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RAPID COMMUNICATION

SEXUAL ISOLATION AND CUTICULAR HYDROCARBON DIFFERENCES BETWEEN *Drosophila santomea* AND *Drosophila yakuba*

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Abstract—*Drosophila santomea* and *Drosophila yakuba* are two sister species inhabiting Saõ Tomé island. Previous studies showed that both species display strong reproductive isolation, although they can produce a few viable hybrids. Our study tried to understand the mechanism of this ethological isolation between two allopatric strains. A strong sexual isolation was confirmed, with a marked asymmetry. Comparisons of latency times to either courtship or copulation suggest that males do not discriminate females, whereas *D. yakuba* females, but not *D. santomea* females, accept their homospecifics more quickly. Cuticular hydrocarbon compositions of both species and sexes were also established with gas chromatography (GC) and GC/mass spectrometry analysis. All have (*Z*)-7-tricosene as their major compound. There are several quantitative differences between species for few minor compounds. The largest difference concerns *n*-heneicosane, which is more abundant in *D. santomea* than in *D. yakuba* flies (up to seven times more between males). A similar quantitative difference was also found in a pair of sympatric strains. Furthermore, *D. yakuba* males artificially perfumed with *n*-heneicosane were discriminated negatively by *D. yakuba* females, suggesting a role for this compound in the sexual isolation between these two species.

Key Words—*Drosophila santomea*, *Drosophila yakuba*, reproductive isolation, courtship, hydrocarbon pheromone.

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INTRODUCTION

Mate selection consists of both choosing a conspecific and identifying the opposite sex. For that purpose, the selection is made on the basis of courtship traits that are characteristic of each species. *Drosophila*'s courtship corresponds to a stereotypic sequence of behaviors involving visual, tactile, acoustic, and chemical stimuli (Ewing, 1983). Specifically, cuticular pheromones are involved in mate stimulation and discrimination (Jallon, 1984; Cobb and Jallon, 1990; Ferveur, 2005). These cuticular hydrocarbons (CHCs) are not very volatile compounds that are perceived only at short distances and may stimulate or inhibit potential partners. In the melanogaster subgroup, they may differ markedly between sexes as in *Drosophila melanogaster*, or be similar as in *Drosophila simulans* (Jallon, 1984; Jallon and David, 1987).

Drosophila santomea and *Drosophila yakuba* are two sister species living on the island of Saô Tomé (Lachaise et al., 2000). Coyne et al. (2002) showed a strong reproductive isolation between them, with similar amplitude for sympatric and allopatric strains. Nevertheless, both species can mate with each other, and hybrids are viable. It was concluded that the two species were recently isolated and that reproductive isolation resulted from a prezygotic barrier, possibly an ethological one (Llopart et al., 2002). *D. santomea* flies lack the abdominal dark pigmentation present in *D. yakuba* flies, but Llopart et al. (2002) showed that this visual cue is not important for interspecific mate discrimination.

In the present study, we first compared the possible behavioral temporal parameters in homospecific and heterospecific matings and then analyzed CHCs, which could be potentially involved in sexual selection between the two species.

METHODS AND MATERIALS

D. santomea flies originated from strain Car 1490-1, collected above the hybrid zone, at an altitude of 1,490 m on Saô Tomé. *D. yakuba* flies are issued from strain SJ 14, collected below the hybrid zone at low altitude. Two other strains collected in the hybrid zone were also used for chemical analysis: strain STO5 for *D. santomea* and SA1 for *D. yakuba*.

Both species were reared on the usual cornmeal–malt medium at 25°C under 12:12 light/dark conditions. All flies were sexed, isolated under carbon dioxide anesthesia within 12 hr after imaginal emergence, and maintained in glass vials (25 ml). Flies were 4-d-old when used for experiments. All tests were run in the morning, between 0900 and 1300 hours, under dim electric light in a temperature-controlled room.

Mating tests were set up in a chamber (2.15 cm diam × 4 mm height) with a glass roof, which could be divided into two parts by a removable separation; thus, each sex could be introduced on either side, and flies could habituate before removing the separation, at time 0. In each test, three females and three males were put together and observed during 45 min. In addition to the percentage of first copulations succeeding the first courtship (percentage of mating success), two temporal parameters were recorded: latency time to the first courtship for males that initiate courtship and latency time to the first copulation for females that accept or do not accept the males.

