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Specificity of the crowding response in the *Brachionus plicatilis* species complex

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Abstract

Crowding chemicals influence a wide variety of life history traits in zooplankton communities. In the rotifer *Brachionus*, sexual reproduction (mixis) is induced by a chemical signal produced by the rotifers that accumulates during population growth. The specificity of the reaction to the mixis induction signal could play a central role in maintaining reproductive barriers between closely related sympatric species. Using cross-induction assays between different species, we tested whether this signal has diversified in the *Brachionus plicatilis* species complex. We found that closely related, as well as more distant species in this complex, could induce mixis in each other. This suggests that there are no species barriers in sex induction and that the mixis signal did not diversify for several million years during the evolution of the *Brachionus plicatilis* complex. This apparent stasis is remarkable because pre- and postmating isolation is common in this species complex and, due to its cosmopolitan distribution, species often occur in sympatry.

Chemical signals play a key role in intra- and interspecies communication of aquatic animals. Crowding chemicals, for example, influence a variety of life history traits, such as feeding, growth, age at first reproduction, or the induction of bisexual reproduction, and are thus important determinants of population dynamics (Kirk 1998; Yoshinaga et al. 1999; Mitchell and Carvalho 2002). Previous studies provided mixed evidence with regard to the specificity of such signals. Some studies with zooplankton found that crowding chemicals operate across species (Hobak and Larsson 1990; Carmona et al. 1993), whereas others demonstrated species specificity (Gilbert 1963, 2003). Intuitively, one would expect specificity to evolve when heterospecific signals interfere with important life history processes, i.e., when responding to a heterospecific cue would reduce fitness by triggering an inappropriate physiological response. In this case, selection would favor divergence of crowding signals during speciation. Accordingly, distantly related species should be less responsive to heterospecific crowding signals than to those of closely related species. The objective of this study was to examine this prediction in an explicitly phylogenetic context for representatives of the *Brachionus plicatilis* species complex.

Crowding chemicals that induce bisexual reproduction are known in cyclical parthenogens, such as cladocerans or rotifers. Cyclical parthenogens may reproduce for generations by female parthenogenesis and only occasionally switch to sexual reproduction (Stross and Hill 1965; Wallace and Snell 2001). Chemicals that trigger the induction of sex and regulate its timing can thus be important contributors to reproductive isolation. Inducing sexual reproduction clearly implies a cost for cyclical parthenogens, because they sacrifice rapid asexual population growth for the production of males and resting eggs (Serra and King 1999). Therefore, sex should only be induced when there are enough conspecifics present so that successful fertilization is likely (Gilbert 2003; Serra et al. 2005).

In the genus *Brachionus*, mixis (i.e., sexual reproduction) is induced by a chemical that accumulates during crowding (Gilbert 1963; Carmona et al. 1993; Stelzer and Snell 2003). Recent work has shown that this is a protein produced by the rotifers themselves (Snell et al. unpubl. data). The association of sexual reproduction with high population densities is probably related to the fact that *Brachionus* males cannot locate females from a distance and rely solely on chance encounters (Snell and Garman 1986). The mixis signal triggers parthenogenetic (amictic) females to produce sexual (mictic) daughters, which then produce either resting eggs or males, depending on whether they have been fertilized or not. Specificity of the mixis signal was examined by Gilbert (2003), who compared mixis induction between two American strains and one Australian strain of the freshwater rotifer *Brachionus calyciflorus*. He found that the Australian strain did not induce mixis in the American strains, which suggests that the mixis-inducing signal is differentiated between these strains. However, the comparison was done only in one direction (Australian inducing American strains) and the phylogenetic relationship between the two strains remained unclear. The strains from the two locations were morphologically different, showed mating isolation, and hence are likely distinct species. In this study, we examined interspecific divergence of the mixis signal in the *Brachion-*

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us plicatilis species complex, an ancient species complex of several million years age for which detailed phylogenetic information is available (Gomez et al. 2002; Derry et al. 2003).

