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Incipient speciation in *Drosophila melanogaster* involves chemical signals

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The sensory and genetic bases of incipient speciation between strains of *Drosophila melanogaster* from Zimbabwe and those from elsewhere are unknown. We studied mating behaviour between eight strains – six from Zimbabwe, together with two cosmopolitan strains. The Zimbabwe strains showed significant sexual isolation when paired with cosmopolitan males, due to Zimbabwe females discriminating against these males. Our results show that flies' cuticular hydrocarbons (CHs) were involved in this sexual isolation, but that visual and acoustic signals were not. The mating frequency of Zimbabwe females was highly significantly negatively correlated with the male's relative amount of 7-tricosene (%7-T), while the mating of cosmopolitan females was positively correlated with %7-T. Variation in transcription levels of two hydrocarbon-determining genes, *desat1* and *desat2*, did not correlate with the observed mating patterns. Our study represents a step forward in our understanding of the sensory processes involved in this classic case of incipient speciation.

Sensory signals are used by animals to assess the sex, species and reproductive status of their potential mate[1]. The intraspecific divergence of such signals between populations can increase their sexual isolation and ultimately lead to distinct species[2]. Although *Drosophila melanogaster* has a global distribution, several strains found in some geographic areas show non-random mating indicating partial sexual isolation[3–5]. The strongest documented case of intraspecific sexual isolation in this species is found between strains from Zimbabwe and those from other geographic areas (Cosmopolitan strains). In particular, Zimbabwe females rarely mate with Cosmopolitan males whereas Cosmopolitan females show a high mating frequency with both Zimbabwe and Cosmopolitan males[6,7].

Several *D. melanogaster* natural strains vary in their cuticular hydrocarbons (CHs), some of which play a pheromonal role[8]. Most females produce high levels of 7,11-dienes (CHs with two double bonds on carbons 7 and 11), whereas the females from several Western African strains (such as the Tai strain from Ivory Coast[9,10]) and from the Caribbean[11] produce high amounts of 5,9-dienes and low amounts of 7,11-dienes. However, males of Tai-like strains still produce high levels of 7-monoenes (with a single desaturation on C7) and no or very low levels of 5-monoenes[9,12]. These compounds can act as pheromones: male-predominant 7-tricosene (7-T) stimulates female mating but inhibits male courtship, while 7,11-dienes enhance male preference[13–16]. Other compounds may also be involved: it has been suggested that 5-tricosene (5-T) inhibits male courtship initiation, while the role of 5, 9-dienes is unclear – these compounds were initially thought to decrease female attractivity as shown by mating levels, but this was not confirmed[10,17].

In *D. melanogaster*, the production of CHs with a first desaturation either on C7 or on C5 depends on the activity of *desat1* and *desat2*, respectively. These are two closely linked genes that code for carbon-specific desaturase enzymes[17–19]. The *desat2* gene is not expressed in Cosmopolitan strains, thus explaining the absence of 5, 9-dienes in Cosmopolitan females[20]. The molecular structure of the *desat2* gene is apparently related to the degree of sexual isolation in Zimbabwe females[21], but nothing is known of the sensory signal(s) involved in this case of incipient speciation[22,23]. The *desat1* gene has a pleiotropic effect on both the production of cuticular pheromones and their discrimination[24,25]. These findings raise the tantalizing possibility that *desat1* and *desat2* are also involved in the perception of C7- and C5-desaturated hydrocarbon pheromones.

After eliminating visual and auditory signals as sources of sensory discrimination between strains, we explored the relationship between the sexual isolation of Zimbabwe and Cosmopolitan strains and (i) their cuticular

pheromones and (ii) the transcript levels of *desat1* and *desat2*. After investigating the mating patterns between six Zimbabwe and two Cosmopolitan strains and their CH profiles, we chose one Cosmopolitan and one Zimbabwean strain to represent each group and explored the role of CHs in depth. We showed the partial involvement of CHs in this case of sexual isolation by perfuming experiments and explored the genetic basis of the cuticular hydrocarbons variation between these two strains by comparing the level of *desat1* and *desat2* transcripts.

Results

Zimbabwe females tend not to copulate with cosmopolitan males. We paired males and females from six strains from Zimbabwe (Z1-6) and two cosmopolitan strains (Arkansas-Louisiana, (AL) from the USA and Dijon (Dij) from France) in all 64 possible combinations and we measured their mating frequencies for one hour. This revealed significant assortative mating patterns (Figure 1): AL and Dij females tended to show higher levels of mating with AL and Dij males than with Z2–Z6 males, while Z2-6 females mated more frequently with Z2-6 males than with AL and Dij males (Figure 1A). To quantify the degree of sexual isolation, we calculated the joint isolation index (I_{psi}) for each of the 64 crosses between the eight types of fly[26]. Overall, the index was highly significant (Total I_{psi} = 0.17, G_{49df}= 561.5, p <0.0001; Table S1).

Surprisingly, Z1 flies behaved like AL and Dij flies (I_{psi}=0.04; t_{2755df}=0.64, p=0.52; Figure 1B). These flies come from the Zimbabwe capital (Harare) and show a cuticular hydrocarbon profile close to that of AL and Dij flies (see below). Cosmopolitan-type flies

