

Female mate recognition and sexual isolation depending on courtship song in *Drosophila sechellia* and its siblings

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In *Drosophila sechellia*, females accept males that sing heterospecific songs less than those that do not sing, whereas in *D. melanogaster* and *D. simulans*, females accept males that sing heterospecific song more than those that do not sing. Here we studied the sexual isolation of *D. sechellia* and its siblings using interspecific hybrids to reveal the mechanisms underlying female mate recognition. The females of hybrids mated more with winged males of the parent species than with wingless ones, suggesting that the discrimination against heterospecific songs by *D. sechellia* females is recessive. Female preference for courtship songs seems to be inherited additively or semidominantly. In addition, we examined female receptivity without the stimuli of courtship songs by comparing the mating frequencies between the crosses using wingless males and found that it is also inherited additively or semidominantly.

Key words: courtship songs, *Drosophila*, female choice, interspecific hybrids, sexual isolation

INTRODUCTION

Female mate recognition plays an important role in the sexual isolation of *Drosophila*. During courtship, both sexes exchange information using several modalities, such as visual, olfactory, and acoustic modalities (Ewing 1983). Courtship in *Drosophila* is one of the best subjects to study the genetic basis of behavior. However, the genetic basis of female mate recognition responsible for sexual isolation is not yet clarified (e.g., Tan 1946, Zouros 1981, Kyriacou and Hall 1982, Coyne 1996, Noor 1997).

The *D. melanogaster* complex consists of four closely related species: *D. melanogaster* and *D. simulans*, which are distributed worldwide, and *D. sechellia* and *D. mauritiana*, which are found in the islands of Seychelles and Mauritius, respectively (Lachaise et al. 1988). *Drosophila sechellia* and *D. mauritiana* diverged allopatrically and they share an ancestor with cosmopolitan *D. simulans* (Lachaise et al. 1988). *Drosophila sechellia* has a specialized food preference for *Morinda citrifolia*, although the other species are generalists (Lachaise et al. 1988, R'Kha et al. 1991, Higa and Fuyama 1993). The courtship behavior of the four species of the *D. melano-*

gaster complex has been well described (Spieth 1952, Manning 1959, Cobb et al. 1985, 1986, 1988, 1989). The courtship song emitted by male wing vibration (Shorey 1962) is one of the most significant signals in mating and sexual isolation in the four species of the *D. melanogaster* complex (Bennet-Clark and Ewing 1969, Kyriacou and Hall 1982, Ritchie et al. 1999). The mean interpulse interval of courtship song of *D. sechellia* is 85 ms, which is the longest among the four species of the *D. melanogaster* complex (Cobb et al. 1989). *Drosophila melanogaster*, *D. simulans* and *D. mauritiana* also have species-specific interpulse intervals of 30–35 ms, 50–55 ms and 35–50 ms, respectively (Ewing and Bennet-Clark 1968, Kyriacou and Hall 1980, Cowling and Burnet 1981, Cobb et al. 1989).

In *D. melanogaster*, *D. simulans* and *D. mauritiana*, females mate with heterospecific males in the song-present-condition more than in the no-song-condition, whereas the females of *D. sechellia* mate with *D. melanogaster* or *D. simulans* males in the no-song-condition more than in the song-present-condition (Tomaru et al. 2000, Tomaru and Oguma 2000). It seems that female mate recognition depending on courtship songs in *D. sechellia* is different from that in *D. melanogaster*, *D. simulans* and *D. mauritiana*, and the discrimination against heterospecific songs in *D. sechellia* may have appeared only in this lineage during the history of the speciation of

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the *D. melanogaster* complex. Here, we studied the sexual isolation of *D. sechellia* and its siblings using interspecific hybrids to reveal the mechanisms underlying female mate recognition depending on courtship songs. Our primary interest is whether hybrid females reject *D. melanogaster* or *D. simulans* males that sing. If this is true, the discrimination against heterospecific songs by *D. sechellia* is dominant, as reported in *D. ananassae* (Doi et al. 2001). If this is not true, we should examine carefully whether or not it is recessive to its sibling species.

