 6% (ITS) to 13% (*cox*1).

Table 1. Results of eight mating-test experiments using strains of *Brachionus calyciflorus* from Florida (FL), Georgia (GA), Texas (TX) and Australia (AUS)

Experimental and statistical test		Strain and clone		Mating test		
		Male	Female	Number of replicates	p (cir)	p (cop)
1		GA 5	FL 40	5	1.00	1.00
		FL 40	GA 3	5	1.00	1.00
2	a	AUS 5	FL 23	8	0.49^{**}	0^{**}
	a		AUS 5	8	1.00	0.94
	b	FL 23	AUS 5	8	0.91^{*}	0^{**}
	b		FL 23	8	1.00	0.85
3	c	AUS 20	FL 40	4	0^{**}	
	c		AUS 20	4	1.00	1.00
		FL 40	AUS 20	4	1.00	0
4			Fl 40	19	0.83	0.65
5	d	TX 1	FL 40	4	0.88^{NS}	0^{*}
	d		TX 1	3	1.00	0.83
6	e	TX 1	FL 51	4	0.75^{NS}	0^{**}
	e		TX 1	4	1.00	0.81
		FL 40	TX 1	4	1.00	0
	f	FL 51	TX 1	4	0.94^{NS}	0**
	f		Fl 51	4	1.00	0.88
			FL 40	4	1.00	1.00
7		TX 1	FL 40	4	0.75	0
		FL 40	TX 1	7	1.00	0
8	g	TX 1	AUS 20	7	0.89^{NS}	0^{**}
	g		TX 1	5	0.90	0.67
	h	AUS 20	TX 1	7	0.25^{**}	0^{**}
	h		AUS 20	7	0.86	1.00

Probabilities (p) are of male circling behavior (cir) after an encounter with a female, and copulation (cop) following circling behavior. Pair-wise comparisons (Mann–Whitney tests) of probabilities within experiments are indicated by letters (a–h); superscripts indicate statistical significance: NS ($p > 0.05$), *($p \leq 0.05$), **($p \leq 0.01$).

Table 2. Summary results for probabilities (p) of male circling behavior (cir) after encounter with a female, and copulation (cop) following circling behavior, in mating-test experiments using strains of *Brachionus calyciflorus* from Florida (FL), Texas (TX) and Australia (AUS)

Male strain	Female strain					
	FL		TX		MAUS	
	p (cir)	p (cop)	p (cir)	p (cop)	p (cir)	p (cop)
FL	0.96 (0.04)	0.85 (0.07)	0.98 (0.02)	0	0.96 (0.05)	0
TX	0.79 (0.04)	0	0.97 (0.03)	0.77 (0.05)	0.89	0
AUS	0.25 (0.25)	0	0.25	0	0.95 (0.05)	0.98 (0.02)

Values are means (±1 SE) when more than one experiment was conducted (see Table 1).

Discussion

Affinities among the four strains of B. calyciflorus

The results of the mating tests showed that the Florida and Georgia strains of *B. calyciflorus* readily cross-copulate (Table 1). The ability of such crosses to result in the production of viable fertilized eggs and hybrids is probable but has not been determined. The DNA analysis demonstrated the similarity of these two strains (Table 3).

In contrast, the Florida, Texas and Australian strains did not cross-copulate and thus were completely reproductively isolated. The distinctiveness of these strains is consistent with their considerable geographic isolation, with the unique morphology of the Australian strain (Figure 1), and with the inability of a high density of females of the Australian strain to induce mictic-female production in the Florida and Georgia strains (Gilbert, 2003). The DNA analysis confirms the genetic distinctiveness of the Florida, Texas and Australian strains. There were from 95 to 132 nucleotide differences between each of these strains in the *cox*1 gene and ITS region (Table 3). Consistent with its geographic separation and distinct morphology, the Australian strain was more genetically different from the Florida and Georgia strains than was the Texas strain.

