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ARTICLE

Sexual isolation between *Drosophila simulans* and *D. mauritiana*: *D. simulans* females do not discriminate against intact-wing *D. mauritiana* males

Masatoshi Tomaru[1] · **Emi Hattori**[2] · **Hirokazu Yamada**[2] · **Yuzuru Oguma**[2]

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Abstract

Female mate choice plays an important role in sexual isolation. The present study examined sexual isolation using *D. simulans* and *D. mauritiana* in conditions where females had no opportunity to compare males versus where females were able to choose males. The sound produced by wing vibration in males (courtship song) affects female receptivity in *Drosophila*. Females of both species copulated with intact conspecific males more than intact heterospecific males and wingless conspecific and heterospecific males. *Drosophila mauritiana* females copulated only with intact conspecific males within 30-min observations period without comparing other males, suggesting that absolute criteria are used for decision-making to accept courting males. Females of *D. simulans* copulated with intact *D. mauritiana* males as well as wingless *D. simulans* or *D. mauritiana* males in no-choice conditions. In a choice situation, *D. simulans* females copulated with intact *D. mauritiana* males as well as wingless *D. mauritiana* males when the females were courted by both types of males, suggesting that *D. simulans* females accept intact *D. mauritiana* males as if they are mute. Females of *D. simulans* copulated with intact *D. simulans* males as well as intact *D. mauritiana* males when they were courted by males of either type, whereas they copulated with intact *D. simulans* males more than intact *D. mauritiana* males in true choice situations. These results suggest that females make a comparative review of courting males before accepting a male and that conspecific song is a factor in criteria affecting female selectivity.

Keywords Context dependence · *Drosophila simulans* · *Drosophila mauritiana* · Female mate choice · Courtship song · Sexual isolation

Introduction

Sexual selection and sexual isolation are one of the most important mechanisms in the process of species formation, not only in sympatry but also in allopatry (Coyne and Orr 2004; Ritchie 2007). During courtship, both sexes exchange signals and examine whether to accept the individual of the other sex as her/his mating partner. Males, females, or both sexes may choose mates (Cotton et al. 2006). Thus, the context of courtship is an important factor affecting mate choice. Although mate choice by males plays a role (Edward and Chapman 2011; Roberts and Mendelson 2017), the greater importance of female mate choice is clear in the evolution of mating systems and sexual isolation (Coyne and Orr 2004; Ritchie 2007; Moehring and Boughman 2019). Asymmetry in sexual isolation has been reported between closely related species in different taxa (Kaneshiro 1976, 1980; Watanabe and Kawanishi 1979; Ryan and Wagner 1987; Hoikkala and Kaneshiro 1993; Arnold et al. 1996; Deering and Scriber 2002; Coyne et al. 2005; Svensson et al. 2007; Goetze and Kiørboe 2008; Tinghitella and Zuk 2009; Wyman et al. 2011, 2014, 2016). Males of the derived species are not accepted by females of the ancestral species due to loss of some courtship elements in the derived species after splitting

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✉ Masatoshi Tomaru
tomaru@kit.jp

[1] Department of Drosophila Genomics and Genetic Resources, Kyoto Institute of Technology, Saga-Ippongi-cho, Ukyo-ku, Kyoto 616-8354, Japan

[2] Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

from the ancestral species (Kaneshiro 1976, 1980; Hoikkala and Kaneshiro 1993; Tinghitella and Zuk 2009), although this pattern cannot be generalised (Wasserman and Koepfer 1980; Arnold et al. 1996). A completely opposite result was also reported (Watanabe and Kawanishi 1979) where females of the derived species do not mate with males of the ancestral one.

Asymmetry is found in sexual isolation between *Drosophila simulans* and *D. mauritiana*; the copulation frequency of *D. mauritiana* females, the derived species, and the ancestral *D. simulans* males is lower than that of the reciprocal cross (Watanabe and Kawanishi 1979). *Drosophila simulans* is widely distributed in temperate and tropical regions, whereas *D. mauritiana* is distributed on the Mauritius Islands. *Drosophila mauritiana* was also collected on Rodriguez Island, where the species may have been introduced by human activity. No *D. simulans* is found on the islands on which *D. mauritiana* occurs (Lachaise et al. 1988; Ashburner et al. 2005). Therefore, the distributions of these two species are allopatric. They are generalist, domestic species, and ecologically similar to cosmopolitan *D. melanogaster* (Lachaise et al. 1988). *Drosophila simulans* tends to be found further from human habitation than *D. melanogaster* and not to be found in environments rich in ethanol (Ashburner et al. 2005). They are sibling species along with *D. sechellia*. Females of interspecific hybrids are fertile, whereas hybrid males are sterile (Lachaise et al. 1986; Ashburner et al. 2005). The clade of these three species is the sister species of *D. melanogaster* (Lachaise et al. 1986, 1988; Ashburner et al. 2005). Courtship behaviours among these species are very similar (Robertson 1983; Cobb et al. 1988, 1989). A male orients himself toward a female, taps her body, and vibrates his wing(s) to generate a courtship song. When she moves, he follows her with or without vibrating his wings. Then, he licks her genitalia and mounts her to attempt to copulate. Females are thought to assess males during courtship. When a female avoids male courtship, she moves away, kicks him, or flutters her wings. When she accepts him, copulation is observed; genital coupling lasts 10–20 min and sperm are transferred from the male to the female during copulation. In *Drosophila*, female cuticular hydrocarbons play roles as sex pheromones eliciting male courtship (Jallon 1984; Cobb and Jallon 1990) and courtship song, a sound produced by wing vibration by males, is a signal affecting female receptivity (Shorey 1962; Bennet-Clark and Ewing 1969; Tomaru and Yamada 2011). A species-specific male courtship signal is a courtship song with a species-specific interpulse interval: 55 ms in *D. simulans*, 45 ms in *D. mauritiana*, 80 ms in *D. sechellia*, and 34 ms in *D. melanogaster* (Shorey 1962; Bennet-Clark and Ewing 1969; Cowling and Burnet 1981; Robertson 1983; Cobb et al. 1989). Artificial song playback experiments revealed that females prefer songs with conspecific parameters in *D. melanogaster* and *D. simulans* (Bennet-Clark and Ewing 1969; Kyriacou and Hall 1982; Ritchie et al. 1999). Although the genetic basis of female acceptance has been studied between *D. simulans* and *D. mauritiana* (Coyne 1989, 1992; Moehring et al. 2004), its relation to song recognition is still unclear.

In the crosses between *D. simulans* and *D. mauritiana*, males from both species court females belonging to any of the species (Cobb et al. 1988; Tomaru et al. 2000). This is because the major cuticular hydrocarbons of females are identical between these species (Cobb and Jallon 1990). Females of *D. mauritiana* copulated with conspecific intact males, but did not with wingless ones (who cannot produce the courtship song) or intact-wing or wingless *D. simulans* males (Robertson 1983; Tomaru et al. 2000). In the reciprocal cross, *D. simulans* females copulated with heterospecific intact-wing males as well as wingless ones. In contrast, we have shown previously that females of some *Drosophila* species discriminate against intact heterospecific males more than wingless conspecific males, suggesting that heterospecific songs can be a species discriminator. (Tomaru et al. 1995, 1998, 2000, 2004; Tomaru and Oguma 2000; Doi et al. 2001). Previously reported studies, however, examined song effects only in no-choice conditions where a female has no opportunity to choose a different type of male. As several studies have shown, it is important to examine whether or not a chooser has the opportunity to directly compare mating partners can affect their mating preferences. Coyne et al. (2005) examined sexual isolation between *D. santomea* and *D. yakuba* in four types of choice situation (no-choice, female-choice, male-choice, and multiple-choice) and concluded that interspecific copulation was observed less when flies could choose partners between conspecific and heterospecific, compared to the no-choice situation, suggesting discrimination by females. Although they pointed out that discrimination must be examined by observing courtship behaviour, they observed interspecific courtship only in a no-choice situation, not in a choice situation. Hoikkala and Aspi (1993) compared female responses in different conditions when females had an opportunity to choose a courting conspecific male (one female was courted by two males) or no opportunity (one female was courted by one male), and they concluded that females choose normal males much more than wing-clipped ones using both absolute and relative criteria, compared with when only an absolute criterion is used in species of the *D. virilis* group. Cobb et al. (1988) studied the courtship of *D. simulans* females and males of *D. simulans*, *D. mauritiana*, and their hybrids in a choice situation. However, they analysed the data where a female was courted by two or more males together with those by one male. In conditions with a choice of males, it is expected that females will prefer males with conspecific rather than heterospecific songs. Such a discrimination against heterospecific males may be revealed in the situation where a female was courted by both an intact heterospecific male and a wingless male.