The *Brachionus plicatilis* species complex is a well-studied example of cryptic speciation (Serra et al. 1998; Ciros-Perez et al. 2001; Gomez et al. 2002). *B. plicatilis* was initially described as one species on the basis of morphology, but allozyme variation, karyotyping, and experiments on mate choice soon suggested a division into two species (Segers 1995). Further studies revealed that the biological diversity is even greater (Gomez and Serra 1995; Ortells et al. 2000). At least eight lineages were recognized in the *B. plicatilis* complex, all of which could be ascribed the species status according to variation in CO1 and internal transcribed spacer 1 (ITS1) sequences (Gomez et al. 2002), and there are likely more (Suatoni et al. pers. comm.). This is supported by numerous studies that documented premating and postmating isolation between different strains of the *B. plicatilis* complex (e.g., Fu et al. 1993; Gomez and Snell 1996; Ortells et al. 2000), as well as the absence of hybridization in the field (Gomez et al. 2000; Ortells et al. 2000).

In this study, we performed bidirectional comparisons of mixis induction between various clones representing different species in the *B. plicatilis* complex. We presented amictic females from one clone with medium conditioned by females of a different clone and measured the level of mixis induction (percent sexual daughters). We expected reduced mixis induction between species and that this effect would be strongest in the most evolutionarily distant lineages in the *B. plicatilis* complex.

Materials and methods

We used five clones of *B. plicatilis*, designated RUS, AUS, L1, IR2, and HAW. All these clones were available in our lab and had been used in earlier studies (Rico-Martinez and Snell 1995; Gomez and Snell 1996; Snell and Stelzer in press). The clones designated RUS, AUS, and L1 were originally collected from the Azov Sea (Russia), Obere Halbjockchlacke (Austria), and Torreblanca marsh (Spain), respectively, and maintained in the lab for many years as resting eggs. The clones designated IR2 and HAW were collected at Indian Rocks Beach, Florida, and Hawaii, respectively. Resting eggs were hatched and single females were isolated and cloned to produce the lineages that we used for our experiments. All clones had been cultured asexually for several months before the experiments; thus, maternal effects that occur only few generations after hatching can be neglected (Hagiwara and Hino 1989; Gilbert 2002). Rotifers were cultured in artificial seawater (Instant Ocean) with salinity of 18 and enriched with F/2 nutrients (Guillard and Ryther 1962). They were fed the green algae *Tetraselmis suecica*. The standard experimental temperature was 25°C.

To determine the phylogenetic association of our rotifer clones, we sequenced the ribosomal ITS1 and compared it with published sequences of an existing phylogeny of the *B. plicatilis* complex (Gomez et al. 2002). To obtain genomic DNA, 1-liter mass cultures were grown to densities of ~50 rotifers mL^{-1}. Rotifers were starved for 24 h by exchanging the culture medium with algae-free medium 2–3 times, and concentrated with 50-μm mesh sieves. DNA was extracted from ~50 μL concentrated biomass using the DNeasy extraction kit (QIAGEN). We amplified the complete ITS1 using the primers III (5′-CACACCGCCCGTCGCTACTAC CGATTG-3′ and VII (5′-GTGCGTTCGAAGTGTCGA TGATCAA-3′) from Palumbi (1996). Polymerase chain reactions (PCRs) were performed with 50 ng template, 2.5 μL 10× Titanium Taq PCR Buffer, 0.5 μL 50× Titanium Taq Polymerase (Clontech Inc.), 5 pmol of each primer, 5 nmol of each Nucleotide, and water to a total volume of 25 μL. Reactions were amplified using the following cycle conditions: one cycle of 1 min denaturing at 95°C, 25 cycles of 30 s at 95°C, 20 s at 50°C, 1 min at 70°C, and one cycle of 3 min extension at 72°C. Both strands were PCR sequenced at the sequencing facility of the School of Biology (Georgia Institute of Technology, Atlanta).