Using molecular characters, a variety of morphologically described cosmopolitan species have been found to be complexes of cryptic species. These species, although morphologically similar, typically have 5% or greater sequence divergence – the level typically found to occur among related species across a wide variety of taxa (Avise, 1994; Moriyama & Powell, 1996; Avise & Walker, 1999). Within the Family Brachionidae, DNA sequence divergences between species range from 8 to 24% and from 22 to 29% for different genera (Gómez et al., 2002; Derry et al., 2003, Walsh & De La Riva, unpublished). Among the closely related *B. plicatilis*, *B. ibericus*, and *B. rotundiformis*, lineages show >12% sequence divergence (Gómez et al., 2002). Many of the strains in the *B. plicatilis* species complex have recently been designated as separate species (Segers, 1995; Ciros-Pérez et al., 2001). This complex includes some co-occurring strains that are demarcated by morphological differences, sequence divergence and behavioral reproductive isolation.

Table 3. Genetic distance among *B. calyciflorus* strains

Strain	Georgia	Florida	Texas	Australia
A. ITS – 735 bp				
Georgia	–	5	32	46
Florida	0.00685	–	28	42
Texas	0.04391	0.03844	–	42
Australia	0.06371	0.05824	0.05795	–
B. *Cox*1 – 694 bp				
Georgia		0	66	82
Florida	0.00000	–	67	87
Texas	0.10258	0.09654	–	90
Australia	0.12727	0.12536	0.12968	–
C. Combined – 1429 bp				
Georgia	–	5	98	128
Florida	0.00685	–	95	129
Texas	0.04391	0.03844	–	132
Australia	0.06371	0.05824	0.05795	–

ITS (A) and *cox*1 (B) sequence distances are calculated as uncorrected "*p*" below the diagonal, and as absolute number of differences above the diagonal. Combined distances are shown in (C). Genbank accession numbers are: Georgia DQ071668 (ITS), DQ071672 (*cox*1); Florida DQ071669 (ITS), DQ071673 (*cox*1); Texas DQ071670 (ITS), DQ071674 (*cox*1); and Australia DQ071671 (ITS), DQ071675 (*cox*1).

Sequence divergence among the strains of *B. calyciflorus* examined in the present study ranged from 0–13% depending on the region sequenced. Among the three strains reproductively isolated from one another (Florida, Texas, Australia), sequence divergences were about 6% for the ITS region and 9–13% for the *cox*1 region. Similarly, among strains of the *B. plicatilis* species complex, Gómez et al. (2002) reported sequence divergences ranging from 0 to 20%. *Cox*1 sequence divergence of the four *B. calyciflorus* strains was nearly identical to within-clade divergences in this region for the *B. plicatilis* complex (0–12%). Levels of variation in our ITS sequences (0–6.8%) are slightly higher than those reported by Gómez et al. (2002) for *B. plicatilis* complex strains (0–2%), most likely due to our inclusion of the more variable ITS2 region. In addition, using the molecular clock calculations in Gómez et al. (2002), the Australian strain of *B. calyciflorus* has been diverging from the other lineages for at least 3 million years. This is ample time for the evolution of reproductive isolating mechanisms, and is consistent with the reproductive isolation observed.

Mating behavior in Brachionus

When Florida males encountered Texas or Australian females, or when Texas males encountered Australian females, there was a very high probability of intense and prolonged circling behavior which never progressed to copulation (Tables 2 and 3). Absence of copulation in these tests cannot be attributed to the condition of the males or females, because the probability of copulation was very high in control crosses with males and females of the same strain (Tables 1 and 2). An implication of these results is that mating bioassays for determining affinities or genetic relatedness should not rely on male precopulatory circling behavior. Male circling may be a meaningful criterion when the probabilities of this behavior in between-group crosses are lower than those in within-group crosses, but it is clear that high probabilities in between-group crosses do not necessarily mean close affinities.