The present study aimed to clarify sexual isolation in female-choice conditions using *D. simulans* and *D. mauritiana*, and also examine the effects of courtship song by removing the wings of males. A *D. simulans* female was confined with two males from four male types: intact or wing-removed *D. simulans* or *D. mauritiana* males. We focused on the context of the courtship: the order of courtship and the presence of a second male on courtship and copulation success in relation to the characteristics of males (species and wing condition). We observed courtship in crosses between a single female and two males in six choice conditions: two conspecifics, one conspecific and one heterospecific, or two heterospecifics, with intact or removed wings.

Materials and methods

Drosophila stocks

Drosophila simulans Ogasawara and *D. mauritiana* G35 were used (Tomaru et al. 2000). Each stock was derived from a single wild-caught female and has been kept in the laboratory for more than 10 years. They were grown on glucose–yeast–cornmeal–agar medium at 23–25 °C with a 14:10 h light:dark regime (lights on 7:00–21:00).

Virgin females and males were collected without anaesthesia within 10 h after eclosion. They were maintained separately in groups of 5–10 flies in a vial for 4–5 days until use for the mating test. All observations were made in the first half (9:00–14:00) of the light period in a room constantly regulated at 23–25 °C and relative humidity of 50–70%.

No-choice tests: song effects on females without opportunity to compare with another male

Two or three days before a test, we removed the wings of male flies with microscissors under carbon dioxide anaesthesia for 3 min. Approximately half the flies had their wings removed; the rest were used as intact-wing male controls. All males experienced 3 min of anaesthesia, but females were un-exposed. On the next day (4- or 5- day-old males and females), a pair of a female and a wingless or intact male was introduced into a glass mating chamber (15 mm in diameter and 3 mm depth). We observed mating behaviour by eye for 30 min and recorded the time until courtship began (courtship latency) and the time until copulation began (copulation latency). The courtship was recorded when the male fly displayed typical courtship elements, such as tapping or wing vibration. We defined copulation as genital coupling that continued for more than 5 min. All eight possible combinations of the crosses (two female types: *D. simulans* or *D. mauritiana* females and four male types: intact or wing-removed *D. simulans* or *D. mauritiana* males) were observed simultaneously; eight different types of pairs were observed in an identical observation period (8 pairs/set). One to four sets of observations were made on each day. We repeated this 54 times for each set.

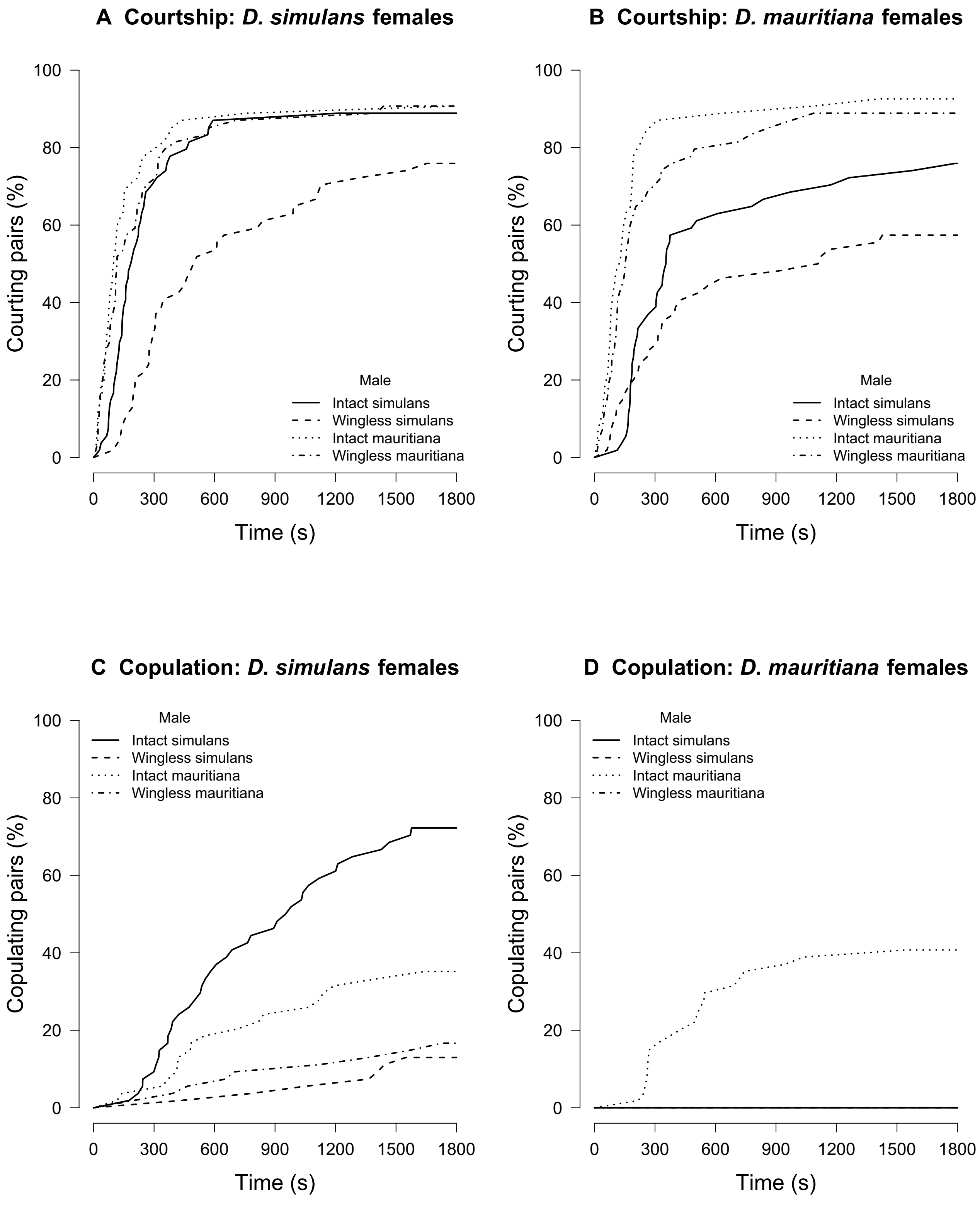
Test of effects of medium colour

To distinguish between flies more easily, male flies were fed with coloured media and then used in the female-choice test (described below). To test the effect of medium colour on copulation, single pair crosses with intact males fed with coloured media and intact females fed with normal cornmeal, and glucose media were conducted. Two days before a test, all male flies were anaesthetized for 3 min without manipulation. They were transferred to coloured medium (glucose, agar, and food colouring, blue or red) or to medium without food colouring (glucose and agar) in the evening of the day before a test. On the next day (4 or 5 days old), a pair of a *D. simulans* female and a *D. simulans* or *D. mauritiana* male (no colouring, blue or red) were introduced into a glass mating chamber (15 mm in diameter and 3-mm depth). We then observed mating behaviour by eye for 30 min. We recorded the time until courtship began (courtship latency) and the time until copulation began (copulation latency). All six possible combinations of the crosses (*D. simulans* females and six male types: *D. simulans* or *D. mauritiana* males with one of the three colours) were observed simultaneously; six different types of pairs were observed in an identical observation period (6 pairs/set). One to four sets of observations were made on each day. We repeated this 26 times for each set.