MATERIALS AND METHODS

Drosophila Stocks *Drosophila sechellia* SS78, *D. melanogaster* Canton-S and *D. simulans* Ogasawara were used (Tomaru et al. 2000, Tomaru and Oguma 2000). Each strain was derived from one wild-caught female. Flies were grown on glucose-yeast-cornmeal-agar medium at $24 \pm 1^\circ\text{C}$ in a 14:10 h light:dark regime (lights on 7:00 – 21:00). To produce interspecific hybrid females, virgin *D. melanogaster* females and *D. sechellia* males were crossed (*melanogaster-sechellia* hybrids). These hybrids were grown at $22 \pm 1^\circ\text{C}$ during the larval and pupal stages to prevent temperature-sensitive pupal death, as in the hybrids of *D. melanogaster* and *D. simulans* (Sturtevant 1929). Hybrids between *D. simulans* females and *D. sechellia* males were also produced (*simulans-sechellia* hybrids); these hybrids were not subjected to a low culture temperature.

Crossability Tests Virgin females and males were collected without anesthesia within 10 h of eclosion. They were maintained separately in groups of 10 in a vial for 4 to 5 days, at $24 \pm 1^\circ\text{C}$, until use. Two days before the experiments, the wings of male flies were cut with micro-scissors under carbon dioxide anesthesia for 3 min. About half the flies had their wings removed; the rest were used as winged male controls. All the males were subjected to 3 min of anesthesia, whereas none of the female flies was subjected to anesthesia.

From 9:00 – 14:00, 10 females and 10 wingless or winged males were introduced into a vial (30 mm diameter \times 105 mm high) with food. After 0.5, 2, 5 or 96 h, we anesthetized the flies and removed the males. The females were dissected within 8 h and examined for sperm in their spermathecae and ventral receptacle. We repeated each cross five or more times. In some crosses, the differences in mating frequency between wingless males and winged males could be detected in a short duration, but in other crosses a longer duration was required (Tomaru et al. 2000). Thus on the basis of the preliminary crossability tests, we selected an appropriate duration for each crossability test.

If the mating frequencies of the wingless males were identical among the females, we could identify the effects

of songs as the differences between the mating frequencies of the winged males. However, when the mating frequencies of the wingless males were different among the crosses, it is better to compare the mating frequencies of winged males considering those of wingless males. On the basis of the mating frequencies of winged and wingless males, we calculated a song effect index (Yamada et al. 2002), that is, (mating frequency of winged males – mating frequency of wingless males) / (mating frequency of wingless males). A positive index shows that the females accepted the winged males more than the wingless ones, whereas a negative index shows that the females rejected the winged males more than the wingless ones.

Statistical Tests A chi-square test with Cochran correction (Zar 1984) was applied to detect the differences in the mating frequency between the crosses of winged and wingless males. To examine the mating frequencies across the species and hybrids, we made pairwise comparisons using a chi-square test with Cochran correction or a Fisher exact probability test by the sequential Bonferroni technique (Rice 1989) with $k = 3$.

RESULTS

Effects of courtship songs Females of *D. sechellia* mated with heterospecific winged males less than with wingless ones (Tables 1-2 and 1-3). In the other crosses, females of pure species and hybrids mated with winged males more than with wingless ones, but this was not significant in the intraspecific crosses of *D. melanogaster* (Table 1-4) and the 2-h crosses of *D. simulans* females and *D. sechellia* males (Table 1-7).

Since hybrid females did not reject *D. melanogaster* or *D. simulans* males that sing courtship songs (Tables 1-10 and 1-12), it seems less likely that the discrimination against heterospecific songs by *D. sechellia* females is dominant. However, we cannot conclude that the trait is recessive without carefully examining the female mate recognition. We first compared the effects of *D. sechellia* courtship songs across the females of pure species. Next we examined the female preference for *D. sechellia* songs across the females of different species and hybrids with the help of song effect indices. Then we examined the receptivity of the females across all the species and hybrids.

Females of pure species and males of *D. sechellia*

The indices of the effects of *D. sechellia* songs (Table 1) were the highest in *D. sechellia* females (∞), the second highest in *D. melanogaster* females (1.53) and the lowest in *D. simulans* females (0.55). In the crosses with winged *D. sechellia* males (Table 2-1), the mating frequency of *D. sechellia* was the highest, that of *D. melan-*

Table 1. Effects of courtship song on mating frequency for females of pure species and hybrids