To analyze fly CHCs for each strain of each species and each sex, ten 4-d-old virgin flies were anesthetized, bathed individually in heptane, and their extracts analyzed in a PerkinElmer AutoSystem GC chromatograph or a Fisons MD800 (under electron impact at 70 eV) gas chromatography/mass spectrometry (GC/MS) apparatus, both equipped with a BP1 SGE capillary column (length 25 m, internal diam 0.22 mm, film thickness 0.1 μm) and at the same temperature program (180–270°C, 3°C/min) (Rouault et al., 2004). The relative proportions of the areas of the various peaks were calculated (in percent) and compared. An external standard, *n*-hexacosane, which is naturally absent in fly cuticle, was used in a given amount, and the area under each peak was compared to that of the standard to determine its absolute amount.

To perfume *D. yakuba* males or females, 3-d-old individuals were anesthetized under carbon dioxide and 0.2 μl of an acetonic solution containing 2 mg/ml *n*-heneicosane were applied topically on each fly, corresponding to more than twice the dose present on *D. santomea* flies. Perfumed flies stood 24 hr before experiments started. Control individuals received acetone alone, which evaporates quickly. Experiments with perfumed flies were done under the same conditions as previously described. Mating success was recorded over a 45-min period. After the experiment, all flies were chemically analyzed by GC.

For statistical comparisons, a Mann–Withney *U* test was applied to compare temporal parameters and a chi-square test for mating success.

RESULTS AND DISCUSSION

Table 1 presents the results of mating experiments within or between two strains of either species, *D. santomea* (Car 1490-1) and *D. yakuba* (SJ 14). They show that homospecific matings are preferred to heterospecific ones: 54% homospecific matings for *D. santomea* and 48% for *D. yakuba*. This confirms the existence of a behavioral reproductive barrier between the two sister species. Second, when comparing heterospecific matings, there is an asymmetrical preference for copulations of *D. yakuba* females with *D. santomea* males

TABLE 1. DIFFERENT PARAMETERS RECORDED DURING 45 MINUTES FOR INTRASPECIFIC AND INTERSPECIFIC MATING EXPERIMENTS

Crossing	Number of Tests	Mating success (%)	Time in minutes ± SD: Latency to first Courtship	Time in minutes ± SD: Latency to first Copulation
FM san	63	54	2.2 ± 0.4	13.4 ± 2.7
FM yak	105	48	1.2 ± 0.2	14.5 ± 1.8
Fsan-Myak	114	4	1.8 ± 0.6	9.7 ± 4.2
Fyak-Msan	99	11	2.2 ± 0.4	24.0 ± 3.3

F, Female; M, male; san, *D. santomea*; yak, *D. yakuba*.
Three males and three females are present in the mating chamber.

(Fyak–Msan, 11%) compared to 4% for *D. santomea* female–*D. yakuba* male pairs.

The courtship latency times of each type of male are not significantly different in the presence of either female, neither for *D. santomea* males ($P = 0.69$) nor for *D. yakuba* males ($P = 0.45$). This suggests that males are equally attracted by the two types of females and, therefore, do not discriminate between them.

When latency times to copulations are compared between females, there are differences. *D. yakuba* females accept homospecific males more quickly than *D. santomea* males ($P = 0.012$). The difference with *D. santomea* females is not significant, however, between the two types of males ($P = 0.49$). Thus, the temporal differences of male courtship and female acceptance suggest that females are the choosy sex and discriminate between the males.

Figure 1 presents the CHC composition of each sex within each species. We observed no qualitative differences between the two species; they all presented similar GC profiles, consisting of 15 peaks. GC/MS analysis led to the identification of long-chain hydrocarbons shown in Figure 1. Peaks 1, 2, 12, and 15 were reported neither in males nor females of the *D. yakuba* continental strain (from Cameroon) described by Jallon and David (1987). Their mass spectra show molecular peaks with *m*/*z* respective values of 296, 310, 380, and 408; moreover, compounds 7, 11, and 14 display mass spectra with M-43 peaks, which suggests a methyl branching on carbon 2. The repeated comigrations of tricosene peaks 3 and 4 and pentacosene peaks 8 and 9 with synthetic (*Z*)-9-tricosene, (*Z*)-7-tricosene, (*Z*)-9-pentacosene, and (*Z*)-7 pentacosene, respectively, suggest (*Z*)-9- and (*Z*)-7- for double bond positions A and B, respectively. Thus, (*Z*)-7-tricosene is the most abundant CHC, whichever sex and species, as in all "monomorphic" species of the melanogaster subgroup (Jallon and David, 1987). There is no significant difference in the absolute