To provide amictic females for the experiments, 8–10 females of the different clones were precultured individually in 25 mL medium in polystyrene Petri dishes at food concentrations of 500–1,000 *Tetraselmis* cells μL^{-1} with daily transfers into fresh medium. Under these conditions, *B. plicatilis* reproduces exclusively asexually (Stelzer and Snell 2003). After culturing the animals for at least three generations under these conditions, juvenile offspring (0–8 h) were collected from different mothers and randomly distributed among the experimental treatments (conditioned medium from different *B. plicatilis* strains).

Conditioned medium was prepared fresh each day during the experiment from exponentially growing 10-liter mass cultures of the different rotifer clones. Five days prior to the first harvest, these mass cultures had been inoculated with 400–500 females. Rotifer densities at the days of harvest were at least five individuals per mL, but usually ranged between 10 and 100 individuals per mL. Mixis in the mass cultures during the days of harvest ranged between 20% and 95%. To prepare conditioned medium, about 1 liter of medium was taken from each culture and rotifers were removed with a 50-μm sieve. After 45 min of centrifugation at 12,000 × g at 20°C, the supernatant was carefully decanted and filtered through 0.2-μm nylon membranes. Finally, *Tetraselmis* algae were added from dense cultures to a concentration of 500–1,000 cells μL^{-1}. We tested mixis induction between different clones in two experimental trials. In the first trial, we used the clones AUS, IR2, and HAW and tested all possible combinations of test clone versus conditioned medium. Similarly, in a second trial, we tested all possible combinations between the clones RUS, AUS, and L1. In each trial, negative controls for each clone were established by using fresh F/2 medium instead of conditioned medium.

For each treatment, eight experimental animals were individually exposed to 25 mL conditioned medium for 2 d. On the second day, they were transferred into conditioned medium that was freshly prepared from the same mass culture. During this 2-d period, each female usually produced about four to eight daughters. Those were removed daily and isolated individually into wells of 24-well polystyrene plates, each filled with 1 mL of F/2 medium containing *Tetraselmis* at a density of about 500 cells μL^{-1}. Two days later, their

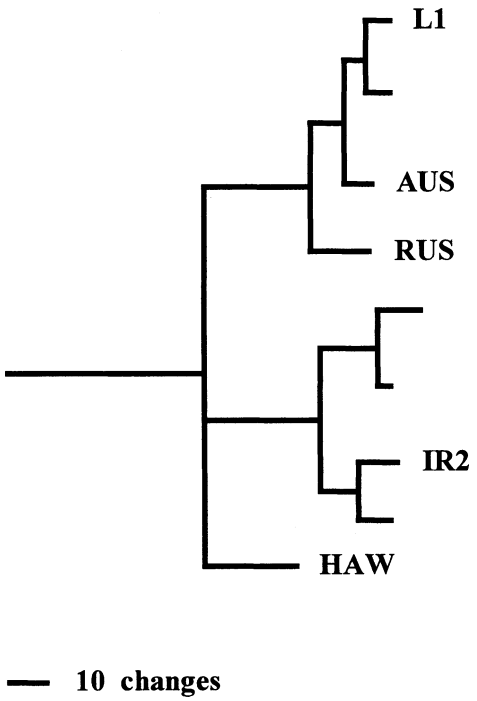

Fig. 1. Clones used in this study and their phylogenetic relationship within the *Brachionus plicatilis* species complex. Phylogenetic tree drawn from the ITS1 maximum-parsimony tree of Gomez et al. (2002). Gomez et al. (2002) used the same primers as described in our Materials and Methods and amplified the complete ITS1 region. Phylogenetic analysis was implemented with PAUP*4.0b4a (Swofford 1998) after multiple alignments with CLUSTAL X. Gaps were treated as fifth base. For more details on the procedure, see Gomez et al. (2002). Outgroups and nodes with bootstrap support <85% are not shown.

reproductive type was determined by the offspring that they produced. Daughters were classified as either mictic, if they produced males, or amictic, if they produced females.

We used the nonparametric Kruskal–Wallis test, corrected for ties, to compare the percentages of mictic daughters in the different treatments for each focal clone. In cases where the Kruskal–Wallis test yielded a significant value, pairwise comparisons were done with the Conover–Inman test to identify homogeneous groups of treatments (Software: StatsDirect). Homogeneous groups were defined as the treatments that were not significantly different among each other, but differed significantly from other treatments ($p < 0.05$).