Female	Male	Mating frequency (%)	N	Crossing time (h)	χ^2_c	Song effect index
1. <i>D. sechellia</i>	Wingless <i>D. sechellia</i>	0.0	80	2	103.918 ***	∞
	Winged <i>D. sechellia</i>	78.8	80			
2. <i>D. sechellia</i>	Wingless <i>D. melanogaster</i>	22.9	70	96	15.065 ***	-0.94
	Winged <i>D. melanogaster</i>	1.4	70			
3. <i>D. sechellia</i>	Wingless <i>D. simulans</i>	8.3	120	96	10.435 **	-1
	Winged <i>D. simulans</i>	0.0	120			
4. <i>D. melanogaster</i>	Wingless <i>D. melanogaster</i>	64.0	50	0.5	2.380	0.22
	Winged <i>D. melanogaster</i>	78.0	50			
5. <i>D. melanogaster</i>	Wingless <i>D. sechellia</i>	25.0	80	2	22.970 ***	1.53
	Winged <i>D. sechellia</i>	63.3	79			
6. <i>D. simulans</i>	Wingless <i>D. simulans</i>	53.2	79	0.5	4.513 *	0.31
	Winged <i>D. simulans</i>	69.6	79			
7. <i>D. simulans</i>	Wingless <i>D. sechellia</i>	14.3	140	2	2.900	0.55
	Winged <i>D. sechellia</i>	22.1	140			
8. <i>D. simulans</i>	Wingless <i>D. sechellia</i>	22.0	100	5	10.051 **	0.95
	Winged <i>D. sechellia</i>	43.0	100			
9. <i>mel-sec</i>	Wingless <i>D. sechellia</i>	4.3	69	2	6.702 **	3.65
	Winged <i>D. sechellia</i>	20.0	70			
10. <i>mel-sec</i>	Wingless <i>D. melanogaster</i>	1.7	60	2	11.644 ***	11.76
	Winged <i>D. melanogaster</i>	21.7	60			
11. <i>sim-sec</i>	Wingless <i>D. sechellia</i>	10.0	60	2	49.811 ***	6.46
	Winged <i>D. sechellia</i>	74.6	59			
12. <i>sim-sec</i>	Wingless <i>D. simulans</i>	28.3	60	2	45.245 ***	2.16
	Winged <i>D. simulans</i>	89.7	58			

mel-sec: hybrids from crosses between *D. melanogaster* females and *D. sechellia* males.

sim-sec: hybrids from crosses between *D. simulans* females and *D. sechellia* males.

χ^2_c : chi-square value with Cochran correction.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ogaster the second highest, and that of *D. simulans* the lowest. In the crosses with wingless *D. sechellia* males (Table 2-2), the mating frequencies of the *D. melanogaster* and *D. simulans* females were significantly higher than that of *D. sechellia* females.

Preference for *D. sechellia* songs To examine the preference for *D. sechellia* songs, the comparison of the mating frequencies of winged *D. sechellia* males across the females of all the species (Tables 2-3 and 2-5) seems not appropriate, since mating frequencies significantly differed across the females in the crosses using wingless *D. sechellia* males. Therefore using the song effect indices from the 2-h crosses, we compared the effects of *D. sechellia* songs across the females.

In the crosses of the females of *D. sechellia*, *D. melanogaster* and their hybrids, the indices of the effects of *D. sechellia* songs (Table 1) were the highest in the *D. sech-*

ellia females (∞), the second highest in the hybrid females (3.65) and the lowest in the *D. melanogaster* females (1.53), suggesting that *D. sechellia* songs affect *D. sechellia* females more to accept a courting male, *D. melanogaster* females less and hybrids intermediately. In the crosses of the females of *D. sechellia*, *D. simulans* and their hybrids, the indices of effects of *D. sechellia* song (Table 1) were the highest in *D. sechellia* females (∞), the second highest in hybrid females (6.46) and the lowest in *D. simulans* females (0.55), suggesting that *D. sechellia* songs affect more *D. sechellia* females, less *D. simulans* females and intermediately hybrids. Therefore, the preference for *D. sechellia* songs in hybrid females is intermediate between their parent species.