Female-choice tests: song effects on females with an opportunity to compare with another male

Females of *D. simulans* and males of *D. simulans* and *D. mauritiana* were used in the female-choice tests; females of *D. mauritiana* were not used in the female-choice tests. Because no copulation in the cross between *D. mauritiana* females and wingless males or heterospecific males was observed in the no-choice test (see "Results" section, Figs. 1, 2), we thought that it would not be productive using *D. mauritiana* females to compare the differences in copulation frequencies between conspecific and heterospecific males in the female-choice situation in the 30-min observation.

Two or three days before a test, we removed the wings of male flies with microscissors with carbon dioxide anaesthesia for 3 min. Approximately half the flies had their wings


A Courtship: D. simulans females
Courting pairs (%)
Time (s)
0
20
40
60
80
100
0
300
600
900
1200
1500
1800
Male
Intact simulans
Wingless simulans
Intact mauritiana
Wingless mauritiana
B Courtship: D. mauritiana females
Courting pairs (%)
Time (s)
Male
Intact simulans
Wingless simulans
Intact mauritiana
Wingless mauritiana
C Copulation: D. simulans females
Copulating pairs (%)
Time (s)
Male
Intact simulans
Wingless simulans
Intact mauritiana
Wingless mauritiana
D Copulation: D. mauritiana females
Copulating pairs (%)
Time (s)
Male
Intact simulans
Wingless simulans
Intact mauritiana
Wingless mauritiana

◂**Fig. 1** Mating dynamics of no-choice tests of the cross between *D. simulans* and *D. mauritiana*. **a** Cumulative frequency of courting pairs during 30-min observation using *D. simulans* females. Solid line: intact *D. simulans* males, dashed line: wingless *D. simulans* males, dotted line: intact *D. mauritiana* males, and dashed-dotted line: wingless *D. mauritiana* males. **b** Cumulative frequency of courting pairs during 30-min observation using *D. mauritiana* females. Males are indicated as in (**a**). **c** Cumulative frequency of copulating pairs during 30-min observation using *D. simulans* females. Males are indicated as in (**a**). **d** Cumulative frequency of copulating pairs during 30-min observation using *D. mauritiana* females. Males are indicated as in (**a**)

removed; the rest were used as intact-wing male controls. All males experienced 3 min of anaesthesia, but females were un-exposed. Male flies were transferred to coloured medium (glucose, agar, and food colouring, blue or red) in the evening of the day before a test. The medium colours were randomly selected to feed flies. On the next day (4 or 5 days old), a *D. simulans* female and two males (different colours) were introduced into a mating chamber (15 mm in diameter and 3-mm depth). We then observed them by eye for 30 min and recorded the time until courtship began (courtship latency) and the time until copulation began (copulation latency) for each male. We distinguished the two types of males by their coloured midgut. Because not all the males courted, we classified pairs into three categories: (1) both males courted, (2) only one male courted, and (3) no males courted. Pairs in which no male courted were omitted (category 3) from the analysis. Six cross types (*D. simulans* females and two from four male types: intact or wing-removed *D. simulans* or *D. mauritiana* males) were observed simultaneously for $N = 50$; six different types of pairs were observed in an identical observation period (6 pairs/set). One to four sets of observations were made on each day. We repeated this 50 times for each set. Additional crosses of intact *D. simulans* males that were distinguishable by their colour (blue or red) were the controls for colouration ($N = 30$); seven cross types were observed simultaneously for $N = 30$; seven different types of pairs were observed in an identical observation period (7 pairs/set). One to four sets of observations were made on each day. We repeated this 30 times for each set. In total, six cross types were observed in $N = 80$, and the control for coloured media was $N = 30$.

Statistical analysis

In the no-choice test, a Chi-square test of independence for contingency table (Zar 2010) was applied for frequency data. We did not use a continuity correction for Chi-square tests to avoid excessive conservativeness. In the no-choice test for medium colour, when one of the cells (more than 20% of eight cells) of the expected value table was less than 5, a 2 by 3 Fisher's exact probability test for contingency table was applied. When a significant association was detected using the Chi-square test, a two-sided 2 by 2 Fisher's exact probability test with mid-*P* adjustment was used for multiple pairwise comparisons with correction by Holm's methods (Holm 1979). As a test of independence for a 2 by 2 contingency table in this study, a two-sided Fisher's exact test with mid-*P* adjustment was used in accordance with the recommendation of Ruxton and Neuhäuser (2010). A 2 by 2 Chi-square test without continuity correction and a Barnard exact test (Barnard 1947), the other recommendations by Ruxton and Neuhäuser (2010), were also used and reported in the supplemental materials. For time data (courtship latency and copulation latency), a Bartlett's test (Zar 2010) was used to test the homogeneity of variances. Then, when there was no significant heterogeneity between variances, analysis of variance (ANOVA) followed by Tukey HSD test was applied (Zar 2010). When there was significant heterogeneity between variances, a Kruskal–Wallis test (Zar 2010) followed by nonparametric Tukey-type multiple comparisons (Zar 2010) with Benjamini–Hochberg adjustment (Benjamini and Hochberg 1995) was applied. In the no-choice test for medium colour, a nonparametric Kendall rank correlation coefficient (τ) (Siegel 1956) between the observation time and time parameters (courtship latency and copulation latency) of pooled data was calculated because of non-normal, skewed distribution. A parametric Pearson product–moment correlation coefficient (r) (Zar 2010) was also calculated and reported in the supplemental materials. Power analysis based on Cohen (1988) was made using parameters for large effect size ($w = 0.5$ for Chi-square test, $f = 0.4$ for ANOVA, and $f^2 = 0.35$ for correlation coefficient). Because Cohen (1988)'s power of ANOVA is for a balanced ANOVA, we calculated powers using minimum n and maximum n in a group.

In the female-choice test, we examined four issues: (1) Which male type courted first? Was there a departure from the 1:1 ratio of the number of courting male types? (2) When one of the male types courted, did the other male type also court? Did the order of courting affect whether the second male type courted or not? (3) Was the copulation success of a male type affected by the courtship of the other male type? Were there any differences in the frequency of copulation success between male types, when both male types courted? (4) Was the copulation success of a male type affected by the presence of the other male type? Were there any differences in the frequency of copulation success between male types, when only one male type courted? The structure of analysis of the female choice is shown schematically in Fig. S1. A binomial test (Zar 2010) was used to test the departure from the 1:1 ratio of the number of courting males (1). A two-sided Fisher exact probability test of independence for contingency table with mid-*P* adjustment was applied for the frequency of occurrence of courtship by both males or either male (2). To examine which