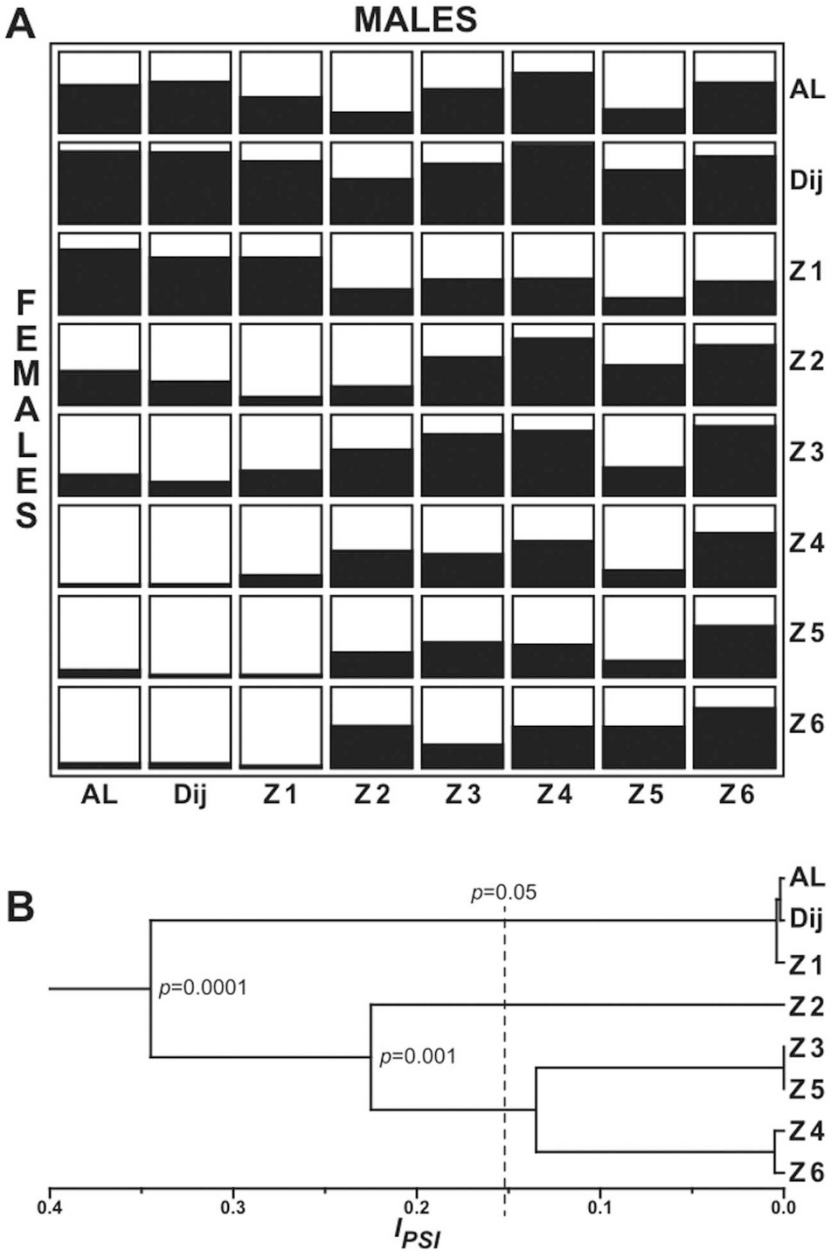


Figure 1 | (A) Mating frequency of single pairs of male and female flies from AL, Dij and Z1–Z6 strains, shown as a percentage. Couples were observed for 60 minutes. Each box represents a cross between the genotype of the female (rows) and of the male (columns). The relative surface of the square filled is proportional to the mating frequency. n=34–156. **(B) Dendrogram of a hierarchical cluster analysis (unweighted pair-group average linkage method) of the eight cosmopolitan-type and Zimbabwean strains, comparing their mating frequencies.** Branch length is proportional to the pairwise I_{psi} (see Materials and Methods). The eight strains are divided in three significant clusters (p=0.05): the first one (''Cosmopolitan-type'') include the AL, Dij and Z1 flies, the second (Zimbabwean) include the Z3 to Z6 flies, whereas the Z2 flies differ from these two groups.

have previously been described in African urban centres, presumably due to human transport[27], so on the basis of the CH data we classified Z1 as part of the Cosmopolitan group. As expected in strains showing incipient speciation, the distribution of mating in crosses involving Zimbabwe strains Z2–Z6 and those grouped as 'Cosmopolitan' (AL, Dij and Z1) revealed a strong asymmetric sexual isolation (I_{psi}=0.35; t_{2755df}=4.16, p=0.0001, Figure 1B. All IA_{psi} values for crosses between 'Cosmopolitan' and Zimbabwe flies were significant (p=0.05; Table S1), due to the relative absence of mating between Zimbabwe females and Cosmopolitan males.

We investigated the sensory effects responsible for the observed mating patterns by studying the courtship behaviour of the Z6 and AL strains (crosses are always given as female x male). We chose these strains because they showed the most extreme differences in mating pattern and in cuticular hydrocarbon profile (see below), and can be taken as exemplars of this case of incipient speciation. First, we explored whether the tendency to positive assortative mating in Z6 females was due to altered male behaviour. Z6 females induced significantly higher levels of courtship in AL males than did AL females (Figure 2A), indicating that Z6 females were highly attractive to AL males. This in turn suggests that the tendency towards isolation is due to female discrimination by Z6 females of a sensory stimulus provided by the AL males, not due to altered male behaviours. This was confirmed by the fact that Z6 females more frequently showed intense ovipositor extrusion (generally considered as a rejection response[28]) in response to AL males than to Z6 males (X_2 = 7.615, p = 0.022). The cumulative mating curves for all four crosses involving these two strains revealed significant differences: over the first 10 minutes, AL x AL pairs showed a significantly higher mating level (23%) than Z6 x Z6 or AL x Z6 pairs (10%), whereas Z6 x AL pairs already showed a significantly lower mating level (Figure 2B). This indicates that the factor(s) that lead Z6 females not to mate with AL males are detected from the very earliest moments of the encounter between male and female.

The nature of the stimulus provided by the male. We first investigated whether the Z6 females were discriminating against a visual signal provided by the AL males, by painting the females' eyes. This unusual procedure was required in order to make the females blind while enabling the males to behave normally. It had no effect on the courtship intensity of sighted males compared to crosses with sighted females, indicating that the males were not discriminating against the females because of the paint on their eyes, and the Z6 x AL cross still induced the highest courtship index (Figure S1). However, sight-deprived Z6 females still showed virtually no mating with AL males, whereas mating frequencies in the other three crosses were all >40% (Figure S1). A similar mating pattern was obtained under red light where both partners are virtually blind: 48% AL and 30% Z6

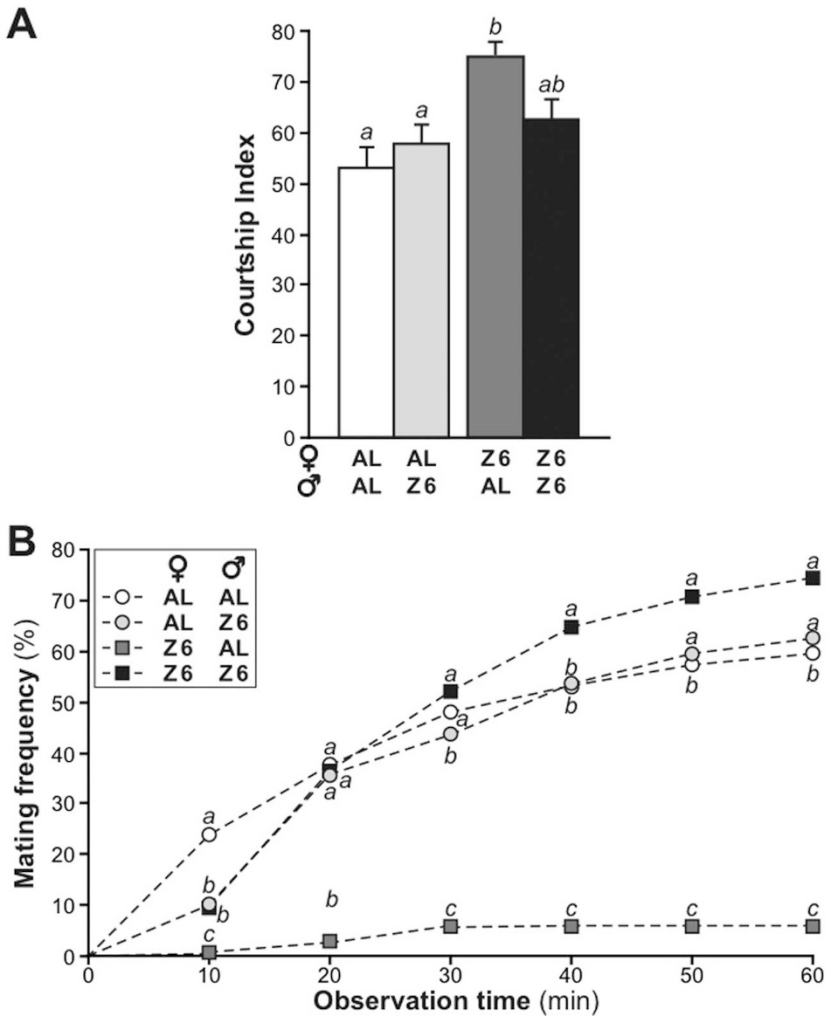


Figure 2 | (A) Mean (± SE) courtship indices in all four crosses between AL and Z6 flies. Courtship was defined as the time spent by a male showing wings vibration, female genitalia licking, circling and copulation attempt[51]. Pairs of flies were observed for 10 minutes. n = 48–54. Letters indicate significant differences. **(B) Cumulative mating curves in all four crosses between AL and Z6 flies.** The total observation period lasted for 60 min. n = 96–156. Letters indicate significant differences.

males mated with AL females, compared to 2% and 41%, respectively with Z6 females. We conclude that male visual signals are not responsible for the discrimination shown by Z6 females.