In a second experiment, conducted at the University of Muenster, we used the RUS clone as the focal animals and exposed them to conditioned water from (1) their own clone, (2) the AUS clone, (3) the brine shrimp *Artemia salina.* The *Artemia* culture consisted of 4–6-d-old nauplii and had a population density of 3.2 individuals per mL. *Artemia* was grown under the same conditions as the rotifers (temperature, food algae, medium), and conditioned medium was prepared in the same way. As a negative control, standard F/2 medium was used. Experimental animals were exposed to conditioned water for 4 d instead of only 2 d in the Atlanta experiments (with daily transfers to freshly prepared conditioned medium). This prolonged exposure lowered the threshold for mixis induction and increased the overall sensitivity of our mixis assay (C.P.S. pers. obs.). For logistic reasons, some conditions were slightly different from the Atlanta experiments: Conditioned water was prepared by filtration through GF/C glass-fiber filters (Whatman), and *Nannochloropsis oculata* was used as food algae. None of these changes had any substantial effects on mixis in *Brachionus* (C.P.S. pers. obs.).

Table 1. Mixis induction among clones of the *Brachionus plicatilis* complex. Percent mixis in offspring of the experimental animals (mean and SE). NEG = untreated culture medium.

Induced clone	Conditioned water			
	NEG	AUS	IR2	HAW
AUS	0 (0)	30.2 (8.3)	28.6 (7.5)	42.5 (15.5)
IR2	0 (0)	25.2 (8.9)	51.5 (14.2)	35.7 (6.5)
HAW	0 (0)	17.2 (5.6)	33.4 (13.4)	22.1 (3.9)
	NEG	RUS	AUS	L1
RUS	3.6 (3.4)	33.4 (7.6)	21.7 (9.5)	8.3 (4.2)
AUS	0 (0)	51.5 (11.2)	64.0 (6.1)	37.0 (6.9)
	NEG	RUS	AUS	ARTEMIA
RUS	5.9 (3.1)	43.0 (5.7)	42.6 (4.6)	9.3 (2.5)

Results

According to their ITS1 sequences, our clones could be unequivocally assigned into positions of an existing phylogeny of the *B. plicatilis* complex (Gomez et al. 2002). Figure 1 shows a sketch of this phylogeny and the positions of our five clones. The clones AUS, IR2, and HAW represent the three main branches in the phylogenetic tree described by Gomez et al. (2002), and thus resemble the three species that are currently discriminated on the basis of fine morphology (Ciros-Perez et al. 2001). The clones L1, AUS, and RUS represent three different species within one main branch (Fig. 1). Gomez et al. (2002) labeled these lineages as *B. plicatilis* sensu strictu, Austria, and Manjavacas, respectively. Three of our sequences were identical to already published sequences: AUS (GenBank Accession Number AF387208), RUS (AF387218), and IR2 (AF387222), whereas two sequences had high similarity to published sequences: L1 (99% identical with AF387201), and HAW (97% identical with AF387240). Our sequences for L1 and HAW have been deposited in GenBank under the accession numbers DQ004843 and DQ004844.

For the five different clones, RUS, AUS, L1, IR2, and HAW, we tested whether conditioned medium from one clone would induce mixis in others. In all cases, we observed significantly higher mixis induction than the negative control (Tables 1, 2). There were no differences in the strength of mixis induction among conditioned media from different clones, as they usually formed homogeneous groups after pairwise comparisons (Table 2). Hence, there was no trend that medium conditioned by clonemates would induce more mixis than conditioned medium from a different clone. The only exception to this pattern was observed in the RUS

Table 2. Statistical analysis of mixis induction in clones of the *Brachionus plicatilis* complex. Results of the Kruskal–Wallis test and the post hoc Conover–Inman test on mixis induction of conditioned media from different clones (AUS, IR2, HAW, RUS, L1) and the untreated culture medium (NEG). Groups of conditioned media that did not differ significantly ($p > 0.05$) from each other are clustered in brackets.