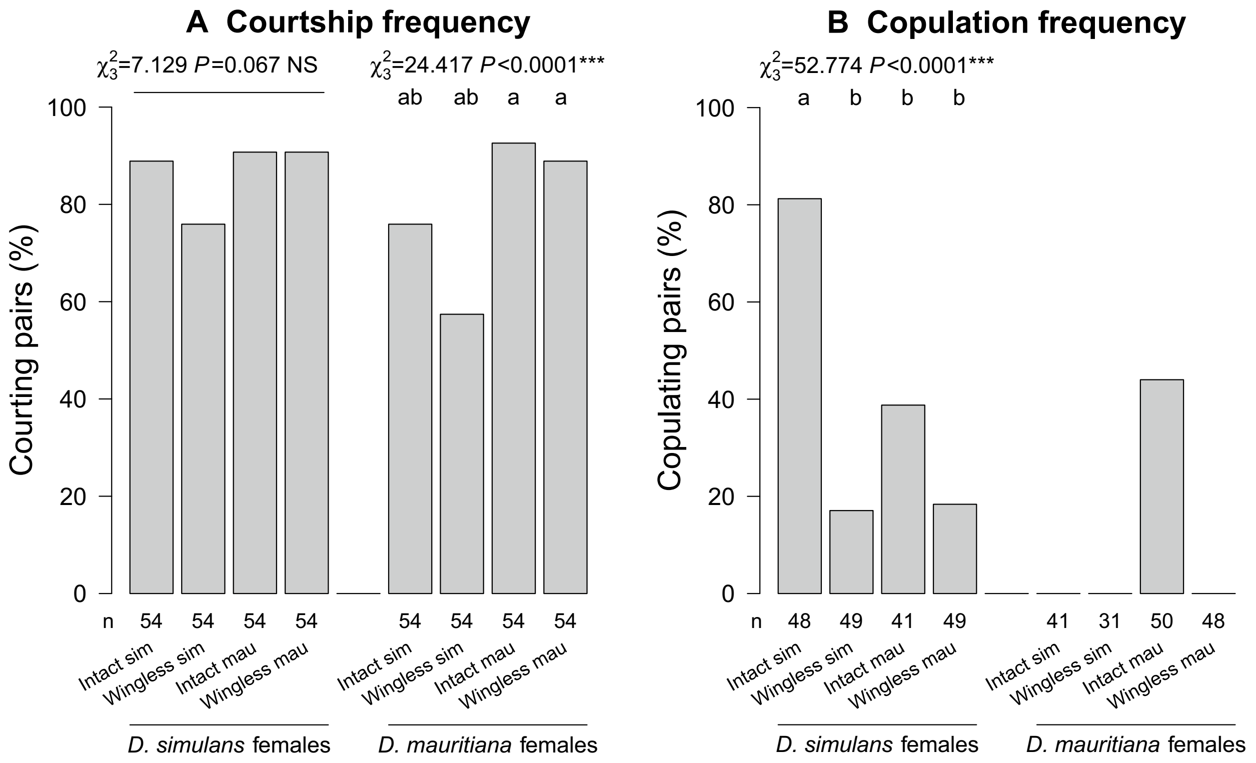


Fig. 2 Courtship and copulation frequency in the no-choice tests of the cross between *D. simulans* and *D. mauritiana*. **a** Courtship frequency. There were no significant differences between the four crosses with *D. simulans* females (Chi-square test $P = 0.067$), but differences were significant with *D. mauritiana* females (Chi-square test $P < 0.001$). Bars with the same letter are not significantly different at $\alpha = 0.05$ by multiple pairwise comparison with correction by Holm's methods for crosses with *D. mauritiana* females. **b** Frequency of copulating pairs among courting pairs. There were significant differences between the four crosses with *D. simulans* females (Chi-square test $P < 0.001$). Bars with the same letter are not significantly different at $\alpha = 0.05$ by multiple pairwise comparison with correction of Holm's methods. In the crosses with *D. mauritiana* females, only intact *D. mauritiana* males copulated. *n* number of courting pairs

male types copulated more than another type, the pair of which both male types courted and those where either male types courted were analysed separately. Departure from the 1:1 ratio of the copulation frequency was tested using a binomial test (Zar 2010) for the pairs in which both male types courted (3) and by a two-sided Fisher's exact probability test of independence for contingency table with mid-*P* adjustment for the pairs in which either male type courted (4). For time data (courtship latency and copulation latency), latencies of $n \leq 2$ were omitted. A Bartlett's test (Zar 2010) was used to test homogeneity of variances, and then, ANOVA followed by Tukey HSD test (Zar 2010) or Kruskal–Wallis test (Zar 2010) followed by nonparametric Tukey-type multiple comparisons (Zar 2010) with Benjamini–Hochberg adjustment (Benjamini and Hochberg 1995) was applied. To avoid the loss of power of multiple comparisons (Zar 2010), we used multiple comparisons even when ANOVA concluded no significant difference. Power analysis (Cohen 1988) was also made using parameters for large effect size ($g = 0.25$ for binomial test, $w = 0.5$ for Chi-square test, and $f = 0.4$ for ANOVA). R statistical software (R Core Team 2018) was used for analysis.

Results

No-choice tests: song effects on females without opportunity to compare with another male

The time course of occurrence of courtship in the no-choice test during 30-min observation is shown in Fig. 1a and b. In the crosses of *D. simulans* females, the frequency of occurrence of courtship did not differ between the four types of males (Chi-square test, $\chi_3^2 = 7.129$, $P = 0.068$, with statistical power of > 0.999, Fig. 2a; Table S1). Courtship latencies of intact-wing males of *D. mauritiana* and wingless males of *D. mauritiana* overlapped with intact-wing males of *D. simulans* (Fig. S2A; Tables 1, S2). Wingless males of

Table 1 Courtship latency and copulation latency in the no-choice test

Females	Males	Number of observed pairs	Courtship latency (s)				Copulation latency (s)			
			Mean	±	SE	(*n*)	Mean	±	SE	(*n*)
D. simulans	Intact *D. simulans*	54	229.4 ^{b}	±	29.1	(48)	730.1 ^{a}	±	65.9	(39)
	Wingless *D. simulans*	54	518.0 ^{a}	±	63.9	(41)	1147.3 ^{a}	±	155.5	(7)
	Intact *D. mauritiana*	54	168.5 ^{c}	±	39.2	(49)	719.3 ^{a}	±	100.0	(19)
	Wingless *D. mauritiana*	54	216.1 ^{bc}	±	42.1	(49)	909.4 ^{a}	±	183.9	(9)
			Bartlett's test				Bartlett's test			
			Statistic = 21.789				Statistic = 1.281			
			df = 3				df = 3			
			$P < 0.001$***				$P = 0.734$ NS			
			Kruskal–Wallis test				ANOVA			
			$\chi^2_3 = 48.159$				$F_{3,70} = 2.204$			
			$P < 0.001$***				$P = 0.0952$ NS			
D. mauritiana	Intact *D. simulans*	54	424.9 ^{a}	±	62.2	(41)		–		(0)
	Wingless *D. simulans*	54	438.9 ^{a}	±	73.5	(31)		–		(0)
	Intact *D. mauritiana*	54	172.1 ^{b}	±	34.9	(50)	520.5	±	70.0	(22)
	Wingless *D. mauritiana*	54	229.0 ^{b}	±	36.6	(48)		–		(0)
			Bartlett's test							
			Statistic = 18.452							
			df = 3							
			$P < 0.001$***							
			Kruskal–Wallis test							
			$\chi^2_3 = 41.716$							
			$P < 0.001$***							

Values followed by the same superscript letter are not significantly different by nonparametric multiple comparisons of Benjamini–Hochberg methods or by Tukey's HSD test.

n number of courting pairs or copulating pairs

***Significant differences ($P < 0.001$)

D. simulans, however, courted significantly later than other males (Fig. S2A; Tables 1, S2). In the crosses of *D. mauritiana* females, the frequencies of occurrence of courtship differed significantly (Chi-square test, $\chi^2_3 = 24.417$, $P < 0.001$, Fig. 2a), but overlapped between the four types of males (Fig. 2a; Table S1). Courtship latencies in the conspecific crosses, both intact and wingless males, were shorter than those in the heterospecific ones (Fig. S2A; Tables 1, S2). Although there were significant variations of courtship latencies between male types (Fig. S2A; Tables 1, S2), males courted conspecific and heterospecific females regardless of their wing condition (Fig. 2a).