We next studied whether the Z6 females were using male courtship song to discriminate against the AL males. Recordings of courtship song from AL and Z6 males revealed slight differences between the two strains (Figure 3A). However, ablation of male wings and playback of synthetic song mimicking these differences showed that this difference in song played no role in mate discrimination by Z6 females. Song was important to mating success, because ablation of male wings abolished mating in all but the AL x AL cross (mating = 20%), but mating was restored when wingless male flies were accompanied by playback of song typical of either their own strain or a heterotypic male's song (Figure 3B). Strikingly, >65 % of AL males mated with AL females when accompanied by playback, with no significant difference between the two playback types, suggesting that AL females are highly stimulated by any type of *D. melanogaster* courtship song. The persistence of very low mating of both kinds of female with Z6 wingless males accompanied by song playback indicates that the wings of these males are important either for the stimulation or detection of females, or both. The sensory modalities involved are unknown but we presume them to be primarily chemical. Lastly, no Z6 females mated with AL males, irrespective of the type of playback, suggesting Z6 females were not using male song to discriminate against AL males. To test this hypothesis, we ablated female aristae, thereby deafening the females[29–31]. This procedure significantly reduced mating by AL and Z6 females, but only in homotypic crosses (Figure 3C). This indicates that AL females were equally stimulated by the acoustic and/or the chemical signals produced by male wings whereas Z6 females relied more on the chemical than on acoustic signals when paired with homotypic males. We conclude that another sensory signal, probably chemical, is responsible for the tendency to isolation in the Z6 x AL cross.

Variation in cuticular hydrocarbons (CHs) has previously been implicated in this assortative mating[5–7], but no decisive evidence has been found. We found significant and complex CH variation between these strains, which generally paralleled the differences between the two groups of strains suggested by the mating patterns (Z2-6 vs Z1, AL and Dij) (Table S2). The most extreme CH differences were observed between Z6 and AL flies (Figure 4). Z6 females produced more C5-desaturated CHs (5-tricosene (5-T), 5-pentacosene (5-P) and 5-heptacosene, 5, 9-hepta- and nona-cosadienes), whereas AL females produced more of the equivalent C7-desaturated CHs (7-tricosene (7-T), 7-pentacosene and 7-heptacosene, 7, 11-hepta- and nona-cosadienes). Similarly, Z6 males produced significantly more 5-T than 7-T, while the opposite was true in AL males. Multivariate analysis (Figure 5) showed variability within Zimbabwe males – Z2, Z3 and Z4 showed an intermediate position between Z5 and Z6 males with high levels of 5-T + 5-P + *n*-heptacosane and Z1, AL and Dij males (with high levels of 7-T + *n*-tricosane). Even though Z2–Z6 males showed inter-strain variation for the percentage of 5-T, they all showed a significantly higher percentage of 5-T than Z1, AL and Dij males. Virtually all these compounds have previously been suggested to play a pheromonal role in *D. melanogaster* courtship[10,13–15].

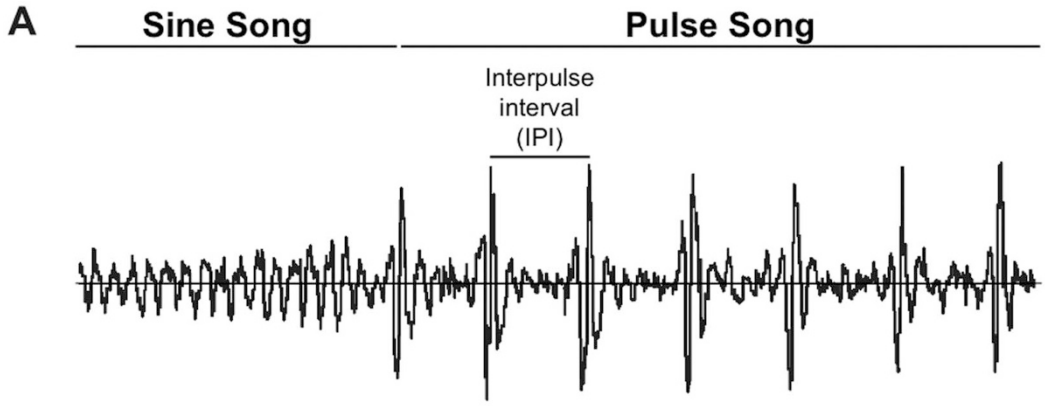
This combined profile of male and female CHs in Zimbabwe strains is unlike the profiles of either cosmopolitan or other African strains; although the C5:C7 ratio found in Z2–Z6 females is similar to that found in Central African strains, the high levels of 5-T shown by Z2–Z6 males are unique[4,9,10,20]. This unique combination may be a local adaptation to environmental parameters such as latitude and/or temperature[22,32,33].

The role of male hydrocarbons in isolation. To investigate whether male CHs are used by females in selecting a mate, we perfumed *desat1* mutant males, which lack any of the CHs described here[34]. AL or Z6 males were used as donors, to produce *desat1**AL* and *desat1**Z6* males respectively. The perfuming procedure altered the CH profile of *desat1* males to a profile between those of *desat1* males and the donor (Figure 6B). Perfuming *desat1* males with either Z6 or AL males significantly increased their success with AL females, with no difference between the treatments (Figure 6A). Strikingly, AL females showed higher mating frequencies than Z6 females in all three crosses. We conclude that AL females are relatively indifferent to the precise cuticular cocktail carried by a male fly, as long as it contains some of the relevant compounds. However, Z6 females showed a significant increase in mating only when paired with *desat1**Z6* males, but not with *desat1**AL* males. This shows that Z6 females are positively stimulated by the CH profile of Z6 males, and suggests that CH male profile and female preferences are involved in this case of incipient speciation. However, the levels of mating shown in the Z6 x *desat1**Z6* cross (Figure 6A) were substantially lower than those in the unmanipulated homotypic cross (Z6 x Z6 – Figure 3C), indicating that the pheromonal profile of the perfumed males was still not optimal for Z6 females.