Female receptivity without song stimuli In the crosses using wingless males, it can be assumed that mating frequency is not affected by courtship songs. To

Table 2. Comparison of mating frequencies across females in crosses with *D. sechellia* males

Male	Female (Mating frequency (%))			χ^2_c for pairwise comparisons		
	<i>sec</i>	<i>mel</i>	<i>sim</i>	<i>sec</i> vs. <i>mel</i>	<i>sec</i> vs. <i>sim</i>	<i>sec</i> vs. <i>sim</i>
Crosses with females of pure species						
1. Winged <i>D. sechellia</i>	78.8	63.3	22.1	4.404 *	65.199 *	35.704 *
2. Wingless <i>D. sechellia</i>	0.0	25.0	14.3	22.857 *	11.646 *	3.301
Comparison between females of <i>D. sechellia</i> , <i>D. melanogaster</i> and their hybrids and males of <i>D. sechellia</i>						
	<i>sec</i>	<i>mel-sec</i>	<i>mel</i>	<i>sec</i> vs. <i>mel</i>	<i>mel-sec</i> vs. <i>sec</i>	<i>mel-sec</i> vs. <i>mel</i>
3. Winged <i>D. sechellia</i>	78.8	20.0	63.3	4.404 *	49.562 *	28.149 *
4. Wingless <i>D. sechellia</i>	0.0	4.3	25.0	22.857 *	1.687	11.631 *
Comparison between females of <i>D. sechellia</i> , <i>D. simulans</i> and their hybrids and males of <i>D. sechellia</i>						
	<i>sec</i>	<i>sim-sec</i>	<i>sim</i>	<i>sec</i> vs. <i>sim</i>	<i>sim-sec</i> vs. <i>sec</i>	<i>sim-sec</i> vs. <i>sim</i>
5. Winged <i>D. sechellia</i>	78.8	74.6	22.1	65.199 *	0.166	47.421 *
6. Wingless <i>D. sechellia</i>	0.0	10.0	14.3	11.646 *	P = 0.0053 [†]	0.473

The mating frequencies are from Table 1.

sec: *D. sechellia*, *mel*: *D. melanogaster*, *sim*: *D. simulans*.

mel-sec: hybrids from crosses between *D. melanogaster* females and *D. sechellia* males.

sim-sec: hybrids from crosses between *D. simulans* females and *D. sechellia* males.

χ^2_c : chi-square value with Cochran correction.

[†]Fisher exact probability.

A chi-square value (or Fisher exact probability) with an asterisk is significant at the 0.05 level after sequential Bonferroni correction ($k = 3$).

examine female receptivity without the song stimuli, we compared mating frequency across the females of the crosses with wingless *D. sechellia* males. In the crosses of the females of *D. sechellia*, *D. melanogaster* and their hybrid, the mating frequency of the hybrid females was intermediate between those of *D. sechellia* and *D. melanogaster* females (Table 2-4), but those of the hybrid and *D. sechellia* did not significantly differ. In the crosses of the females of *D. sechellia*, *D. simulans* and their hybrid, the mating frequency of *D. sechellia* females was the lowest; the hybrid females had an intermediate mating frequency between those of their parent species (Table 2-6), although it was not significantly different from that of *D. simulans* females.

The comparison across the females of the crosses with wingless *D. melanogaster* or *D. simulans* males will provide useful information, although the crossing times were different among the crosses. The wingless *D. melanogaster* males mated more with the *D. melanogaster* females (64.0%, 0.5-h cross, Table 1-4) but less with *melanogaster-sechellia* hybrid (1.7%, 2-h cross, Table 1-10) and *D. sechellia* females (22.9%, 96-h cross, Table 1-2). In the crosses with wingless *D. simulans* males, the mating frequency of *D. sechellia* females was the lowest (8.3%, 96-h cross, Table 1-3), that of the *simulans-sechellia* hybrid females the second (28.3%, 2-h cross, Table 1-12) and *D. simulans* females the highest (53.2%, 0.5-h cross, Table 1-6). It seems that the *D. melanogaster* or

D. simulans females accept the wingless males more than the *D. sechellia* females.

DISCUSSION

The *D. sechellia* females mated with the winged *D. sechellia* males more than with the wingless ones, whereas they mated with the winged heterospecific (*D. melanogaster* and *D. simulans*) males less than with the wingless ones (Table 1). In contrast, the *D. melanogaster* and *D. simulans* females mated with the winged males more than with the wingless ones, even if the males were heterospecific *D. sechellia*. The *melanogaster-sechellia* hybrid and *simulans-sechellia* hybrid females also mated with the winged males of their parent species more than with the wingless ones. It seems that the discrimination against heterospecific songs in *D. sechellia* is recessive in *D. melanogaster* and *D. simulans*, or it is maternally inherited. This is in contrast to the case in which the discrimination against heterospecific songs in *D. ananassae* is dominant in *D. pallidosa* (Doi et al. 2001).