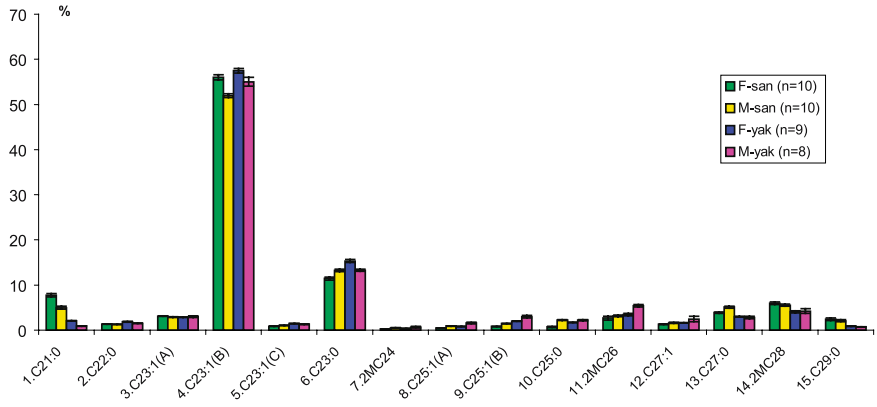

FIG. 1. Cuticular hydrocarbon profiles of 4-d-old virgin females and males of each species, *D. santomea* (strain Car 1490-1) and *D. yakuba* (strain SJ 14). Each peak has been associated with its area percentage over the sum of all CHC peak areas and is presented, with its GC/MS identification, in the order of increasing retention times.

amounts of this compound in the cuticle of either type of female (on average 1265 ± 68 ng per *D. santomea* female and 1281 ± 61 ng per *D. yakuba* female), and these females are not discriminated by either type of males. Thus, it is suggested that in *D. santomea*, (*Z*)-7-tricosene might also play an important role in female sex appeal (Cobb and Jallon, 1990).

There is also no significant difference in the total amounts of CHC in either type of female (2.2 μg/fly) but *D. santomea* males have more CHC (1.6 μg/fly) than *D. yakuba* ones (1.3 μg/fly). If one considers both sexes, there are significant quantitative differences between species for five compounds: *n*-heneicosane (peak 1), (*Z*)-9- and (*Z*)-7-pentacosene (peaks 8 and 9), *n*-heptacosane (peak 13), and *n*-nonacosane (peak 15). The largest difference, however, concerns *n*-heneicosane, which is more abundant in *D. santomea* flies, 3.9 times among females and 6.9 times among males. Analysis of the CHCs of the two other strains, *D. santomea* STO5 and *D. yakuba* SA1 collected in the sympatric zone, shows similar profiles. *n*-Heneicosane is also more abundant in both sexes of this second *D. santomea* strain, although the quantitative difference is not very different from that of the allopatric strains (4.7 times more for females, 5.7 for males).

This raises the possibility that this compound might be used to discriminate between heterospecific flies. To test this hypothesis, *D. yakuba* flies of both sexes were perfumed—or not—with a dose of *n*-heneicosane (400 ng) and presented to (nonperfumed) homospecific flies of the other sex. No significant differences

in mating success were observed between perfumed females and controls crossed with *D. yakuba* males (39 and 34%, respectively). On the other hand, whether perfumed or not, when *D. yakuba* males were crossed with *D. yakuba* females, fewer perfumed males mated with homospecific females: 30.0% compared to 55.5% with nonperfumed ($\chi^2 = 6.251$, $df = 1$, $P = 0.02$). This supports the hypothesis that *D. yakuba* females might discriminate negatively modified *D. yakuba* males that smell like *D. santomea* males.

In summary, our experiments support the classical role of females as the choosy sex in mate selection and suggest that this selection could result from a specific CHC. In *D. melanogaster*, Scott (1994) also showed an intraspecific female choice between males that bear in their cuticle more or less of the major CHC that has evolved among populations (Rouault et al., 2004). Here, females discriminate between heterospecific males using a less abundant compound.

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