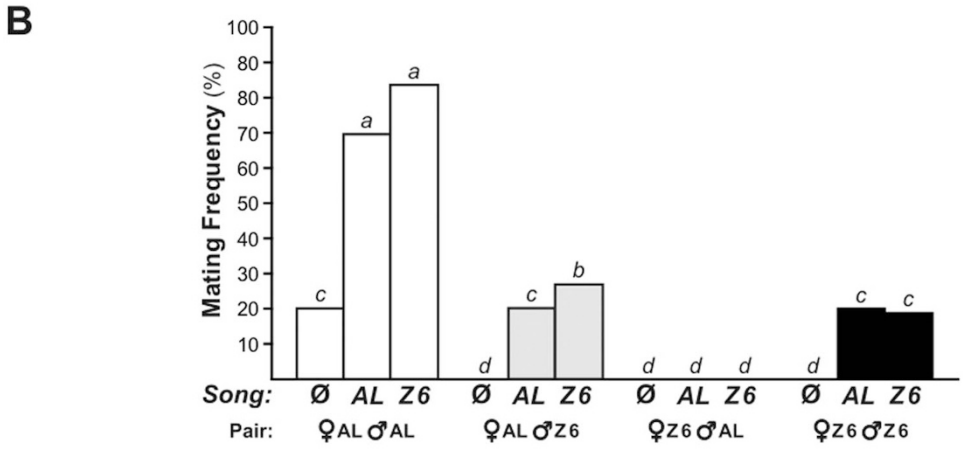
It is possible that the combination of the two profiles (*desat1* males are rich in linear saturated alkanes, and Z6 males are rich in 5-T) induced simultaneous antagonistic effects — repulsion and attraction — on the behaviour of Z6 females. The partial effect of perfuming on mating frequency (20 % instead of 45%) may also be due to the absence or alteration of other unknown pheromonal or non-pheromonal signals in manipulated males. Z6 females were also crossed with AL and Z6 males perfumed with either donor male (Figure S2A). Z6 males showed higher mating frequency than AL males, and the perfume of AL males induced a slightly higher mating response than that seen with Z6 male perfume. The altered female response may be caused by (*i*) the complex combination of the two CH profiles (Figure S2B), (*ii*) the altered distribution of CH topography on the male cuticle[35], and/or (*iii*) altered male behaviour produced by the perfuming procedure[14,46] .

To identify which part of the male cuticular profile might be involved in this case of incipient speciation, we calculated correlation coefficients between the mean cuticular composition of each strain, and its mating performance. The mating frequencies of Z2-6 females were highly significantly negatively correlated with the proportion of 7-T on the cuticle of the males they were paired with ($r = -0.458$, d.f. = 38, $p = 0.003$). For the three cosmopolitan-type females the situation was the complete opposite: there was a significant positive correlation between mating frequency and the proportion of 7-T on the male ($r = 0.429$, d.f. = 22, $p = 0.036$). No significant correlations were found between mating frequency and male %5-T, 5-T:7-T ratio or C5:C7 ratio for either group of females (data not shown). This suggests that Zimbabwe females discriminate against males with a hydrocarbon bouquet containing a high proportion of 7-T, while cosmopolitan-type females are stimulated by 7-T-rich males. We conclude that the differences in cuticular hydrocarbons play a role in the effect. However, this is not the full story: other, as yet unknown, signals may also contribute to the sensory profile that stimulates Z females most.

As an initial exploration of the genetic bases of this variation, we used RT-PCR to measure the levels of the *desat2* transcript and of five *desat1* transcripts[17–19,24,25] in AL and Z1 flies, and in Z6 and Tai (Ivory Coast) flies (Figure 7; data are presented as expression relative to AL flies). Tai flies were included because – they possess a well-studied CH profile that is intermediate between those of Z6 and AL flies. The cuticle of Tai males is mainly covered with 7-monoenes (as in AL males) while Tai females mainly carry 5,9-dienes (as in Z6 females). *desat1* and *desat2* genes are both involved in the desaturation of fatty acids, a key step in the cuticular hydrocarbon biosynthesis pathway[36,37]. Inconclusive findings have linked *desat2* to this case of incipient speciation[21], while *desat1* is involved in the detection of cuticular hydrocarbons as well as their production[24,25]. *desat2*



♂	Sine Song Frequency (Hz)	Pulse Song IPI (ms)	Pulse Song Frequency (Hz)
AL	148.1 ± 2.2	31.9 ± 1.1	236.4 ± 13.3
Z6	150.1 ± 2.2	28.8 ± 1.2	284.6 ± 9.5



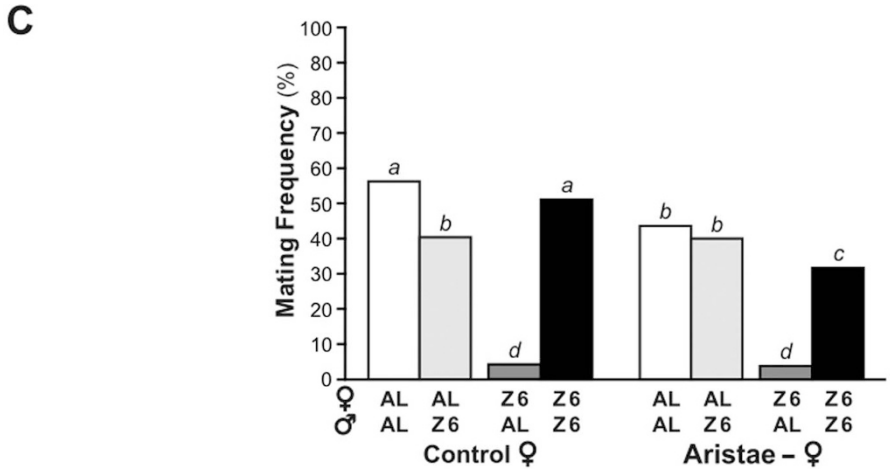


Figure 3 | (A) Composition of the courtship song of AL and Z6 males. n = 10. **(B) Isolation is not due to auditory signals from males.** Mating frequency in all four crosses between AL and Z6 flies was observed for 60 minutes. Males had their wings clipped off and were either allowed to mate in the absence of any song (Ø) or were accompanied by a synthetic song corresponding to AL or Z6 males. n = 5–56. **(C) Isolation is not due to signals detected by the female aristae.** Mating frequency in all four crosses between AL and Z6 flies was observed for 60 minutes. Females were either intact ('Control'), or had their aristae removed ('Aristae -'). n = 28–49. Letters indicate significant differences.