If the rejection of the songs of *D. melanogaster* and *D. simulans* is observed in hybrids, we can conclude that the discrimination against heterospecific songs in *D. sechellia* is dominant. In such a case, the hybrid female judges that the song is heterospecific and she discriminates against the "heterospecific" song; then she decides to

reject the courting male. However, if the rejection is not observed in hybrids, as in our present study, we should carefully examine female mate recognition before we can conclude that the discrimination against heterospecific songs in *D. sechellia* is recessive. If the preference for the *D. sechellia* song is recessive to *D. melanogaster* and *D. simulans* song, the hybrid female judges that the song is conspecific, and then she can copulate well with the winged males of their parent species. The preference function of hybrids is intermediate between those of the parent species if it is additively inherited. *Drosophila melanogaster* and *D. simulans* females mate well with a male whose song parameters are species-specific values (about 34 ms of interpulse interval and 55 s of its fluctuation for *D. melanogaster* and about 55 ms of interpulse interval and 35 s of its fluctuation for *D. simulans*) and they prefer less long or short inter-pulse intervals (Bennet-Clark and Ewing 1969, Kyriacou and Hall 1982). In *D. melanogaster* females, an artificially synthesized courtship song of the *D. sechellia* type enhances mating more than no sound, but less than the *D. melanogaster* type (Ritchie et al. 1999). The indices of *D. sechellia* song effects in the hybrid females were intermediate between those of the parent species (Table 1). In addition, in the crosses of *D. melanogaster* or *D. simulans* males, the song effect indices in the hybrid females were relatively high (11.76 for *melanogaster-sechellia* hybrids and 2.16 for *simulans-sechellia* hybrids, respectively) and comparable to the indices of *D. sechellia* song effects in hybrids (3.65 for *melanogaster-sechellia* hybrids and 6.46 for *simulans-sechellia* hybrids, respectively). Therefore, the female preference for songs in the hybrids seems intermediate between those of the parent species.

Another factor affecting female mate recognition is basic receptivity that is not affected by courtship songs. Assuming that the mating frequency of the cross between females and wingless males indicates the receptivity of the females who did not receive courtship song stimuli, the *D. melanogaster* and *D. simulans* females have higher receptivity than the *D. sechellia* females (Table 2-2). The mating frequencies of the crosses between the females of the hybrids and wingless males were intermediate between those of the parent species (Tables 2-4 and 2-6), suggesting that the hybrids have an intermediate receptivity between those of the parent species. It is therefore likely that basic receptivity is inherited additively or semidominantly.

Although it is possible that wingless *D. sechellia* males court heterospecific females less than conspecific females, the mating frequencies of wingless *D. sechellia* males were higher in the cross with heterospecific females than in that with conspecific females (Table 2-2). During the crossability tests, we often observed that *D. sechellia* males actively courted heterospecific females. Thus the *D. sechellia* males court heterospecific females well. The

wing vibration of males produces sound (Shorey 1962), however, it may also act as a visual signal. The sound receptors of *Drosophila* are arista, feather-like branched bristles extending from the third segment of the antennae. An arista rotates to transmit sound vibration to Johnston's organ in the second segment (Manning 1967, Burnet et al. 1971, Göpfer and Robert 2001). We showed that the decrease of the mating frequency of the wingless males are due to the absence of sound as determined using females whose arista were removed (Tomaru et al. 2000). The female responses of the strains used in this study were found to be species-specific using several strains of these species (Tomaru et al. 2000, Tomaru and Oguma 2000). Since the duration of confinement is longer in some interspecific crosses than in conspecific crosses, the present study may involve many uncontrollable factors affecting female mate recognition. By comparing mating frequency between the winged and wingless males for each experiment between the species and the hybrids, we can control the effects that pheromones and other behavioral factors may play in mate recognition.

We previously proposed that the discrimination against heterospecific songs may be brought about by the reinforcement of reproductive isolation in the *D. auraria* complex (Tomaru et al. 1995). Reinforcement and/or reproductive character displacement is one of the possible driving forces shaping the mate preference function, during the sexual isolation of *D. sechellia* was established. One of the other possible scenarios is that the sexual isolation of *D. sechellia* may have been brought about as a by-product of sexual selection, as proposed in the case of *D. simulans* and *D. mauritiana* (Cobb et al. 1988).

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