Mating dynamics shown in Fig. 1c and d are different from courtship occurrence (Fig. 1a, b). Females of *D. simulans* copulated frequently with intact-wing *D. simulans* males and they copulated less frequently with intact-wing *D. mauritiana* males and wingless males of both species (Chi-square test, $\chi^2_3 = 52.774$, $P < 0.0001$, Fig. 2b; Table S1). However, copulation latencies did not differ between the four types of males (Fig. S2B; Tables 1, S2), suggesting that copulation latency is not a good indicator to evaluate sexual isolation in these species. The issues of sample size and statistical power are discussed in the "Discussion" section. These results suggested that courtship songs from conspecific males are important for females of *D. simulans* to accept a male, although some females accept males without conspecific songs.

Mating dynamics of *D. mauritiana* females (Fig. 1d) are different from those of *D. simulans* (Fig. 1c). Females of *D. mauritiana* accepted only intact-wing *D. mauritiana* males (Fig. 2b). They did not accept conspecific wingless males or intact-wing and wingless *D. simulans* males, suggesting that conspecific courtship song is crucial for *D. mauritiana* females to accept a courting male.

Effect of medium colour

The time course of occurrence of courtship in the no-choice test using males from three different coloured media (no colour, blue, and red) during 30-min observation is shown

Table 2 Courtship latency and copulation latency in the no-choice test for medium colour

Males	Medium colour	Number of observed pairs	Courtship latency (s)			Copulation latency (s)		
			Mean	± SE	(n)	Mean	± SE	(n)
D. simulans	No colour	26	393.8 ^{a}	± 89.3	(24)	528.1 ^{a}	± 83.0	(18)
	Blue	26	306.7 ^{a}	± 57.0	(25)	825.2 ^{a}	± 97.2	(21)
	Red	26	359.4 ^{a}	± 89.0	(25)	742.8 ^{a}	± 100.3	(20)
			Bartlett's test			Bartlett's test		
			Statistic = 5.326			Statistic = 1.228		
			df = 2			df = 2		
			$P = 0.070$ NS			$P = 0.541$ NS		
			ANOVA			ANOVA		
			$F_{2,71} = 0.303$			$F_{2,56} = 2.532$		
			$P = 0.074$ NS			$P = 0.089$ NS		
	Pooled	78	352.8	± 45.5	(74)	706.6	± 56.2	(59)
D. mauritiana	No colour	26	338.2 ^{a}	± 63.2	(23)	1055.0 ^{a}	± 175.9	(7)
	Blue	26	333.8 ^{a}	± 57.2	(25)	774.1 ^{a}	± 101.4	(9)
	Red	26	210.5 ^{a}	± 40.4	(26)	739.0 ^{a}	± 147.6	(9)
			Bartlett's test			Bartlett's test		
			Statistic = 3.810			Statistic = 1.397		
			df = 2			df = 2		
			$P = 0.149$ NS			$P = 0.497$ NS		
			ANOVA			ANOVA		
			$F_{2,71} = 1.870$			$F_{2,22} = 1.385$		
			$P = 0.162$ NS			$P = 0.271$ NS		
	Pooled	78	291.9	± 31.4	(74)	840.1	± 82.3	(25)

Values followed by the same superscript letter are not significantly different by the Tukey HSD test.

n number of courting pairs or copulating pairs

in Fig. S3A and B. There were no significant differences between the colours of media in terms of courtship frequency and courtship latency (statistical powers for courtship frequency are 0.992 in both species and statistical powers for courtship latency are 0.853 and 0.837 in *D. simulans* and *D. mauritiana*, respectively; Fig. S3C, D; Tables 2, S3, S4). Mating dynamics (Fig. S3E, F) were similar to courtship occurrence. There were no significant differences between the colours of media in the copulation frequency (Fisher's exact test $P = 0.720$ with statistical power of 0.990 for *D. simulans* males and $\chi_2^2 = 0.178$, $P = 0.914$ with statistical power of 0.990 for *D. mauritiana* males, Fig. S3G; Table S3) and the copulation latency (statistical powers are 0.726 and 0.307 in *D. simulans* and *D. mauritiana*, respectively; Fig. S3H; Tables 2, S4). The issues of sample size and statistical power are discussed in the "Discussion" section. We concluded that medium colour differences did not significantly affect the courtship parameters examined in this study in *D. simulans* males and *D. mauritiana* males. Thus, we pooled data of different media colours (Table 2). Courtship frequency was identical (94.9%: 74 out of 78) in *D. simulans* males and *D. mauritiana* males. Copulation frequency of *D. simulans* males (79.7%: 59 out of 74) and that of *D. mauritiana* males (33.8%: 25 out of 74) differed significantly (two-sided Fisher's exact test with mid-*P* adjustment, $P < 0.0001$). No significant correlations were found between observation time and time parameters in *D. simulans* males (courtship latency: $\tau = 0.083$, $Z = 1.018$, $P = 0.308$ with statistical power of 0.997; copulation latency: $\tau = -0.108$, $Z = -1.164$, $P = 0.244$ with statistical power of 0.997, Table S5) and *D. mauritiana* males (courtship latency: $\tau = -0.018$, $Z = -0.216$, $P = 0.829$ with statistical power of 0.988; copulation latency: $\tau = 0.179$, $Z = 1.222$, $P = 0.222$ with statistical power of 0.765, Table S5). Scatter plots are shown in Fig. S4.

Female-choice tests: song effects on females with an opportunity to compare with another male

In the female-choice test, a female and two males were confined in an observation chamber. Numbers of occurrence of courtship and copulation in each cross are summarized in Table S6. If there was no difference in males' willingness to court a female, the first courtship will show a 1:1 ratio between the two male types. A binomial test was used to test the departure from the 1:1 ratio (Fig. 3a; Table S7). Of the seven cross types, there were significant differences in departure from the 1:1 ratio in three cross types: wingless *D. simulans* males (crosses 2, 4, 5) courted significantly less than the other types of males. No significant differences were found in the other four cross types (statistical powers are 0.971 or > 0.999, Fig. 3a; Table S7).

After one male courted a female, another male courted the female in some pairs. If males' willingness does not differ, courtship by the second male will be observed at a similar rate between the types. The occurrence of courtship by both males and that by either male was tested using a two-sided Fisher's exact test for contingency table with mid-*P* adjustment (Fig. 3b; Table S8). Again, in the pairs of wingless *D. simulans* males and other-type males (crosses 2, 4, 5), there were significant differences in the occurrence of courtship by both males; when another-type male first courted a female, wingless *D. simulans* males courted less. No significant differences were found in the other four cross types where power analysis showed lack of power in cross 7 (statistical power is 0.722; statistical powers of the other three crosses are > 0.988, Fig. 3b; Table S8). These results suggested that wingless *D. simulans* males have less willingness to court *D. simulans* females than intact *D. simulans* males and intact and wingless *D. mauritiana* males, and that colouration did not affect the number of males courting second (cross 7). The homosexual courtship between the males was observed in some but not all cases in which both males courted; the homosexual courtship did not last long and stopped immediately. No aggression behaviour between them was observed. All males who courted other males courted females.

Figure 3c and d shows copulation frequencies. In the pairs in which both males courted, intact *D. simulans* males copulated significantly more than the other-type males (binomial tests, crosses 1, 2, and 3, Fig. 3c; Table S9). There were no significant differences between male types in the other crosses, where power analysis showed lack of power in cross 7 (statistical power is 0.547; statistical powers of the other three crosses are > 0.854, Fig. 3c; Table S9). In the pairs in which either male courted, intact *D. simulans* males copulated significantly more than wingless *D. simulans* males or wingless *D. mauritiana* males (two-sided Fisher's exact tests for contingency table with mid-*P* adjustment, Fig 3d; Table S10), while they copulated at a similar rate to intact *D. mauritiana* males (cross 1, Fisher's exact test with mid-*P* adjustment, $P = 0.290$ with statistical power of 0.807, Fig. 3d; Table S10). In the remaining four crosses, no differences in copulation frequencies were detected between trials where only one male courted; power analysis showed lack of power in crosses 6 and 7 (statistical powers are 0.649 and 0.490, respectively; statistical powers of crosses 4 and 5 are 0.923 and 0.928, respectively, Fig. 3c; Table S9).