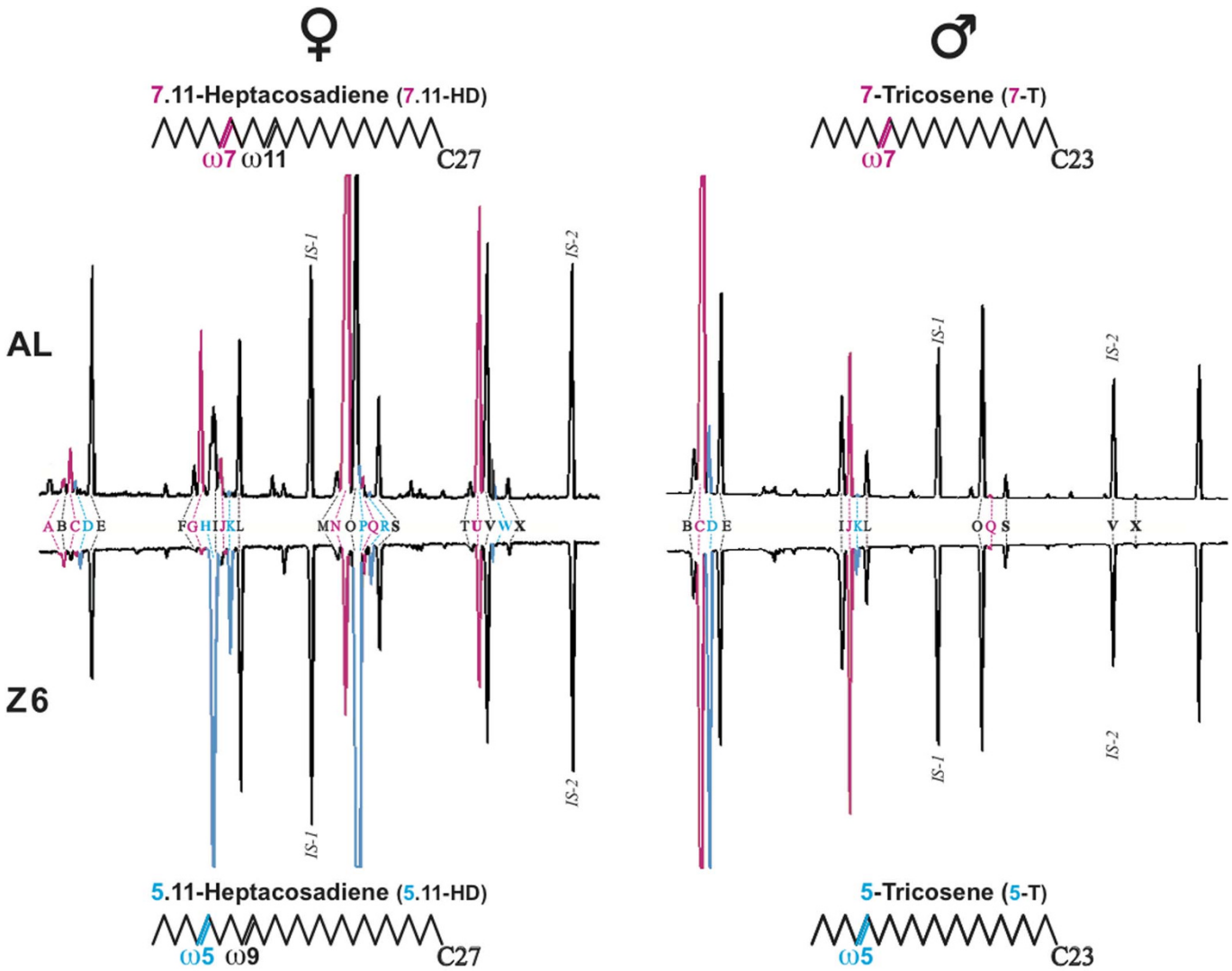


Figure 4 | Zimbabwe and cosmopolitan flies have qualitatively different cuticular hydrocarbon profiles. Typical gas chromatography traces are shown. Peaks were identified by mass spectrometry. Key for compounds: A= 7,11-TD, B=23MeBr+9-T, C=7-T, D=5-T, E=n-C23, F=9,13-PD, G=7,11-PD, H=5,9-PD, I=9-P, J=7-P, K=5-P, L=n-C25, M=9,13-HD, N=7,11-HD, O=27MeBr, P=5,9-HD, Q=7-H, R=5-H, S=n-C27, T=9,13-ND, U=7,11-ND, V=29MeBr, W=5,9-ND, X=n-C29. Compounds highlighted in cyan and magenta are desaturated on C5 and C7, respectively. Detailed hydrocarbon composition is shown on Fig. S2.

transcript levels were significantly higher in the bodies of Z6 and Tai flies than in Z1 and AL flies, with no difference between the sexes (Figure 7). Of the five *desat1* transcripts, RC, RE and RB showed significant inter-strain variation between male strains (Figure 7), but none of the transcripts was significantly different solely in Z6 males. Our data suggest that the *desat2* gene is involved in the C5:C7 ratio that characterises females of both Z6 and Tai African strains. However, the sex-specific difference between the two African strains — low levels of 7-T and high levels of 5-T in Z6 males but not in Tai males — may be further influenced by either (i) a more subtle tissue-specific variation of *desat2* expression in males and/or (ii) a differential variation of RA and RE transcripts between females. Since RA and RE are implicated in hydrocarbon production[25], their interaction with *desat2* transcript may affect male and female CH profiles differently.

Discussion

We have provided evidence that cuticular hydrocarbons are involved in this example of incipient speciation between *D. melanogaster* strains from Zimbabwe and from elsewhere in the world. Furthermore, we have shown that this effect is partially due to female flies reacting in opposite ways to the proportion of 7-T, the main cuticular component of male *D. melanogaster* flies from all strains except Zimbabwe. Zimbabwe females discriminate against cosmopolitan-like males on the basis of the proportion of 7-T in their CH bouquet, while females from other strains are stimulated by higher proportions of this substance. Although we cannot exclude the possibility that correlated signals, for example volatile compounds, are also involved in female discrimination, the fact that transferring AL CHs to *desat1* males did not significantly increase their mating levels with Z6 females, while transfer of the Z6 profile did significantly increase mating (Figure 6), indicates that the % of 7-T as a component of the total CH bouquet may partially account for the observed pattern of isolation in these strains. In male *D. melanogaster*, 7-T plays an inhibitory role in male-male courtship and has been shown to activate bitter gustatory sensory neurons[38]. We hypothesise that in Zimbabwe females there is a similar effect, leading these females to reject 7-T rich males.

Although we found no evidence of quantitative differences in transcription levels of *desat1* between two cosmopolitan lines and two lines from Zimbabwe, it is possible that there is a qualitative difference in the forms of this gene in these two kinds of strain. *desat1*