Gómez & Serra (1995) were the first to warn about using only male circling behavior for affinity testing. They noted that copulations between clonal groups of the *B. plicatilis* complex generally occurred when the probability of initiating circling behavior was high, but that this pattern did not always hold. They observed copulations in some crosses when circling behavior was unlikely, and few copulations in other crosses when the probability of circling was high. Gómez & Serra (1996) found a similarly weak coupling between the probability of male circling and copulation when they examined the effect of female age on male mating behavior. Males initiated circling behavior with old females, although to a lesser extent than with young females, but copulated only with young females.

Prolonged male circling behavior without copulation in some between-strain crosses in *B. calyciflorus* has important implications regarding the control of mating behavior. Clearly, in some cases a strong signal for the initiation of male circling behavior never triggers copulation. Therefore, these two behaviors appear to be separate and may involve the recognition of different female signals by different male receptors. One female signal could initiate male circling behavior after an encounter with a female. A second female signal could induce circling males to copulate.

The signal that induces males to circle females after they contact them is a chemical in the female that is recognized by receptors on the male corona (Gilbert, 1963). This chemical, called a mate-recognition pheromone (MRP), has been isolated in *B. plicatilis* and characterized as a 29 kD glycoprotein concentrated in the female corona (Snell et al., 1988, 1995; Snell, 1998). Recognition of the MRP by a male should depend on the chemical structure of the MRP and the male contact receptors. The signal initiating copulation in males that are circling females could be a different female chemical, or pheromone, detected by male coronal receptors. Thus, in the between-strain crosses where males initiate circling behavior but never copulate, male receptors for copulation may not recognize the copulation pheromone.

Observations of mating behavior in the present study indicate that females circled by males can distinguish males of different strains and actively respond to strains other than their own by retracting their corona. Female coronal

retraction was particularly pronounced when Texas or Australian females were circled by Florida males, and when Australian females were circled by Texas males. Florida, Texas and Australian females rarely retracted their corona when circled by males of their own strain. Also, Florida females rarely retracted their corona when circled by Texas males. The mechanism by which these females can distinguish males of different strains is not known. Females may recognize some male substance via receptors. Alternatively, or in addition, they may retract their corona in response to prolonged male circling. Female coronal retraction during male circling behavior was first noted in *B. plicatilis* by Gómez & Serra (1995).

Absence of copulation between Florida males and Texas or Australian females, and between Texas males and Australian females, cannot be attributed to female coronal retraction. Reciprocal crosses showed that copulation never occurred even when females had a fully extended corona, as in tests with Texas or Australian males with Florida females. Also, Florida males failed to copulate with Australian females when these females were narcotized with carbonated water and unable to retract their corona (Gilbert, unpublished). Finally, female coronal retraction does not necessarily prevent copulation. In some within-strain crosses with the Florida strain, copulation frequently occurred despite female coronal retraction (Gilbert, unpublished). However, retraction of the female corona in many of these cases did interfere with the ability of a male to attach his penis to the female corona and appeared to prevent copulation.

Conclusions

The DNA sequence analysis and mating tests in the present study show that *B. calyciflorus* is a species complex in which some geographically and genetically distinct strains are reproductively isolated from one another. This species complex is similar to one described in more detail for *B. plicatilis* (Gómez et al., 2002). It also is similar to the species complex in the copepod *Eurytemora affinis* (Lee, 2000). However, it is interesting that the mechanism of reproductive isolation is behavioral in *Brachionus* and postzygotic in the copepod. Copepod populations having *cox*1 sequence divergences ranging from 0.15 to 17.1% cross-mated but showed hybrid breakdown in the *F1* or *F2* generations (Lee, 2000).

Observations of mating behavior in the present study indicate that the signals and responses leading to copulation in rotifers may be more complex than previously suspected. Male circling behavior and copulation appear to be two separate behaviors and may involve the recognition of separate female chemicals by different male receptors. Furthermore, females may play an active role in mating. They can recognize males of different strains, and can interfere with copulation attempts by retracting their corona.

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