In different courtship contexts of cross 1 (cross of *D. simulans* females, intact *D. simulans* males, and intact *D. mauritiana* males), differences in copulation frequencies were found. When both males courted (44 pairs), intact *D. simulans* males copulated (28 pairs) significantly more than intact *D. mauritiana* males (6 pairs); its ratio is 28:6 or 4.66 (Fig. 3c; Tables S11, S12). On the other hand, there was no significant difference in copulation frequencies when either male courted (70% by intact *D. simulans* males and 50% by intact *D. mauritiana* males: Fig. 3d; Table S11); its ratio is 0.7:0.5 or 1.4 (Table S12). These differences suggested that conspecific copulations are enhanced in situations where females can choose males.

Courtship latencies of first-courting males (Fig. S5; Table S11) were significantly faster than the other males in some crosses (crosses 1, 3, 5), but no differences were detected in the other crosses (statistical powers are < 0.605 in four cross types; Fig. S5; Tables S13, S14). *D. mauritiana* males were faster courters; in the no-choice test, courtship latencies were significantly faster than *D. simulans* (Fig. S2A; Table 1). Although copulation latencies cannot be statistically compared between male types due to the small sample size, except for the cross using intact *D. mauritiana* males and wingless *D. mauritiana* males, there is no marked difference between male types (statistical power is 0.282; Fig. S5; Tables S13, S14).

Discussion

The present study examined sexual isolation between *D. simulans* and *D. mauritiana* in relation to courtship song. The removal of wings makes males virtually mute; thus, females lose the opportunity to obtain one of the most important signals to judge courting males. Females of *D. mauritiana* accepted intact-wing conspecific males but not other types of males, whereas *D. simulans* females accepted intact-wing conspecific males and did not discriminate against intact-wing *D. mauritiana* males in no-choice situations. However, females of *D. simulans* copulated with intact *D. simulans* males more than intact *D. mauritiana* males in true choice situations. It may be possible that sexual isolation between *D. simulans* females and *D. mauritiana* males is enhanced in a situation where females can choose a male.

Statistical analyses showed many non-significant differences in the present study. To evaluate type II error, statistical power analysis was performed in accordance with Cohen (1988). Because small powers, such as less than 0.8, were calculated in several cases, especially in time parameters of the female-choice test (Tables S2, S4, S8, S9, S10, S14), we should mention that type II errors may be included in no significant differences. In contrast, significant differences

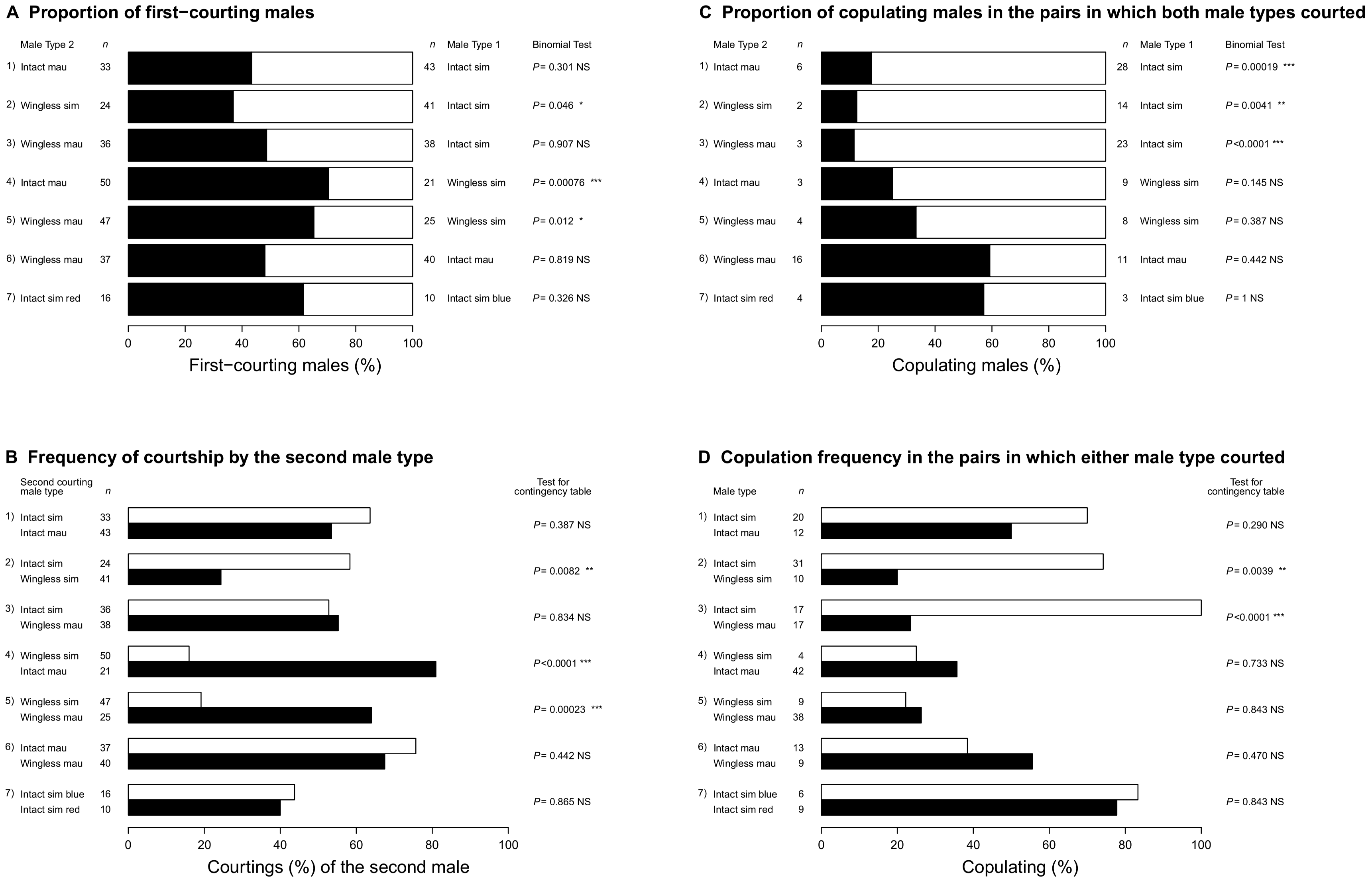
Fig. 3 Courtship and copulation in the *D. simulans* female-choice tests. Seven cross types were tested in the female-choice test of *D. simulans* ▸ females and two types of males. Males were: (1) an intact *D. simulans* male (intact sim) and an intact *D. mauritiana* male (intact mau), (2) an intact *D. simulans* male (intact sim) and a wingless *D. simulans* male (wingless sim), (3) an intact *D. simulans* male (intact sim) and a wingless *D. mauritiana* male (wingless mau), (4) a wingless *D. simulans* male (wingless sim) and an intact *D. mauritiana* male (intact mau), (5) a wingless *D. simulans* male (wingless sim) and a wingless *D. mauritiana* male (wingless mau), (6) an intact *D. mauritiana* male (intact mau) and a wingless *D. mauritiana* male (wingless mau), (7) two intact *D. simulans* males coloured blue (intact sim blue) or red (intact sim red). Structure of the analyses is shown in Fig. S1. **a** Proportion of first-courting males. *n* number of courting males shown on each side of the bar. Binomial test: Departure from the 1:1 ratio between male types was tested. See also Table S4. **b** Frequency of courtship by the second male type, when one of the male types courted. *n* number of the first-courting males. Note that the male type shown on the left is the opposite (the second courting) male type. Test for contingency table: differences in the frequency of courtship occurrence, whether another male courted were tested by two-sided Fisher's exact probability tests with mid-*P* adjustment. See also Table S5. **c** Proportion of copulating males in the pairs in which both male types courted. *n* number of copulating males shown on each side of the bar. See also Table S6. Binomial test: departure from the 1:1 ratio between male types was tested. **d** Copulation frequency in pairs in which either male courted. The copulation frequencies of male type 1 and male type 2 are separately shown. *n* number of courting males. Test for contingency table: the results of the two-sided Fisher's exact test with mid-*P* adjustment. See also Table S7