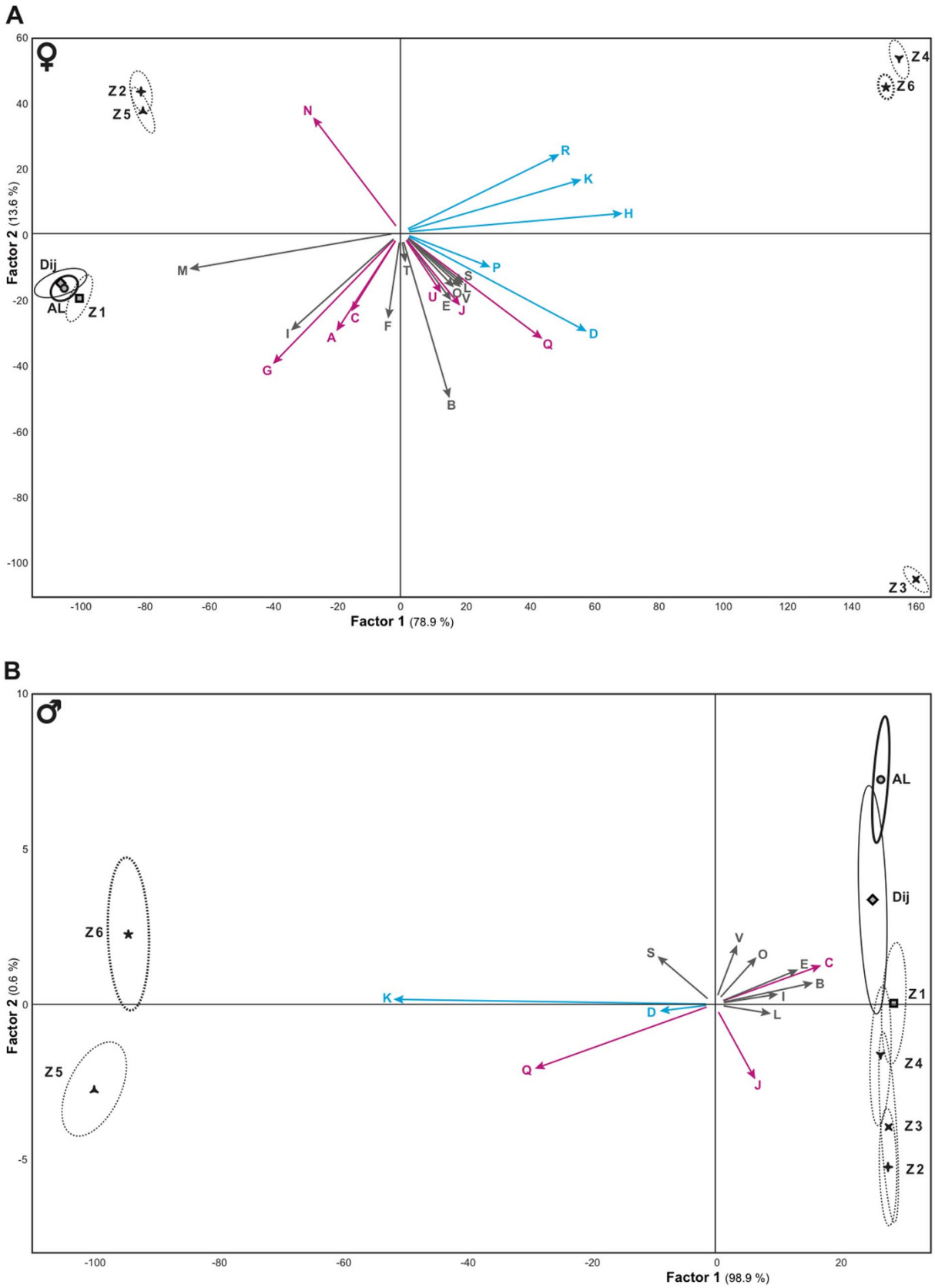


Figure 5 | Discriminant analysis of female (A) and male (B) flies of all eight strains, projected onto the first two principal components. Probability ellipses correspond to 95% probability. The various components in the cuticular cocktail (A-X) were also projected onto these components (arrows) to give a sense of the dimensionality. For each sex, we conducted a forward stepwise discriminant analysis (with an entry threshold value of $p=0.05$ and a removal threshold value of $p=0.10$) using the additive/log ratio transformed proportion of the CHs) using the absolute amount of the uncorrelated CHs as quantitative variables and the strain as a qualitative variable. Magenta arrows indicate C7 compounds, cyan arrows indicate C5 compounds, black arrows indicate others compounds. For legends, see figure 4.