Induced clone	Kruskal–Wallis test		Groups after Conover–Inman test
	H	*p*	Conditioned media
AUS	9.29	0.0257	(NEG), (AUS, IR2, HAW)
IR2	13.61	0.0035	(NEG), (AUS, IR2, HAW)
HAW	11.43	0.0096	(NEG), (AUS, IR2, HAW)
RUS	10.81	0.0128	(NEG, L1), (AUS, RUS)
AUS	19.08	0.0003	(NEG), (L1), (RUS, AUS)
RUS	40.38	<0.0001	(NEG, ARTEMIA), (AUS, RUS)

clone, which did not respond with significant mixis induction when treated with conditioned medium from the L1 clone. In the AUS clone, conditioned medium from the L1 clone significantly induced mixis, although to a lesser extent than medium from the RUS or AUS clones (Tables 1, 2). Unfortunately, the L1 clone inexplicably performed poorly during our experiments. L1 individuals as well as mass cultures showed high mortality and slow growth rates. In some experiments, only a few experimental animals survived, so we did not analyze mixis induction for all comparisons of the L1 clone.

To examine whether the mixis factor could be a general metabolic waste product, we tested the effect of seawater conditioned by *Artemia salina* on mixis induction in the RUS clone. In this experiment, our sample size was much higher than in the previous experiments ($n = 22$ vs. $n = 8$), as we wanted to ensure that we could also detect very weak effects. Despite these efforts, we did not detect a significant effect of *Artemia*-conditioned medium on mixis induction in *Brachionus* (Table 2, bottom row), although conditioned water from the RUS and AUS clones had highly significant effects (Kruskal–Wallis test, $T = 41.37$, $p < 0.0001$; Conover–Inman test: NEG vs. ARTEMIA, $p = 0.3633$). In this experiment, there were also no significant differences in the strength of mixis induction with conditioned medium from the reference clone (RUS) versus conditioned medium from a different clone (AUS).

Discussion

Our results clearly demonstrate that there is little differentiation in mixis induction signals among the tested clones. In most cases, mixis in the focal clones could be induced by conditioned medium from any other clone. There were also no notable differences in the levels of mixis arising from treatments with conditioned medium from clonemates versus different clones. According to their ITS1 sequences, our clones represent five different species of the eight putative species of the *B. plicatilis* complex (Gomez et al. 2002). Therefore, this study provides evidence for the lack of differentiation in the mixis induction signal in the *B. plicatilis* complex. Moreover, it suggests that there has been no divergence of the mixis signal for more than approximately 10 million years, which is the estimated time when the *B. plicatilis* complex presumably started to diverge (Gomez et al. 2002).

Observing a lack of differentiation in mixis induction in the *B. plicatilis* species complex led us to test whether a phylogenetically very distant animal like the brine shrimp *Artemia* might also be able to induce mixis in rotifers. We tested this by exposing *B. plicatilis* to medium conditioned by the crustacean *Artemia salina*, which occurs also in saline water bodies. We found no effect of *Artemia*-conditioned medium on mixis induction in our RUS clone. Mixis levels were not significantly higher than in unconditioned medium. This result contradicts an earlier study by Carmona et al. (1993), who found that *Artemia*-conditioned water significantly increased mixis rates in *B. plicatilis*. However, the experimental design of Carmona et al. (1993) did not rule out a potentially confounding factor. They measured mixis rates in populations, not in individually cultured females, as we did. Given the high *Brachionus* densities in their *Artemia* treatment, it is quite likely that rotifer crowding, not *Artemia* chemicals, induced mixis.