between crosses were found in the copulation frequency, even when copulation latency did not differ. We consider that statistical tests on courtship and copulation frequencies discussed here are reliable with sufficient statistical power (> 0.8). Coyne et al. (2005) reported that copulation latency as well as copulation frequency significantly differ in sexual isolation between *D. santomea* and *D. yakuba*, where the sample size is not so large ($N = 2–22$). We consider that the copulation latency was not a good indicator to detect decision-making of females in the species studied here compared with copulation frequency.

Drosophila mauritiana females accept intact-wing *D. mauritiana* males but not other types of males

Females of *D. mauritiana* copulated with intact conspecific males (44.0%), but they did not with wingless ones (Figs. 1, 2; Table S1). However, direct observation in mass mating conditions (crosses between 10 females and 10 males) showed that 12.2% of *D. mauritiana* females copulated with wingless conspecific males in 30 min, where the copulation frequencies of intact ones are 45.1% (Tomaru et al. 2000). It may be possible that multiple and repeated courtships by several males in the mass mating condition lower the acceptance threshold of females without receiving a conspecific song. The previous studies also examined the interspecific cross between *D. mauritiana* females and *D. simulans* males; *D. mauritiana* females were inseminated by intact *D. simulans* males more than by wingless *D. simulans* males in mass mating conditions (Tomaru et al. 2000). Robertson (1983) reported low insemination rate (5%) in the cross between *D. mauritiana* females and intact *D. simulans* males after 48-h confinement. It is possible that only a few *D. simulans* males courted *D. mauritiana* females, leading to low insemination. In the present study, we observed the courtship behaviour of single pairs of this cross directly by eye and found that both intact and wingless *D. simulans* males as well as *D. mauritiana* males courted *D. mauritiana* females and that only intact *D. mauritiana* males succeeded in copulation in the no-choice situation (Figs. 1, 2). These observations suggested that conspecific courtship song is one of the most important signals for *D. mauritiana* females to accept the courting males and that heterospecific (*D. simulans*) courtship song does not positively affect the receptivity of *D. mauritiana* females in the 30-min observation period. Because no intact and wingless *D. simulans* males copulated with *D. mauritiana* females in single pair crosses within 30-min observation, we were unable see whether the courtship song of *D. simulans* males had a negative effect on *D. mauritiana* females.

Courtship song is an important signal for copulation success in *D. mauritiana* in the no-choice situation, without reference signals (heterospecific song). Therefore, the decision of *D. mauritiana* females seems to be made mostly using absolute criteria, as seen in *D. montana* and *D. ezoana* (Hoikkala and Aspi 1993). In some species, females copulated more with wingless heterospecific males, but less with intact-wing ones, for example, in the *D. auraria* complex (Tomaru et al. 1995, 1998), *D. sechellia* (Tomaru et al. 2000; Tomaru and Oguma 2000; Tomaru et al. 2004), and *D. ananassae* (Doi et al. 2001). This was not the case in the cross between *D. mauritiana* females and *D. simulans* males in the 30-min observation of the present study. However, this does not simply mean that the presence of species-specific courtship song is essential in *D. mauritiana* females. In the cross between *D. mauritiana* females and *D. simulans* males or *D. sechellia* males, females were inseminated by wingless males after 2-h or 48-h confinement, although less than intact males (Tomaru et al. 2000). These differences in insemination rates were due to courtship song, not the presence of wings in males, which was confirmed using deaf females (arista removal): intact *D. mauritiana* females were inseminated by intact *D. simulans* males or *D. sechellia* males more than aristaless females after 2-h or 48-h confinement (Tomaru et al. 2000). Air-transmitted courtship songs seemed to affect females to accept courting males; aristaless females may perceive courtship songs through substrate vibrations caused by flight muscle contractions in males as


A Proportion of first−courting males
Male Type 2
n
n
Male Type 1
Binomial Test
1) Intact mau 33 43 Intact sim P= 0.301 NS
2) Wingless sim 24 41 Intact sim P= 0.046 *
3) Wingless mau 36 38 Intact sim P= 0.907 NS
4) Intact mau 50 21 Wingless sim P= 0.00076 ***
5) Wingless mau 47 25 Wingless sim P= 0.012 *
6) Wingless mau 37 40 Intact mau P= 0.819 NS
7) Intact sim red 16 10 Intact sim blue P= 0.326 NS
0 20 40 60 80 100
First−courting males (%)
C Proportion of copulating males in the pairs in which both male types courted
Male Type 2
n
n
Male Type 1
Binomial Test
1) Intact mau 6 28 Intact sim P= 0.00019 ***
2) Wingless sim 2 14 Intact sim P= 0.0041 **
3) Wingless mau 3 23 Intact sim P<0.0001 ***
4) Intact mau 3 9 Wingless sim P= 0.145 NS
5) Wingless mau 4 8 Wingless sim P= 0.387 NS
6) Wingless mau 16 11 Intact mau P= 0.442 NS
7) Intact sim red 4 3 Intact sim blue P= 1 NS
0 20 40 60 80 100
Copulating males (%)
B Frequency of courtship by the second male type
Second courting male type
n
Test for contingency table
1) Intact sim 33
Intact mau 43
P= 0.387 NS
2) Intact sim 24
Wingless sim 41
P= 0.0082 **
3) Intact sim 36
Wingless mau 38
P= 0.834 NS
4) Wingless sim 50
Intact mau 21
P<0.0001 ***
5) Wingless sim 47
Wingless mau 25
P= 0.00023 ***
6) Intact mau 37
Wingless mau 40
P= 0.442 NS
7) Intact sim blue 16
Intact sim red 10
P= 0.865 NS
0 20 40 60 80 100
Courtings (%) of the second male
D Copulation frequency in the pairs in which either male type courted
Male type
n
Test for contingency table
1) Intact sim 20
Intact mau 12
P= 0.290 NS
2) Intact sim 31
Wingless sim 10
P= 0.0039 **
3) Intact sim 17
Wingless mau 17
P<0.0001 ***
4) Wingless sim 4
Intact mau 42
P= 0.733 NS
5) Wingless sim 9
Wingless mau 38
P= 0.843 NS
6) Intact mau 13
Wingless mau 9
P= 0.470 NS
7) Intact sim blue 6
Intact sim red 9
P= 0.843 NS
0 20 40 60 80 100
Copulating (%)

shown in other *Drosophila* species (Fabre et al. 2012; Mazzoni et al. 2013).