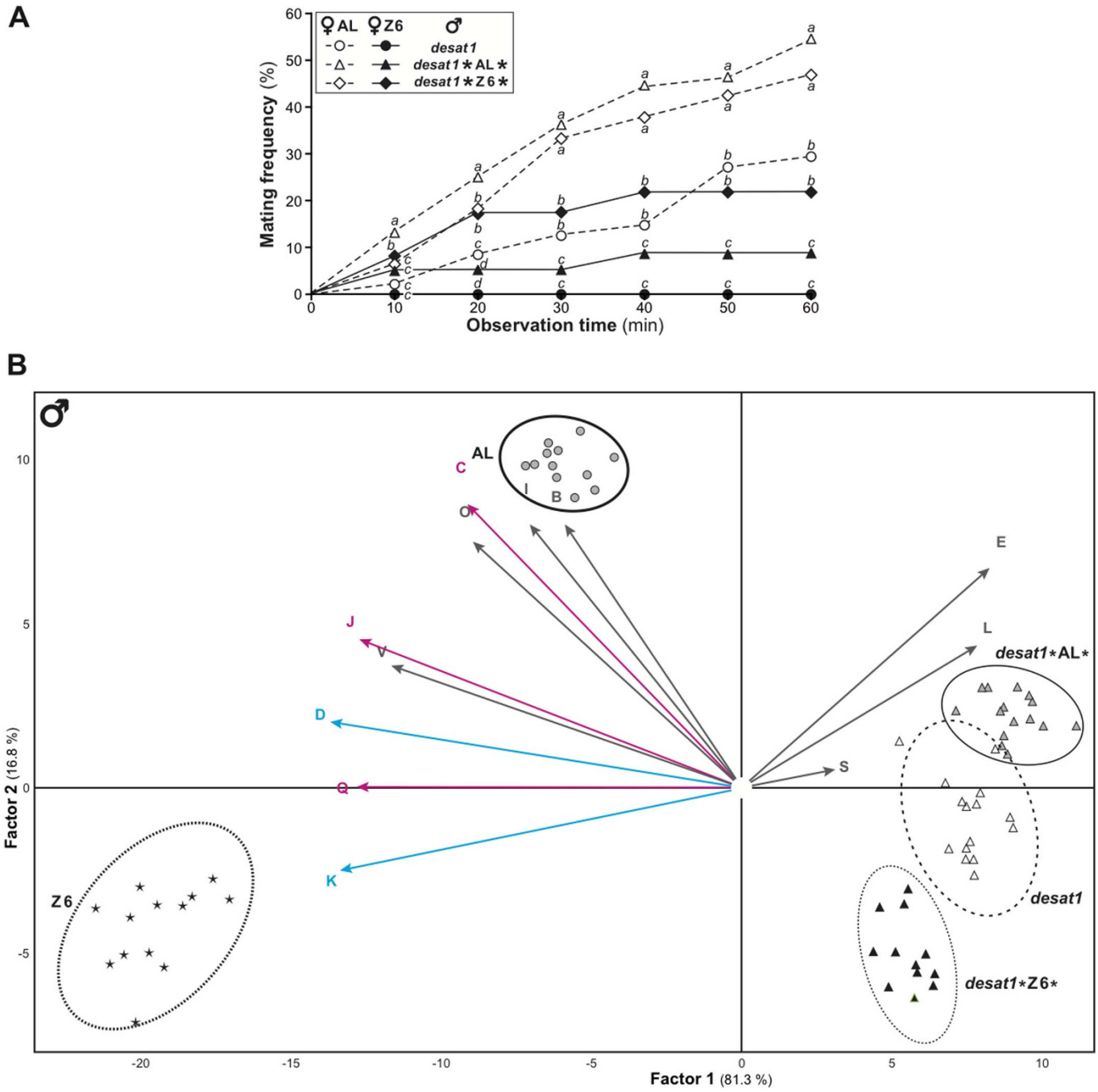


Figure 6 | (A) Chemical signals are partially responsible for isolation. Cumulative mating curves for AL and Z6 females with *desat1* males that were either unmanipulated (*desat1*) or received hydrocarbons from AL males (*desat1**AL*) or from Z6 males (*desat1**Z6*). n=48–80. Letters indicate significant differences. **(B) Discriminant analysis of male flies from AL, Z6 and *desat1* strains, and from males that had pheromone transfers (*desat1**AL* and *desat1**Z6*), projected onto the first two principal components.** Equiprobable ellipses correspond to 95% probability. The various components in the cuticular cocktail were also projected onto these components (arrows) to give a sense of dimensionality. We conducted a forward stepwise discriminant analysis (with an entry threshold value of $p=0.05$ and a removal threshold value of $p=0.10$) using the additive/log ratio transformed proportion of the CHs) using the absolute amount of the uncorrelated CHs as quantitative variables and the strain as a qualitative variable. Magenta arrows indicate C7 compounds, cyan arrows indicate C5 compounds, black arrows indicate others compounds.

simultaneously controls both the production and the detection of some *Drosophila* sex pheromones[24,25]. This occurs through different promoter regions[25] that drive its expression in oenocytes (abdominal tissues that control pheromone production)[24,39]; and in brain neurons involved in pheromone perception[24,25]. Zimbabwe females may carry a specific form of this gene or of its promoter(s) that result in altered responses. We also need to explore, both at the genetic and tissue levels, the functional relationship between *desat1* and *desat2* genes in terms of their combined effects on the intraspecific variation for production and perception of pheromones.

Our data help answer a conundrum that has existed for over 15 years[6,23] but they do not fully resolve this case of incipient speciation. The fact that AL females apparently respond significantly to manipulation of the male's courtship song, whereas Z6 females did not, suggests that there may be further differences between these strains, which may add to the pheromonally-driven isolation

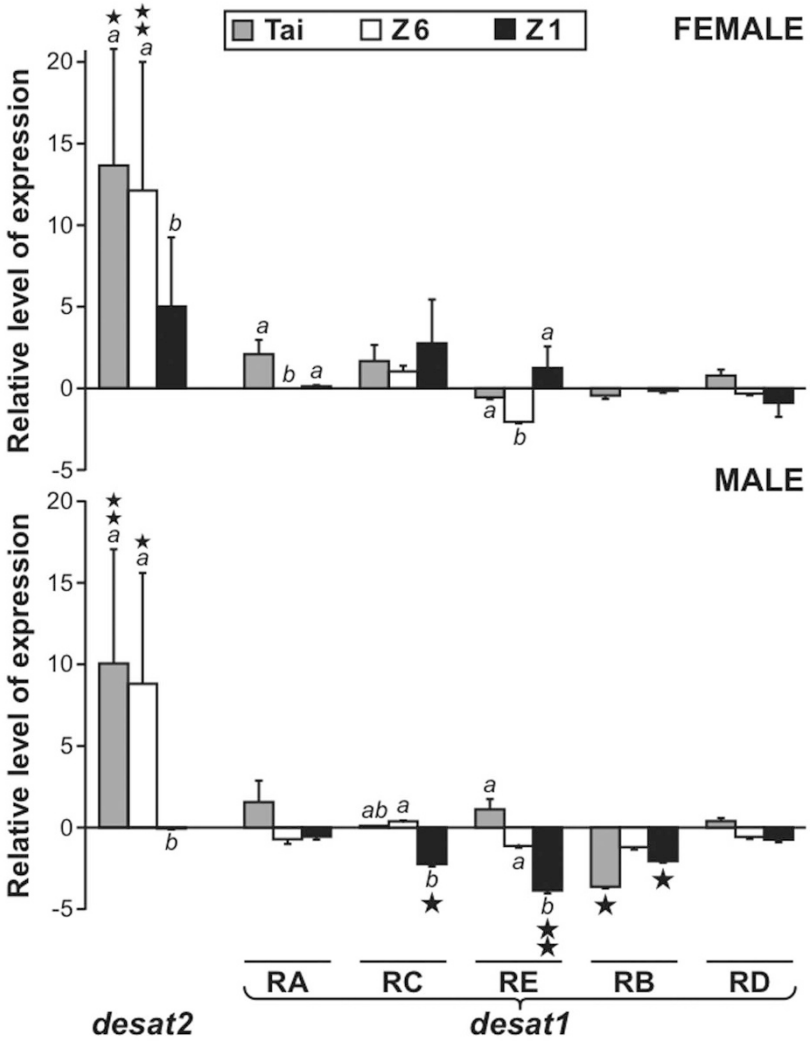


Figure 7 | Transcription levels of *desat1* and *desat2* do not directly correlate with mating patterns. RT-PCR was carried out on the transcription levels of *desat1* and *desat2* for male and female flies from Tai (Ivory Coast), and Z6 and Z1; differences in comparison to AL flies are shown as stars above/below bars (*: $P<0.05$; **: $P<0.01$). Significant differences in transcript levels ratio between Z1, Z6 and Tai strains (shown as different letters above/below bars) were detected with the Relative Expression Software Tool (REST, REST-MCS beta software version 2[47]) where the iteration number was fixed at 2000.

we have demonstrated here. Our findings indicate the potential importance of sensory processes in speciation, and lay the basis for a full understanding of this classic case of incipient speciation.

Methods

Strains and fly husbandry. All *D.melanogaster* strains were raised on yeast/cornmeal/agar medium and kept at 24 ± 0.5°C with 65 ± 5% humidity on a 12 : 12 light/dark cycle. The Zimbabwe and the AL lines were provided by Professor Jerry Coyne (University of Chicago, Illinois). The Zimbabwe lines were collected in 1990 either in the Wildlife Reserve of Sengwa for Z3, Z5 and Z6 lines, or in Harare, the Zimbabwe capital, for Z1, Z2 and Z4 lines. For the sake of simplicity, we modified the names of the Zimbabwe strains used here. The original names were Z1=H23, Z2=H18, Z3=S6, Z4=H42, Z5=Z53 and Z6=S30. The cosmopolitan line Arkansas-Louisiana (AL) comes from the United States. The Dijon 2000 (Dij) line was collected by Dr. J-F Ferveur in Dijon (France) in 2000. Although some of these strains have been kept in the laboratory for 20 years, our aim was to study incipient speciation rather than whether these strains represent current wild-type variation. More importantly, the performance of these strains was virtually constant compared to results reported in the 1990s[6,7]. It should be noted that after five years of acclimation in the laboratory, wild-type flies show no important changes in CH, courtship or mating[40]. The *desat1* line has a mutation in the *desat1* gene and *desat1* males and females show drastically reduced levels of 7-tricosene (7-T) and 7, 11-heptacosadiene (7, 11-HD), respectively.