Previous studies demonstrated that there is mating isolation among the rotifer clones used in our study. Gomez and Snell (1996) found that L1 males displayed significantly more homogamic copulations when they could choose between their own females and RUS or AUS females. Rico-Martinez and Snell (1995) tested male mating behavior in AUS, RUS, and HAW. They found that males displayed significantly more homogamic than heterogamic mating attempts (i.e., circling around the female's body) in comparisons of AUS versus RUS and AUS versus HAW clones (Rico-Martinez and Snell 1995). The IR2 clone has been tested in the study of Snell and Stelzer (in press), where it was shown that RUS males do not copulate with females of the IR2 clone. Likewise, IR2 males discriminate strongly against AUS and HAW females in both copulations and circling behavior (C.P.S. unpubl. data). Although mating barriers seem well developed in the *B. plicatilis* species complex lineages that have been investigated, we found little evidence of similar differentiation in mixis induction signals.

In the freshwater rotifer *B. calyciflorus*, it has been shown that the mixis signal differs among strains (Gilbert 2003). As these strains originated from two distant locations, Australia and America, it suggests that the mixis signal diverged in allopatry. Scenarios for sympatric divergence of the mixis signal seem equally plausible. Many species of the *B. plicatilis* complex occur in sympatry, although they usually show mating isolation (Ortells et al. 2000, 2003). Ortells et al. (2003) followed the population dynamics of five coexisting *B. plicatilis* species and found evidence for temporal separation, but also provided many examples of extensive seasonal overlap. These periods of overlap may be the conditions that favor divergence of the mixis signal. Usually one species is highly abundant (at a density above that inducing mixis) while another is just establishing a population. The lower abundant species would be at a disadvantage if it responded to the mixis signal of the other species because it would curtail its own asexual population growth prematurely

by inducing sexual females and be unable to find enough mating partners (Serra et al. 2005). A field study of Carmona et al. (1995) provides an example that the sexual periods of two coexisting *B. plicatilis* species can indeed overlap. However, in this study, both species were highly abundant at the time of mixis induction, so it seems unlikely that one species was cross-induced by the other.

Due to its high dispersal capacity, it is conceivable that episodes of local sympatry occurred often during the evolution of the *B. plicatilis* complex. Resting eggs are resistant to desiccation and are readily dispersed by wind or waterfowl. Hence, *Brachionus* species usually have a very widespread distribution. For example, our IR2 clone originated from Florida and its ITS1 sequence was identical to the CALIFORNIA1 clone of Gomez et al. (2002). Both belong to the Almenara group, of which some mitochondrial haplotypes were found in both North America and Spain. Similarly, the lineage Austria (to which our AUS clone belongs) was found in Europe, America, and Asia.

There are reasons to believe that selection for divergence of the mixis signal might be weak in some *Brachionus* populations. Gilbert (2002) described a transgenerational effect on mixis induction in a strain of *B. calyciflorus*: up to several generations after hatching from resting eggs, females were relatively insensitive to the mixis stimulus. Although Gilbert provided stimuli large enough to induce mixis, these females continued to reproduce asexually, and only after several generations responded to the mixis stimulus. This transgenerational effect has been demonstrated in other rotifer species as well (Schroder and Gilbert 2004), although it has yet to be tested in *B. plicatilis.* Nevertheless, if delayed mixis exists in *B. plicatilis,* it could provide a mechanism to prevent a low-abundance species having just emerged from resting eggs from being induced by a highly abundant co-occurring species. Delayed mixis, therefore, could reduce selection pressure for adaptive divergence of the mixis signal.

In conclusion, the signal for mixis induction seems to have remained unchanged for many million years of evolution in the *B. plicatilis* species complex. This is unexpected because it is likely that there are costs associated with the response to heterospecific signals and because divergence in the mixis signal has been demonstrated in the freshwater congener *B. calyciflorus* (Gilbert 2003). This illustrates that there is still much to be learned about the role of these genes in rotifer speciation. The recent discovery that the mixis signal in *B. plicatilis* is a protein (Snell et al. unpubl.) provides the opportunity to study divergence in mixis induction at the molecular level. Knowledge of the molecular structure of the mixis signal will allow a better understanding of evolutionary forces shaping its function and diversity. A better understanding could also be gained by studying mixis induction in a broader phylogenetic context, such as among different species representing the whole genus *Brachionus.*

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