Drosophila simulans females do not discriminate against intact-wing *D. mauritiana* males

Although *D. simulans* females accepted intact *D. mauritiana* males less than intact *D. simulans* males in the no-choice test (Figs. 1, 2), whether or not they discriminated against intact *D. mauritiana* males was unclear. In the no-choice condition, *D. simulans* females accepted wingless *D. simulans* males, intact *D. mauritiana* males, and wingless *D. mauritiana* males, although the copulation frequencies with them were lower than that with intact *D. simulans* males (Figs. 1, 2), as in the mass mating conditions (Tomaru et al. 2000). Conspecific courtship song played a role in *D. simulans* females, but heterospecific (*D. mauritiana*) song seemed to be less important in decision-making to reject courting heterospecific males in *D. simulans* females. Hoikkala and Aspi (1993) reported that the wing-manipulated males copulated less than intact males in choice situation in conspecific crosses of *D. littoralis*, *D. montana*, and *D. ezoana*. When females could choose, *D. simulans* females copulated with intact *D. mauritiana* males as well as wingless *D. mauritiana* males when both males courted (Fig. 3c; cross 6, Tables S9, S12) and when only one male courted (Fig. 3d; cross 6, Tables S10, S12). Intact *D. mauritiana* males were also accepted in combination with wingless *D. simulans* males (Fig. 3c, d; cross 4, Tables S9, S10). These results were consistent with the results in no-choice conditions (Figs. 1, 2). Courting *D. mauritiana* males were not discriminated against by *D. simulans* females when they emitted the species-specific courtship song of *D. mauritiana*, suggesting that this species-specific song of *D. mauritiana* did not negatively affect decision-making of *D. simulans* females to accept courting males.

Because there was no significant difference in copulation frequencies between wing conditions when both males courted (Fig. 3c; cross 6, Table S9), it may be possible that *D. simulans* females accepted intact *D. mauritiana* males as if they were mute. Electrophysiological and behavioural studies showed that *D. simulans* respond to played-back *D. melanogaster*-type songs (Tootoonian et al. 2012; Yoon et al. 2013). Because the species-specific parameter of interpulse interval of *D. mauritiana* (45 ms) is intermediate between those of *D. melanogaster* (34 ms) and *D. simulans* (55 ms) (Shorey 1962; Bennet-Clark and Ewing 1969; Cowling and Burnet 1981; Robertson 1983), there is no reason to consider that the auditory system of *D. simulans* does not respond to the *D. mauritiana* song. It is likely that courtship songs from *D. mauritiana* males were also received by their auditory receptor (Johnston's organ) through feather-like shaped antennal segments, aristae, and that the song information is transmitted to the auditory center in the brain. It seems that the decision is made there; it is not likely that the auditory system could not convert the heterospecific courtship song into an electrical signal in *D. simulans* females.

Hoikkala and Aspi (1993) showed that females used both absolute and relative criteria in intraspecific crosses of the *D. virilis* group. We sought to clarify courtship and copulation in the female-choice situation, in the expectation that there may be context-dependent mate choice. However, in contrast to the previously studied species that showed discrimination against heterospecific males (Tomaru et al. 1995, 1998, 2000, 2004; Tomaru and Oguma 2000; Doi et al. 2001), the present study suggested that females of *D. mauritiana* and *D. simulans* use absolute criteria in conspecific crosses, but no strong evidence in heterospecific cross in *D. simulans* females was found.

Sexual isolation between *D. simulans* females and *D. mauritiana* males may be enhanced by the comparison with other males

In the cross using *D. simulans* females, combination with intact *D. simulans* males and intact *D. mauritiana* males resulted in a different outcome from that with wingless males. In a situation where females can choose a male, an excess of conspecific copulations was observed compared to heterospecific copulations, but not significantly more when females were courted by either males (Fig. 3c, d; cross 1, Table S12). We can interpret that *D. simulans* females chose intact conspecific males compared to intact heterospecific males; it may be possible that sexual isolation is enhanced when both males courted the female and that one of the most important signals is a courtship song.

In the no-choice test, however, it seemed that copulation frequency of intact *D. mauritiana* males was raised in the presence of not courting intact *D. simulans* males (see also the Supplemental Materials 2). Because the sample sizes when either male courted in the female-choice test (Table S10) were smaller than the no-choice test (Table S1), it was partly explained by the fact that the inconsistent significance was brought about by the differences in sample size. In addition, it may also be possible that the presence of a non-courting conspecific *D. simulans* male may positively affect females' willingness to accept another courting male. We did not observe aggression behaviour between the males, but a kind of interaction between them, including homosexual courtship, may affect copulation. The presence of normal males raised copulation success of wing-manipulated males in conspecific cross of *D. littoralis* in the choice situation (Hoikkala and Aspi 1993).

In female-choice conditions, Cobb et al. (1988) showed no discrimination by *D. simulans* females between conspecific, heterospecific (*D. mauritiana*), and hybrid males. It may be possible that their results consisted of different situations where females were courted by two or more kinds of males and by one male; that is, females that could choose males and those that could not.

In sexual isolation between *D. santomea* and *D. yakuba*, Coyne et al. (2005) discussed which choice experiment design is a more realistic model of nature, but did not come to a conclusion. Because *D. santomea* and *D. yakuba* are parapatric in São Tomé island and forming a hybrid zone (Lachaise et al. 2000; Ashburner et al. 2005), these flies may have an opportunity to choose between conspecific and heterospecific. In the field observation, Gromko and Markow (1993) reported that some pairs showing courtship are a *D. simulans* female and a *D. melanogaster* male or vice versa, suggesting that these flies may have an opportunity to choose a partner. In contrast, *D. simulans* and *D. mauritiana* are allopatric and do not encounter each other in nature (Lachaise et al. 1988; Ashburner et al. 2005). Secondary contact may be brought about by human activity, such as the introduction of sika deer (*Cervus nippon*) into Europe where red deer (*C. elaphus*) live (Wyman et al. 2011, 2014, 2016). Although sexual isolation experiments between *D. simulans* and *D. mauritiana* are a kind of artificial situation, these experiments revealed how females discriminate against heterospecific males. In some species, female preference for heterospecifics is partly explained by a directional preference for exaggerated male secondary characteristics (swordtail, Ryan and Wagner 1987; least auklets, Jones and Hunter 1998; estrildid finch, Collins and Luddem 2002). Asymmetry in sexual isolation between *D. simulans* (55 ms interpulse interval of courtship song) and *D. mauritiana* (45 ms) does not seem to be caused by a directional preference for shorter interpulse intervals of courtship song, because both *D. simulans* and *D. mauritiana* females reject *D. melanogaster* males emitting shorter interpulse interval of courtship song (34 ms) (Watanabe and Kawanishi 1979; Robertson 1983; Lachaise et al. 1986; Cobb et al. 1988; Tomaru et al. 2000). It may be explained partly by differences in female preference functions between the species (Ryan and Rand 1993; Ritchie 1996).

Effects of the order of courtship and other factors seem to be less important

The order of courtship seems to be less important for *D. simulans* females to choose males than the characteristics of the courting males (the presence or absence of wing and species). There were no trends that the first-courting males copulated more than the second courting males; the excess in copulation was four cases by the first-courting males, nine cases by the second courting males, and one case by equal number in the pairs where both males courted (Table S6). The courtship order was also not important in *D. littoralis*, *D. montana*, and *D. ezoana* in choice situations (Hoikkala and Aspi 1993). Because all males (intact and wingless ones) experienced 3-min anaesthesia 2 or 3 days before experiment and no females experienced anaesthesia, the effects of anaesthesia were controlled in this study. Both sexes were housed separately in a group at most 10 flies per vial for 4 or 5 days before the experiment. Our results showed that most males courted and copulated well within 30 min (Figs. 1, 2, 3, S3). Although social experience could potentially affect courtship behaviour (Svetec 2005), we believe that it had a minimal impact on courtship and copulation in this study. Wingless *D. simulans* males courted less and copulated less in the female-choice test (Fig. 3). The courtship of *D. simulans* male was enhanced by artificial conspecific courtship sounds (Yoon et al. 2013). The absence of conspecific courtship sounds produced by himself or by another fly may reduce courtship activity of wingless *D. simulans* males, whereas no sound may less affect wingless *D. mauritiana* males.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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