Behaviour. *Mating behaviour.* All flies were isolated under light CO_2 anaesthesia 0–4 h after eclosion. Male flies were held individually in fresh glass food vials for 4 or 5 days before testing. Females were kept for 4 or 5 days in groups of five to ten. Male and female flies used as donors for hydrocarbon transfer were kept in vials in groups of 20 until 4 to 6 days old. All experiments were performed in at 24 ± 0.5°C and 65 ± 5% humidity. Tests were completed over several days and took place 1–4 h after lights on. One male was aspirated (without anaesthesia) under a watch glass used as an observation chamber (1.6 cm^3). After 10 minutes, a virgin female was introduced. Each test was performed for 60 minutes in white light and the overall frequency of copulating pairs was measured for each treatment.

Courtship behaviour. Flies were prepared as for the mating behaviour tests. A single male was aspirated (without anaesthesia) under a watch glass and the virgin female was introduced 10 minutes later. Each test was performed for 10 minutes in white light and the time that male spent courting was measured. We then calculated the courtship index – the percentage of time the male spent courting the female. To make the female blind, we painted both her eyes with black nail varnish ('Miss Helen' n°112), using a small brush. This procedure was performed one hour before the test to allow the varnish to dry. Tests performed in red light produced similar mating patterns (see Results & Discussion). We therefore conclude that painting the eyes did make the flies blind and did not introduce any other confounding sensory variable.

Song recording and playback. 4–5 day old male flies were isolated at eclosion and then introduced into the recording chamber together with a female that had its wings cut off, to prevent female wing-produced sounds from being recorded. Male song was recorded using an Insectavox recorder, linked to a computer. Song was filtered before being visualised on screen using the Audacity programme and analysed with Spike 2 software. AL and Z6 males produced slightly different songs (Figure 3A). Based on these data and a more extensive survey in Colegrave et al.[41] artificial songs were synthesised using Signal (Electronic Design). Each song consisted of an identical pulse song around 300 Hz with an IPI of either 35.5 ms (representing the song of Cosmopolitan flies) or 30.3 ms (representing the song of Zimbabwean flies). Variation in IPI followed a rhythmic cycle[42]. Full details of song synthesis are

described in Ritchie et al.[43]. The day before the test, males were anaesthetised by CO2 and had both wings completely removed. The mating cell, 1.6 cm diameter x 1 cm high, had a mesh surface and was placed on a loudspeaker (LCS 2415) which played back the synthetic song at 80 dB. The whole apparatus was placed in a sound-insulated chamber at 25°C. The song was continually broadcast throughout the duration of the experiment (30 minutes).

Cuticular hydrocarbons. *Extraction and analysis*. Cuticular hydrocarbons (CHs) from 4 day-old male and female flies of the different strains were analyzed by gas chromatography (GC) following hexane extraction and the addition of synthetic C26 and C31 hydrocarbon internal markers, according to standard procedures[44]. Analyses were performed with a Varian CP3380 chromatograph, fitted with a flame-ionization detector, with a CP-sil/5CB capillary column (Varian, 25 m × 0.32 mm ID) and a split-splitless injection system (operating with a split flow of 60 ml/min and a septum purge of 3 ml/min, opening of the split port 30 sec after injection). Hydrogen was used as carrier gas (50 cm/sec velocity at room temperature). The injector and detector temperatures were 260 and 280°C, respectively. The column was held isothermally at 140°C for 2 min, then programmed to increase at a rate of 5°C/min to 280°C. The data were automatically computed and recorded using PC software (Star 5.2, Varian).

Hydrocarbon transfer. We used the method of Coyne et al.[45]. 15 receiver males (*desat1* males) were enclosed with 150 killed donor males (AL or Z6 males) in a food vial overnight before experiment. Using dead donors avoids the problem of potential social interactions that can occur with live donors[46]. To increase the contact between donors and receivers, the plug of the vial was pushed down, leaving a 0.5 cm high space between the food and the plug. Transfer efficiency was tested using gas chromatography.

Flies to be analyzed for their behavioral and chemical characteristics were collected in parallel. We used a highly sensitive detection method (Solid Phase Micro Extraction[47]) to compare the HC profiles in these two samples, and found no variation other than the compounds passively transfered between partners during physical interaction. These compounds are not relevant in the present study, which focused on precopulatory behavior.

Real-Time PCR. We measured the relative amount of the five *desat1* transcripts (RA, RC, RE, RB and RD) and of the *desat2* transcript in 35 to 45 headless bodies of 5-day-old males. RNAs were extracted by the Trizol method (GIBCO BRL) and treated with RNase-free DNase to avoid contamination by genomic DNA. Total RNA (2 µg) was reverse transcribed with the iScript cDNA Synthesis Kit (Biorad). Quantative PCR reactions were performed with the IQ SYBR Green supermix (Biorad) in a thermal cycler (MyIQ, Biorad) according to the procedure recommended by the manufacturer. The qPCR reaction was done in a volume of 20µl, by 40 cycles (95°C for 30 sec, TM °C for 30 sec and 72°C for 30 sec), preceded by 3 min denaturation step at 98°C and followed by a 1 min elongation step at 72°C. TM of the hybridization step depends on the primer pair used (see Houot et al.[25]). Each reaction was performed in triplicate and the mean of the three independent biological replicates was calculated. All results were normalized to the Actine5C mRNA level.

Statistical analysis. *Mating frequency*. For mating frequency comparisons, we used JMATING Software[26] to compute the total I_{psi} and the pairwise I_{psi} and IA_{psi}.

The pairwise I_{psi} were used as a dissimilarity index to achieve an agglomerative hierarchical clustering (unweighted pair-group average linkage method). To analyse cumulative mating frequency, mating levels were recorded every 10 min over 60 min and compared using a chi-square test with a computation of significance by cell.

Courtship Indices were compared with the Kruskal-Wallis test, completed by Conover-Iman's multiple pairwise comparison (two-tailed with Bonferroni correction).

Cuticular profiles. For male and female flies of each strain, the relative amounts of CHs were transformed by additive log-ratio transformation (*alr*)[48] using CoDaPack (v2.01.1)[49] ; the zero values were substituted by 10^{-6} values and the denominator was taken to be the proportion of *n*-C29. For each sex, we carried out a stepwise dis-criminant analysis (DA) (forward, entry threshold value: $p=0.05$ and removal threshold value: $p=0.10$) using the *alr*-transformed CH proportions as quantitative variables and the strain (or perfume) as the qualitative variable.

Except for I_{psi}, statistical analyses were conducted with XLSTAT 2007 (Addinsoft, 2007).

Transcript levels. Statistical analyses were performed using XLSTAT software. Significant differences in transcript levels ratio between genotypes were detected with the Relative Expression Software Tool (REST, REST-MCS beta software version 2[50]) and the iteration number was fixed at 2000. This test is based on the probability of an effect as large as that observed under the null hypothesis (no effect of the treatment), using a randomization test (Pair Wise Fixed Reallocation Randomisation Test©).

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Author contributions

MG, CE and JFF designed the experiments, MG, BH and MGR carried out the experiments, MG, CE, MGR, MC and JFF analyzed the data, MG, CE, MC and JFF wrote the ms, MG and CE made the figures, MG, CE, BH, MGR, MC and JFF reviewed the ms.

Additional information

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